

Full Length Research Paper

Genetic diversity of bovine viral diarrhoea virus in Beijing region, China from 2009 to 2010

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Accepted 16 September, 2013

The genetic diversity of bovine viral diarrhoea viruses (BVDV) from Beijing region, China between 2009-2010, was investigated using a phylogenetic analysis of partial 5'untranslated region (5'UTR) nucleotide sequences from 61 BVDV positive samples, and for 13 selected samples, the N^{pro} region was also investigated. All the sixty-one blood samples were collected from cattle that were BVDV persistently infected (PI) within a BVD eradication program on Beijing farms during 2009 and 2010. Phylogenetic analysis based on 5'UTR sequences indicated that all BVDV positive samples were of the BVDV-1 genotype, but were clustered into four different subtypes: sub-genotype 1b (n = 31), 1m (n = 24), 1c (n = 4) and 1d (n = 2). BVDV-1b and 1m are the most prevalent sub-genotypes in Beijing. The results also demonstrate that in most herds, certain BVDV-1 sub-genotype predominates. It is the first time that BVDV-1d is reported in China with unclear origin. The result of this study will be useful in constructing an effective vaccination plan to further control BVDV in Beijing region.

Key words: bovine viral diarrhoea viruses 1 (BVDV-1), 5'untranslated region (5'UTR), N^{pro}, phylogenetic analysis.

INTRODUCTION

Bovine viral diarrhoea virus (BVDV), a member of the genus Pestivirus, family Flaviviridae, causes significant losses in cattle farming worldwide (Vilcek et al., 2001). BVDV has a positive single-stranded RNA genome, approximately 12.3 kb in length, and encodes an open reading frame (ORF) that is translated into a single polyprotein of approximately 4000 amino acids. The ORF is flanked by untranslated regions (5'UTR, 3'UTR) (Meyers and Thiel, 1996). The 5'UTR highly conserved segments provide sufficient sequence data for reliable genetic classification of new BVDV isolates (Toplak et al., 2004).

Two antigenically distinct genotypes of BVDV (BVDV-1 and BVDV-2) are recognized. BVDV-1 was first described in 1954 and is presently found worldwide, whereas BVDV-2 was discovered in the USA and Canada fifteen years ago (Baker et al., 1954; Ridpath et al., 1994), and has recently been sporadically reported in other countries, such as Belgium (Letellier et al., 1999), Germany (Wolfmeyer et al., 1997), Japan (Nagai et al., 1998), Austria (Vilcek et al., 2003), Argentina (Leandro et al., 2001) and China (Zhu et al., 2009). Both genotypes can cause acute and persistent infections with similar clinical manifestations, however, some highly virulent BVDV-2

Table 1. BVDV positive samples used in this study.

Herd origin	Collection data	Genotype	Sample and Accession number (5'UTR)
Xijiaoyi	2009	BVDV-1b	BJ09-10 (HQ116542),BJ09-16 (HQ116545),BJ09-17 (HQ650843)
	2009	BVDV-1m	BJ09-12 (HQ650842),BJ09-13 (HQ116543),BJ09-15 (HQ116544)
Zhongyi	2009	BVDV-1m	BJ09-02 (HM769723),BJ09-08 (HQ116540),BJ09-09 (HQ116541)
			BJ09-18 (HQ650854),BJ09-19 (HQ650844),BJ09-28 (HQ879781),BJ09-35 (HQ650850),BJ09-38 (HQ650851),BJ09-40 (HQ650857),BJ09-41 (HQ650852),BJ09-43 (HQ650858),BJ09-44 (HQ650859),BJ09-45 (HQ650860),BJ09-46 (HQ650861),BJ09-47 (HQ650862),BJ09-50 (HQ650863),BJ09-52 (HQ650853),BJ09-54 (HQ650855),BJ09-56 (HQ650866), BJ09-57 (HQ650867), BJ09-58 (HQ650868)
Lvhe Ermu	2009	BVDV-1b	
	2009	BVDV-1m	BJ09-33 (HQ650849),BJ09-39 (HQ650856),BJ09-51 (HQ650864),BJ09-55 (HQ650865)
Jinyindao	2009	BVDV-1c	BJ09-27 (HQ116552),BJ09-31 (HQ650847),BJ09-32 (HQ650848)
Changyangsi	2009	BVDV-1b	BJ09-03 (HQ116536),BJ09-04 (HQ116537),BJ09-05 (HQ116538),BJ09-06 (HQ116539)
	2009	BVDV-1b	BJ09-20 (HQ116546),BJ09-29 (HQ650845),BJ09-30 (HQ650846)
Jinxing	2009	BVDV-1c	BJ09-21 (HQ116547)
	2009	BVDV-1d	BJ09-22 (HQ116548),BJ09-23 (HQ116549)
Beijiaosan	2010	BVDV-1b	BJ10-01 (HQ879782),BJ10-02 (HQ879783),BJ10-03 (HQ879784)
Xinpu	2010	BVDV-1m	BJ10-04 (HQ879785),BJ10-05 (HQ879786),BJ10-06 (HQ879787)
Jingyuan	2010	BVDV-1m	BJ10-07 (HQ879788),BJ10-08 (HQ879789), BJ10-09 (HQ879790)
Nankou'er	2010	BVDV-1m	BJ10-10 (HQ879791)
Nankousan	2010	BVDV-1m	BJ10-11 (HQ879792),BJ10-12 (HQ879793),BJ10-13 (HQ879794),BJ10-14 (HQ879795),BJ10-15 (HQ879796),BJ10-16 (HQ879797),BJ10-17 (HQ879798)

strains can also cause a severe haemorrhagic syndrome with high mortality rate (Ridpath et al., 1994). Within BVDV-1, BVDV-1a and BVDV-1b were initially described (Ridpath et al., 1994). Vilcek et al. (2001) collected 78 BVDV isolates from Austria, France, Hungary, Italy, Slovakia, Spain and UK, and their phylogenetic analysis separated BVDV-1 into at least 11 genetic sub-groups (Vilcek et al., 2001). Subsequently, additional BVDV isolates were reported from many countries and regions, and the meaningful genomic regions of these isolates were sequenced and analyzed. Several other BVDV-1 sub-genotypes, named 1l, 1m, 1n, 1o, 1p, were introduced. In China, occurrence of BVDV infection has been observed since 1980, with most BVDV isolates being classified as BVDV-1b (Mahony et al., 2005). Several BVDV-1c were identified in Xinjiang (Huang et al., 2008), and several isolates were clustered into BVDV-1m (Xue et al., 2010). Xue et al. (2010) also reported that several isolates from China were clustered into a new BVDV sub-genotype 1p.

Pestiviruses are known to be highly variable antigenically and genetically (Paton, 1995). When designing and constructing effective vaccination strategies to control BVD, the genetic diversity of circulating BVDV needs to be considered. After screening persistently infected cows in Beijing dairy herds, we investigated the genetic diversity of BVDV obtained in positive serum samples by performing phylogenetic analysis of partial 5'UTR genome

part, and for selected samples the N^{pro} region to check the results was obtained.

MATERIALS AND METHODS

Animals and samples

Animals investigated in this study were from 11 dairy farms in Beijing, which had an area of 16,808 km² and about 150,000 cattle. Before this study, BVDV PI cattle screening under a BVDV eradication program were enforced on 15 dairy farms in Beijing during 2009 and 2010, including about 30,000 cattle (Zhang et al., 2012). Sixty one (61) sera samples were collected from all the PI cattle which had been tested positive in an antigen capture ELISA screening test (HerdCheck BVDV Antigen Test Kit/Serum Plus, IDEXX, Sweden) (Shannon et al., 1991) within the program. These positive samples belong to 11 farms, which are located at 7 districts of Beijing, including Fangshan, Tongzhou, Daxing, Haidian, Changping, Shunyi and Miyun District, and most of Beijing's cattle are kept in these areas. The samples were stored at -70°C. All the sera samples examined in this study are listed in Table 1.

RNA isolation

Total RNA was extracted from sera samples using TIANamp Virus RNA Kit (Tiangen Biotech, China) according to the manufacturer's instructions. The extraction was accomplished with 140 µl of sera and 560 µl of TRIZOL, and the RNA was resuspended in 40 µl of DEPC-treated water. The RNA isolation was performed directly on sera, which excluded the possibility of BVDV contamination during cell culture and the authenticity of the results was upheld (Vilcek et

al., 2001; Xue et al., 2010).

cDNA synthesis and PCR

cDNA synthesis was performed using Quantscript cDNA First-strand Synthesis Kit (Tiangen Biotech, China) with random primers. Subsequently, a 288 bp DNA product was amplified from the 5'UTR using GoTaq Green Master Mix (Promega Corporation, USA) for 35 cycles. Utilized primer, 324 and 326, were widely used to amplify a cDNA fragment for genetic diversity analysis of BVDV isolates on 5'UTR (Vilcek et al., 1994, 2001, 2003). The conditions for amplification were 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. For 13 selected samples, a 428 bp DNA product was amplified from N^{pro} region employing the same method with primers BD1 and BD3 (Vilcek et al., 2001). The conditions for amplification were 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C.

Sequencing and phylogenetic analysis

Amplified products were separated by electrophoresis in 1.5% agarose gel in Tris-Acetate EDTA buffer, and purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany). The resulting DNA were then sequenced in both directions by using primers 324/326 or BD1/BD3 and an ABI 3730XL sequencing device utilizing fluorescent labeled dideoxynucleotide terminators. All the sequences were confirmed as BVDV by blasting the specified sequences against existing sequences in the NCBI Genbank. Nucleotide sequences were aligned using the Clustal W V.12.10. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0, employing the Neighbor-Joining method with 1000 Bootstrap replications (Tamura et al., 2007).

RESULTS AND DISCUSSION

The genetic diversity of BVDV field positive samples was surveyed by determining the nucleotide sequences of the 5'UTR of 61 positive samples and the N^{pro} region of 13 selected samples, obtained in Beijing between 2009 and 2010. The 5'UTR phylogenetic tree was constructed with the nucleotide sequences of the 61 positive samples, and 36 representative strains retrieved from GenBank. The N^{pro} phylogenetic tree was created using the nucleotide sequences of the 13 selected samples and 24 representative strains retrieved from GenBank. The 13 selected samples are clustered into the four sub-genotypes by the 5'UTR phylogenetic tree. We selected these samples, and constructed the N^{pro} tree to independently test the results obtained from the 5'UTR tree.

As expected from previous analyses (Vilcek, 2001; Mahony et al., 2005) both phylogenetic trees produced consistent results (Mahony et al., 2005; Vilcek et al., 2001). All the 61 BVDV field positive samples investigated in this study were of the BVDV-1 genotype (Figures 1 and 2). Of the total, 31 positive samples were of the BVDV-1b sub-genotype, 24 were of the BVDV-1m sub-genotype, 4 were of the BVDV-1c sub-genotype, and two were of the BVDV-1d sub-genotype. To the best of our knowledge, this is the first time BVDV-1d has been

reported in China, and the first time BVDV-1m, BVDV-1b and BVDV-1c has been reported in Beijing. Based on these results, the BVDV-2 genotype was absent in Beijing during this period. From the distribution of 61 positive samples obtained (Table 1), we can see that in most herds (8 out of 11) there is one predominant sub-genotype. The alignment of these sequences reveals that mutations were usually located in two variable regions, 208-228 and 298-328 of the NADL strain, which is consistent with the results obtained by Vilcek et al. (1997).

Xue et al. (2010) identified BVDV-1p from cattle in Beijing, however, in the current study no samples clustered into the BVDV-1p sub-genotype (Xue et al., 2010). Two most prevalent sub-genotypes in Beijing are BVDV-1b and 1m. A Blast search of the NCBI Genbank, indicated that these BVDV-1b and 1m sequences are similar with the strains reported in China previous to this study (Vilcek et al., 1997).

Four positive samples were clustered into the BVDV-1c sub-genotype, which is the predominant sub-genotype in Australia. A Blast analysis of the BVDV-1c positive samples against NCBI Genbank non-redundant database identifies VR924 and VR1000 (Mahony et al., 2005) as the most similar sequences which were both collected from Australia. The two herds exhibiting BVDV-1c as the prevalent sub-genotype, the Jinxing and Jinyindao herds had imported cows from Australia in 2005 and 2006. However, when importing cattle, a BVDV antigen capture ELISA was conducted to confirm that no BVDV PI animal was introduced. So it is an interesting question: how was BVDV-1c introduced? Interestingly, two farms, Zhongyi and Lvhe Ermu, imported cows from Australia without developing BVDV-1c as the prevalent sub-genotype (Li et al., 1983). The self-clearance action of BVDV infection should be considered. In order for BVDV infection to persist in a herd for an extended period of time (without re-introduction), one or more sero-negative animals need to be in early pregnancy while there are PI animals in the herd. Otherwise, self-clearance will occur (Lindberg and Alenius, 1999). Consequently, we should acknowledge that PI animals may not be identified if they are aborted, stillborn, experience an early death or are traded before a testable age.

After blasting, the most similar sequence to the two BVDV-1d positive samples was isolate 2900/83 (Tajima et al., 2001), initially reported in Germany. So far as we know, frozen semens imported from Germany were used in Beijing dairy farms. However, how BVDV-1d was introduced is still unknown and worth further investigation.

BVDV PI cattle screening is a very important step in BVDV eradication programs, but if we want to keep the spread of BVDV under control, designing and constructing effective vaccination strategies is also essential. Data pertaining to BVDV genotypes circulating in Beijing

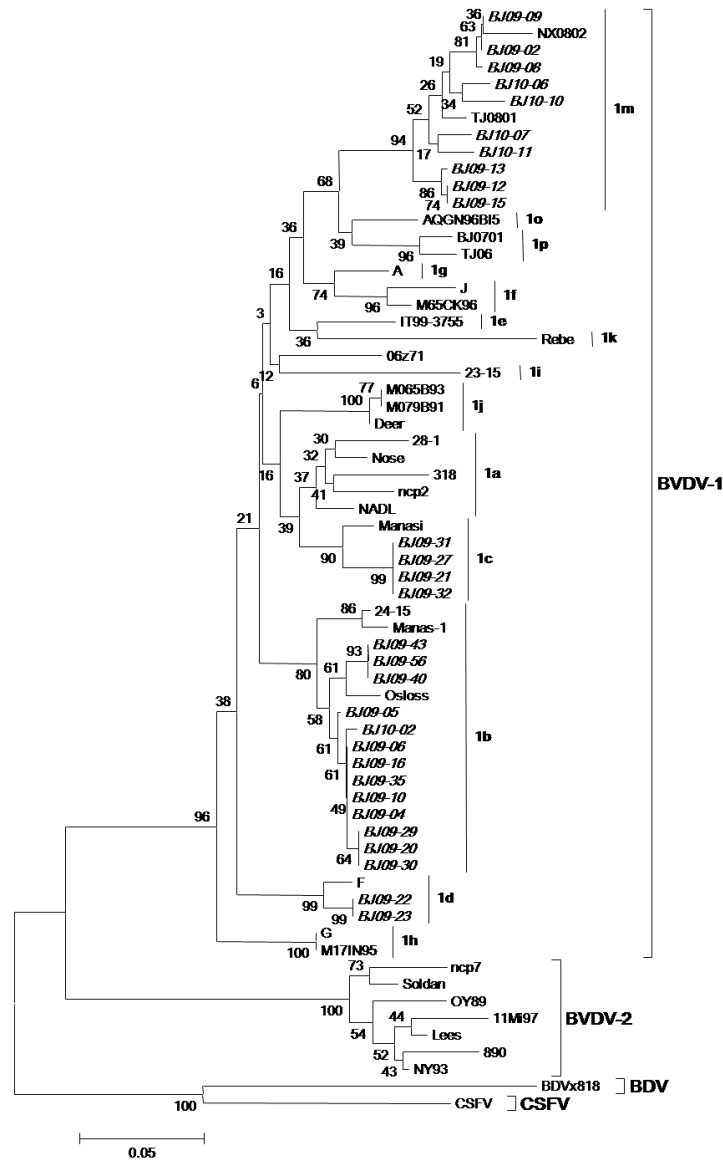


Figure 1. Phylogenetic tree based on amplification of the 5'UTR region (128-372 in NADL) of 61 BVDV positive samples (in italic) and 36 representative strains. 32 positive samples being identical with some samples shown were removed from the tree. The phylogenetic tree was created using the MEGA program by means of Neighbor-Joining. Numbers over the phylogenetic branches indicate the percentage of 1000 bootstrap replicates that support each branch. Bar indicates 0.05 nucleotide substitutions per site. The GenBank accession numbers of the representative strains are as follows: ncp7 (AY443026), Soldan (U94914), 890 (L32886), NY93 (AF039173), 11/Mi/97 (AJ293603), Lees (U65051), OY89 (AB003621), 24-15 (AF298060), 318 (AF298062), ncp2 (AY443027), Nose (AB019670), 28/1 (AF298061), M065B/93 (U97409), M079B/91 (U97410), M65CK/96 (U97456), M17IN/95 (U97431), TJ06 (GU120246), BJ0701 (GU120247), TJ0801 (GU120255), NX0802 (GU120253), Manas-1 (EU555288), Manasi (EU159702), NADL (AJ133739), Osloss (M96687), F (AF298065), IT99-3755 (AJ318616), J (AF298067), A (AF298064), G (AF298066), 23-15 (AF298059), Deer (AB040132), Rebe (AF299317), 06z71 (DQ973181) and AQGN96B15 (AB300691). The tree was rooted by two outgroup: BDVx818 (AF037405) and CSFV (m31768).

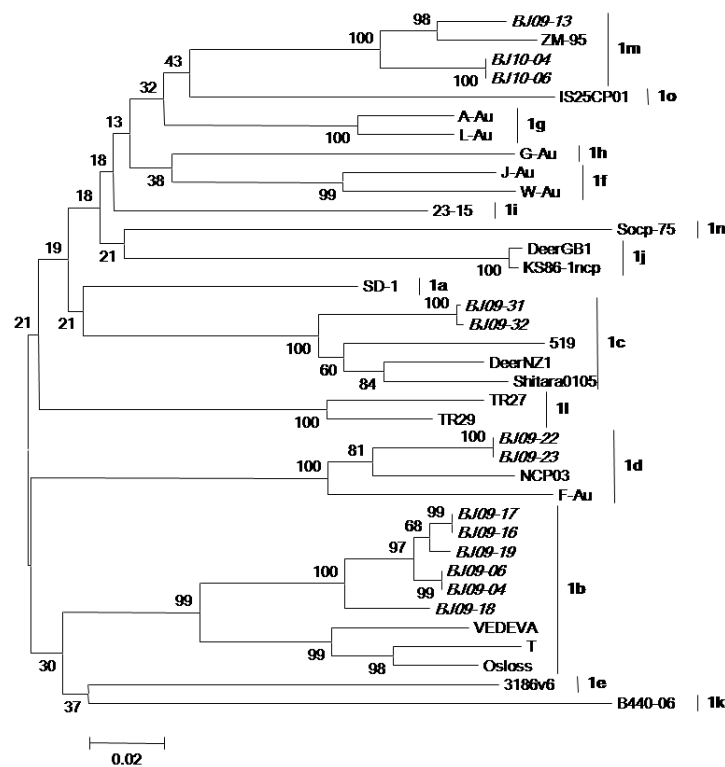


Figure 2. Phylogenetic tree created for the N^{pro} gene region (389-770 in NADL) of 13 selected BVDV positive samples (in italic) and 24 representative strains. This phylogenetic tree was constructed by the same method with the 5'UTR tree. Bar indicates 0.02 nucleotide substitutions per site. The GenBank accession numbers of the 13 selected BVDV positive samples are JN542492-JN542504. The GenBank accession numbers of the representative strains are as follows: SD-1 (M96751), 519 (AF144464), DeerNZ1 (U80903), Shitara0105 (AB359926), F-Au (AF287284), 3186v6 (AF287282), J-Au (AF287286), W-Au (AF287290), A-Au (AF287283), L-Au (AF287287), G-Au (AF287285), 23-15 (AF287279), DeerGB1 (U80902), B440-06 (EU224257), TR27 (EU163975), TR29 (EU163977), Osloss (M96687), VEDEVA (AJ585412), Ncp03 (AB359927), Socp-75 (AB359929), KS86-1ncp (AB078950), IS25CP01 (AB359931), ZM-95 (AF526381) and T (AF287289).

is indispensable for a better understanding of the pathogenesis and epidemiology of BVDV infections. On the basis of this study, the BVDV-1b and BVDV-1m sub-genotypes should be considered first when constructing a vaccine to control the BVDV infections in Beijing. Consequently, further studies need evaluation of the antigenicity of Beijing isolates belonging to different sub-genotypes.

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