

Molecular characterization of *Bovine virus diarrhea viruses* species 2 (BVDV-2) from cattle in Turkey

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Abstract Five BVDV species 2 (BVDV-2) isolates were detected from cattle in Turkey. Phylogenetic analysis was performed based on the 5' untranslated regions (UTRs) and E2 coding gene regions, respectively. The isolates were closely related to BVDV-2a strains from North America and Canada used as references. This is the first report of the detection of BVDV-2 in naturally infected Turkish cattle. It is important to consider BVDV-2 for planning future BVDV control and vaccination programs in Turkey.

Keywords BVDV-2 · Sequencing · Cattle · Turkey

Introduction

Bovine Virus Diarrhea Virus (BVDV) belongs to the *Pestivirus* genus of the *Flaviviridae* family. BVD viruses

are classified into two virus species, namely BVDV-1 and BVDV-2. The differentiation between these two species is based on a comparison of the 5' untranslated region (UTR), N^{pro}, and E2 sequences (Pellerin et al. 1994; Ridpath et al. 1994; Vilcek et al. 2001; Tajima et al. 2001). While BVDV-1 viruses have 11 subtypes (Vilcek et al. 2001) and additionally recent reports showed the existence of the new tree subtypes (Jackova et al. 2008; Nagai et al. 2008), BVDV-2 viruses are classified into two subtypes (2a and 2b) (Vilcek et al. 2001; Ridpath et al. 2000; Flores et al. 2002). The two species cause different clinical signs since they have different antigenic and genomic structures. BVDV-1 strains usually cause a subclinical form, while highly virulent BVDV genotype 2 strains may cause severe disease (hemorrhagic syndrome, HS) characterized by high fever, thrombocytopenia, hemorrhages, and high mortalities (Corapi et al. 1989; Carman et al. 1998; Ridpath and Neill 1998; Ridpath 2005). However, it should be stressed that most BVDV-2 viruses cause an asymptomatic infection (Ridpath and Neill 2000). Additionally, mucosal disease (MD) or persistent infections (PI) with BVDV-2 strains have been described in cattle (Nagai et al. 1998).

Infection with BVDV-2 was first described in North America in the early of 1990s (Corapi et al. 1989; Pellerin et al. 1994; Ridpath et al. 1994). The outbreaks have been particularly devastating in Quebec (Canada), and resulted in the death of 32,000 out of 143,000 (22.4%) cattle (Pellerin et al. 1994). A significant ratio (3–12%) of isolated BVDV strains from several European countries was characterized as BVDV-2 (Tajima et al. 2001; Beer et al. 2002; Letellier et al. 1999). The prevalence of BVDV-2 was 6.6% in Japan (Sakoda et al. 1999). In the USA, however, BVDV-2 was found in 39–43%, with rates up to 95% of the pestivirus isolates from the epidemic outbreak from 1993 to 1995 in

GenBank-accession numbers of BVDV 2 strain of this study: EU542421-EU542423, EU051825, and EU051826 for 5'UTR sequences and GQ144318-GQ144321 for E2 gene region.

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Ontario in east-central Canada (Carman et al. 1998; Bolin and Ridpath 1998; Ridpath et al. 2006).

In Turkey, the etiological role of BVDV for different clinical pictures, the prevalence of persistent infections and seropositivity rates for BVDV in several herds were investigated (Alkan et al. 2000; Oguzoglu et al. 2004). Recently, Turkish BVDV isolates were analyzed on a genetic level, where one sheep isolate was identified as BVDV genotype 2b based on the N^{pro} gene region (Yesilbag et al. 2008); however, BVDV-2 isolates from cattle could not be detected in this previous study.

Here, we report on the genetic characterization of BVDV-2 strains from naturally infected cattle in Turkey, based on the 5'UTR and the E2 region of the genome.

Materials and methods

Samples

In this study, the blood samples of 36 animals over 2 years of age sent for routine control at the end of 2006 and early 2007 from a private dairy farm with 500 cows were used. These animals were originally imported from north-east of the USA in 2004, but since then have been bred at this facility. The veterinarian reported clinical signs in the herd characterized by upper respiratory tract disease in heifers and diarrhea that was sometimes bloody in calves. However, none of the animals sampled ($n=36$) had described any clinical signs at the time of sampling.

Antigen ELISA and RT-PCR

Firstly, whole blood samples were tested for BVDV antigen using a commercial BVDV Antigen ELISA (IDEXX, USA). The test was performed according to the manufacturer's instructions.

From antigen positive samples, total RNA was extracted using a High Pure Viral Nucleic Acid Kit (Roche, Germany), according to the manufacturer's recommendation. cDNA synthesis was carried out using a first strand cDNA synthesis Kit (Fermentas, Lithuania) as described by the manufacturer's protocol. Amplification of cDNAs by PCR was performed using the primer pairs 324/326 for 5'UTR and B11/B32 for E2 gene region, respectively (Vilcek et al. 1997; Couvreur et al. 2002). To determine the biotypes (noncytopathogenic or cytopathogenic) of the field viruses, primers for the pestivirus NS3 gene region were used (Greiser-Wilke et al. 1993). NADL (cytopathogenic reference virus) and 0712/Hann (noncytopathogenic reference virus) are positive control viruses using the determination of biotypes discrimination and provided

kindly by Virology Institute of Hannover Veterinary University, Germany.

Nucleotide sequencing and phylogenetic analyses

The PCR products were purified using the DNA Purification Kit (Roche, Germany) according to manufacturer's instructions. Nucleotide sequencing was performed on a Beckmann Coulter CEQ 8000 using the GenomeLab™ Methods Development Kit. Comparisons, clustering, and alignments of sequences were analyzed with referenced other BVDVs from GenBank using the Bioedit program (version 7.0.5.3) and Clustal X (1.83) (Thompson et al. 1997). Bootstrap analysis was performed using the Mega software program (version 4.0), and values were calculated with 1,000 replicates. Phylogenetic analysis with the Mega software program (Tamura et al. 2007) was performed by inputting a dendrogram produced by the Neighbor-Joining Method for multiple sequence alignments. A sequences distance matrix was constructed using the ClustalW algorithm (Thompson et al. 1994) and calculated divergence and percent identity values of each sequences pair in the current alignment.

Results

Virological results

Six of 36 blood samples were found to be positive for BVDV by BVDV-Ag ELISA. Only two of six cattle could be tested again after 21 days to assess the status of BVDV infection (persistently infected versus transiently viremic), because other infected animals have been eliminated from the herd. These two animals were identified as persistently infected according to the results of the ELISA of the second blood samples.

RT-PCR results and analysis of 5'UTR and E2 coding sequences

The six field pestiviruses were identified as noncytopathogenic by the RT-PCR using NS3 gene region primers (Fig. 1). Five of them (including the two from the persistently infected animals) produced an expected 288 bp amplicons after RT-PCR with 5'UTR-specific panpesti generic primers. The weak positive one sample in ELISA could not produced a good quality 288 bp product by PCR. Sequence analysis of these five amplicons were evaluated using the BLAST program (accession numbers from GenBank for the five nucleotide sequences are EU051825, EU051826, EU542421, EU542422, and EU542423 for 5'UTR).

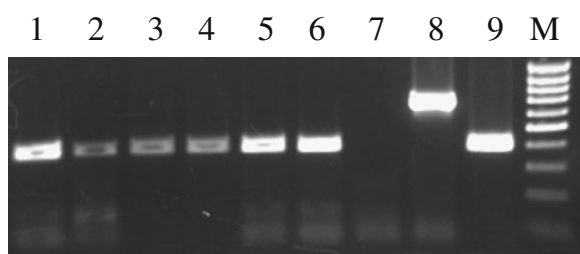


Fig. 1 Lanes 1–6 BVDV antigen ELISA positive samples were detected as noncytopathogenic strains by RT-PCR using the primers for p125 gene region. Lane 7 negative control. Lane 8 NADL, positive control virus for cytopathogenicity (683 bp). Lane 9 0712/Hann, positive control virus for noncytopathogenicity (402 bp). M DNA marker (100 bp, Fermentas, Lithuania)

With four of the five samples, expected 606 bp strong amplicons after RT-PCR with E2 gene region specific primers were obtained and sequenced, their accession numbers from GenBank are GQ144318–GQ144321.

The identity percentage of Turkish BVDV 2 sequences based on 5'UTR were found between 69.6–76.0% and 84.3–97.5% both BVDV1 and BVDV2 referenced on GenBank, respectively (data not shown). Similarly, there were found identity percentage for E2 gene region between 52.0–58.6% and 85.2–96.4% for the same viruses (data not shown).

Phylogenetic tree analysis (Fig. 2) was performed with some BVDV genotype 1 and 2 sequences that are available from GenBank, including viruses from North America, Canada, Japan, and from European countries. The sequence analysis results indicated that the Turkish BVDV strains from cattle were BVDV-2. The results of the phylogenetic tree with bootstrap trials comparing Turkish BVDV-2 sequences with other pestivirus sequences for 5'UTR and E2 gene region are shown in Fig. 2a and b. According to phylogenetic tree, the nucleotide sequences of Turkish BVDV-2 strains have separated a cluster in each other and originated from same branch the other referenced as BVDV-2a.

Discussion

Since the first description in 1946 (Olafson et al. 1946), BVDV infections have been found to occur worldwide and cause significant economical losses. A major problem for the detection, the differential diagnoses, and control programs for BVDV infection is their genetic heterogeneity.

Hemorrhagic syndrome and thrombocytopenia have practical biological importance in distinguishing BVDV 1 and BVDV 2 genotypes. However, it is reported that the

severity of the diseases resulting from BVDV genotype 2 appears to be strain dependent (virulence factor) and that not all BVDV 2 isolates cause clinically severe disease (Ridpath and Neill 2000). In this study, two cattle detected as PI with BVDV genotype 2 had no clinical symptoms which confirms the observations made by Ridpath and Neill (2000). Others (Fulton et al. 2005; Tajima and Dubovi 2005) reported that BVDV is a collection of diverse subgenotypes in the USA with the three major subgenotypes, BVDV-1a, BVDV-1b, and BVDV-2a in the cattle population and all three BVDV subgenotypes were found in persistently infected cattle. These results suggest that the distribution of BVDV biotypes (cp and/or ncp) and subgenotypes should be correlated with the clinical histories and/or pathodiagnoses. It is known that animals persistently infected with BVDV type 2, while continuously shedding highly virulent virus, may appear clinically normal. Similarly, two cattle infected with noncytopathogenic BVDV type 2 in this study were determined as PI. Additionally, PI animals can cause severe acute BVDV outbreaks in herds and produce a cytopathogenic variant from noncytopathic virus by mutation, which can be induced by agent of MD would constitute an epidemiological dead-end (Nagai et al. 1998; Ridpath et al. 2000; Hamers et al. 2001).

Comparing the 5'UTR sequences of five Turkish BVDV type 2 field isolates, the identity rates were estimated to be between 95.1–97.5% by using Clustal W algorithm. Those of the E2 gene region were determined between 92.1% and 98.2%. Thus there appear to be individual differences between these isolates. The similarity rates between the 5'UTR sequences of Turkish BVDV genotype 2 and other reference strains ranged from 69.6% to 97.5% and from 52.0 to 96.4% in the case of the E2 gene.

In recent years, BVDV eradication programs have been used in a lot of countries (Obritzhauser et al. 2005). Thus the molecular characterization of existing BVDV strains in a country has gained significant importance, especially for safe cattle industry. For example, BVDV 2a genotype is notified as predominantly from Korea (Oem et al. 2009).

Yesilbag et al. (2008) characterized a sheep isolate from Turkey, identified as BVDV-2b by using N^{pro} gene region sequences. 5'UTR and E2 gene region sequences of this BVDV-2b isolate are not available on the GenBank, therefore our cattle-derived BVDV2 sequences could not be compared with this virus from Turkey.

However, both 5'UTR and E2 regions sequences of Turkish BVDV-2 field isolates did not cluster with BVDV-2a reference viruses from other countries. The sequences of the Turkish BVDV-2a field isolates showed the highest similarity with BVDV 1373, 24515, and

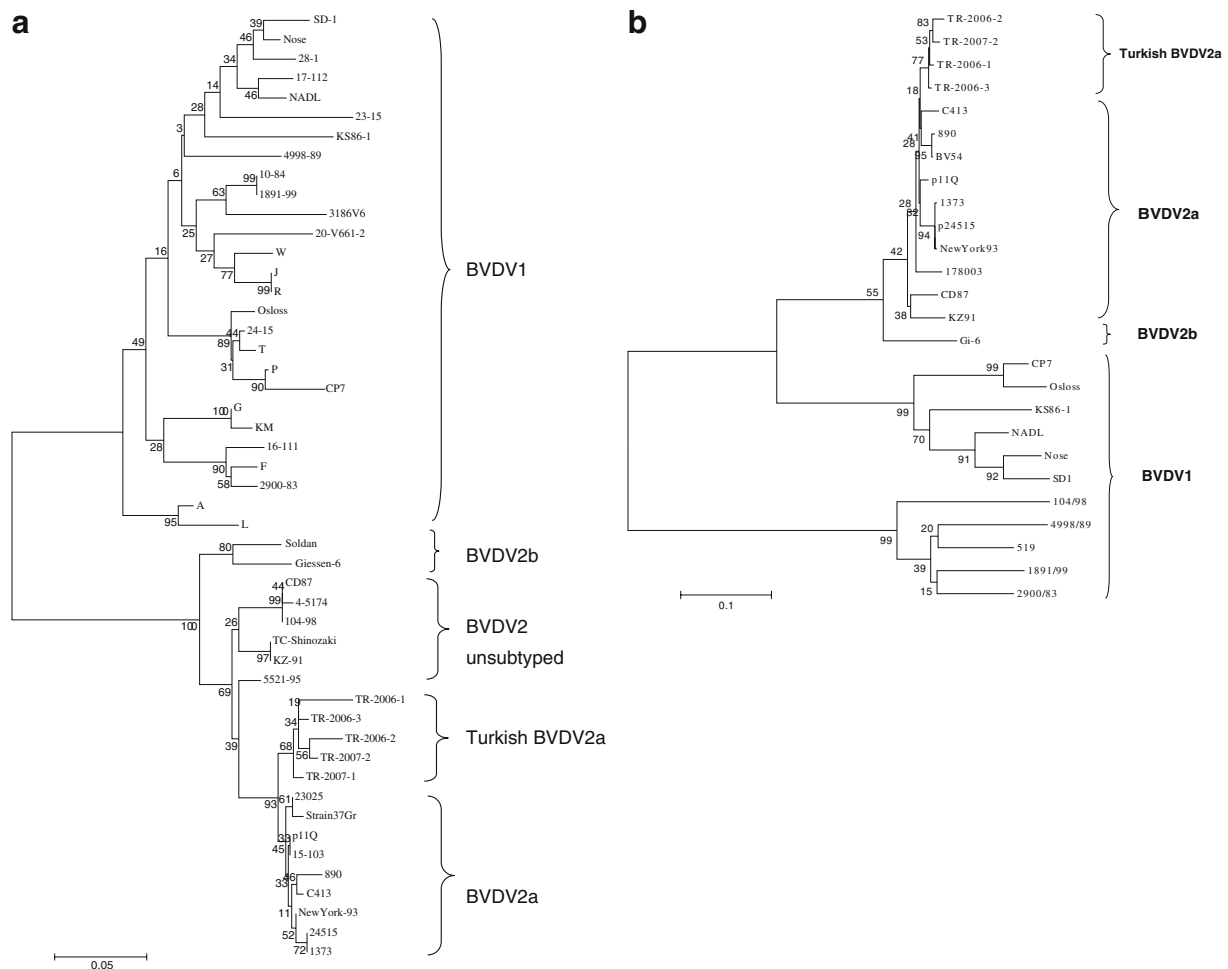


Fig. 2 a The phylogenetic tree were performed using nucleotide sequences of the 5'-UTR gene region of Turkish BVDV type 2a with some following BVDV sequences were downloaded from the GenBank database: **BVDV-1:** AF298054-AF298073 (Vilcek et al. 2001), NADL (M31182), SD-1 (M96751), Nose (AB019670), KS86-1 (AB042713), Osloss (M96687), CP7 (U63479), 2900/83 (AJ304375), 10-84 (AF298054), 4998/89 (AJ304385), 1891/99 (AJ304384), G-AU (AF298066), 23-15 (AF298059). **BVDV-2:** NewYork93 (AF502399), 1373 (AF145967), p24515 (AY149216), 890 (U18059), P11Q (AY149215), BV54 (AY442521), C413 (NC_002032), CD87 (L32887), 104/98 (AJ304381), Gi-6 (AY379547), Soldan (U94914), 4-5174 (AF298063), KZ91-ncp (AB003620), TC Shinozaki (AB042676), Strain37 (EU327594). **Turkish BVDV2 strains:** TR-2006-1 (EU542421), TR-2006-2 (EU542422), TR-2006-3 (EU542423), TR-2007-1 (EU051825), TR-2007-2 (EU051826). There were a total of the 205 nucleotide positions analyzed. The tree was constructed by the neighbor-joining method and indicate percentage of 1,000 bootstrap replicates by using MEGA version 4.0 (Tamura et al. 2007). Bar number of base

substitutions per site. **b** The phylogenetic analysis by using the neighbor-joining method was made based on 410 nucleotide of the E2 gene region of Turkish BVDV type 2a with those downloaded some published following BVDV sequences from the GenBank database. **BVDV-1:** AF298054-AF298073 (Vilcek et al. 2001). NADL (M31182), SD-1 (M96751), Nose (AB033752), KS86-1 (AB033753), Osloss (M96687), CP7 (U63479), 519/93 (AF144610), 2900/83 (AJ302969), 4998/89 (AJ302959), 1891/99 (AJ303001), 104/98 (AJ302961). **BVDV-2:** NewYork93 (AF502399), 1373 (AF145967), p24515 (AY149216), 890 (U18059), P11Q (AY149215), BV54 (AY442521), C413 (NC_002032), CD87 (U44907), Gi-6 (AF144612), KZ91-ncp (AB105687). There is no sequences of the E2 gene region available for 10-84, G-AU, 23-15, Soldan, 4-5174, Strain37 BVDV isolates. **Turkish BVDV2 strains:** TR-2006-1 (GQ114320), TR-2006-2 (GQ114319), TR-2006-3 (GQ114318), TR-2007-2 (GQ114321). The bootstrap values (in %) were calculated from 1,000 replicates with 111 random seeds by using MEGA version 4.0 (Tamura et al. 2007). Bar number of base

p11Q, which had been isolated from BVDV outbreaks in North America at the Ontario Lake in Quebec at the American–Canadian border. These similarities are interesting because the herds sampled in our study included animals imported from the north-east of the USA in 2004 as well as their progeny. It is known that the introduction

of infected animals may play a significant role in the distribution of new infectious agents (or a new strain or genotype) on a herd or even country level. Certified animals free of certain viral infections should be purchased only, or animals should be quarantined and controlled for disease before being introduced in a herd.

However, how and when BVD species 2 viruses were introduced into Turkey cannot be determined.

To summarize, the presence of BVDV type 2a was described in Turkish dairy cattle for the first time and characterizations of these isolates are reported. Further studies are required to investigate the prevalence of BVDV-2 infection in a larger cattle population as well as the role in various clinical conditions and economical losses. The presence of new species is important for the evaluation of current diagnostic protocols and for the development of control programs, such as vaccination. Furthermore, it underlines the necessity to quarantine and test imported cattle thoroughly before introduction into local herds.

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