**Molecular identification and phylogenetic analysis of non-cytopathic bovine viral diarrhea virus isolate obtained from a local respiratory outbreak in Northern Turkey**

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**Summary**

In February 2016, a local respiratory disease outbreak with two fatalities was reported in Samsun, Turkey. A non-cytopathic bovine viral diarrhea virus (ncp-BVDV) was identified from the organ and leucocyte samples of dead juvenile heifers using RT-PCR with specific primers for the NS2/3 gene coding region. The NS2/3 gene of BVDV was sequenced and compared with other published sequences of BVDV. The sequences of our isolate which was named as Samsun TR, had 81-83% nucleotide (nt) identity for BVDV-1. Phylogenetic analysis revealed that Samsun-TR was closely related to LC089875 (Japan), AF526381 (China) and also shared 83% nucleotide(nt) identity with them. The NS2/3 gene sequence of Samsun-TR was deposited in the GenBank database with the accession number of KX428495.

**Keywords:** BVDV, RT-PCR, phylogenetic analysis, nucleotide sequence, ELISA

Bovine Viral Diarrhea Virus that includes two species, BVDV-1 and BVDV-2, is a member of Pestivirus genus within Flaviviridae virus family together with Border Disease Virus (BDV) and Classical Swine Fever Virus (CSFV) (9). BVDV mainly affects dairy and beef cattle which are considered as its natural hosts (8). However, it has been reported that BVDV can infect other even-toed ungulates including sheep, goat, antelope, alpacas, and llamas (5, 8, 18). In some parts of the world, BVDV may be a common cause of border disease in sheep due to interspecies transmission of BVDV between cattle and sheep (12).

BVDV species have two well-known biotypes commonly described as cytopathic (cp) and non-cytopathic (ncp) according to their effects on cultured cells in vitro (9). Ncp biotypes are responsible for the vast majority of BVDV infection in living cattle at some stage of their lives (4, 9). Infection with BVDV is either asymptomatic or causes symptoms including mild enteric and respiratory symptoms, and results in seroconversion to protect from reinfection (9). When ncp BVDV infects fetuses during the first 40-120 days of gestation, a state of immune unresponsiveness may occur, resulting in persistently infected (PI) animals which serve as a lifelong source of infection to herds due to the virus spreading through excretes. Moreover, PI animals are unable to produce an antibody response against the virus and mucosal disease may lead to death if these animals are super-infected by cp biotypes of BVDV (2, 9, 18).

BVDV can be described as a costly disease of cattle due to the calculated annual expenses of more than $48 per cow for developed countries including Denmark, USA and Canada (17). It also has significant negative economic impacts on the livestock industry because of having a broad tissue tropism and infections with a variety of clinical manifestations including respiratory, neurological and reproductive disorders, diarrhea and congenital abnormalities in herds (4, 8). Furthermore, BVDV takes part in bovine respiratory disease complex together with other important viral pathogens including bovine herpes virus type-1 (BHV-1), bovine respiratory syncitial virus (BRSV), bovine parainfluenza virus type-3 (BPIV-3) and bovine adenoviruses (BAV), and
some bacteria. Here we have reported the identification of ncp BVDV from dead or living heifers in a beef cattle farm in the Samsun Province, Northern Turkey.

**Material and methods**

**Case history.** In February 2016, a local outbreak with two fatalities was reported from a beef cattle farm located in the suburb areas near the Samsun Province, Northern Turkey. The two dead animals were juvenile (240 days and 270 days old). Organ samples including a lung and a liver were taken from these cases and sent to a laboratory under a cold chain. We were not able to obtain blood samples from these animals due to clotting. Furthermore, serum, nasal swab and whole blood samples were collected from 15 heifers that were in the same barn and also had respiratory symptoms, including high fever, mucus and nasal/ocular discharges and in appetite. We tried to retract serum and blood samples from infected animals 28 days after the first sampling for retesting the presence of virus and/or seroconversion to from infected animals. Twenty-eight days after the first sampling for retesting the presence of virus and/or seroconversion to from infected animals 28 days after the first sampling for retesting the presence of virus and/or seroconversion to.

**Samples processing.** The organ samples were processed in order to use for nucleic acid extraction. Approximately 30 mg of each organ sample was homogenized by using TissueLyser (Qiagen AG, Switzerland) in 1.8 ml Minimal Essential Medium (MEM) containing 2% penicillin/streptomycin (Sigma, UK). Obtained homogenates were clarified by centrifuging at 9000 × rpm for 15 min. Supernatants were separated into different tubes and stored at –20°C until used.

All blood samples were taken from the vena jugularis using serum and EDTA tubes. Serum samples were centrifuged at 2500 × rpm for 15 min and separated into sterile tubes. The peripheral blood samples in the EDTA tubes were centrifuged at 2500 × rpm for 15 min and separated into sterile tubes. The supernatants were discarded. The pellet was resuspended in 500 µL PBS. The nasal swabs of the animals were put into in 5 ml of PBS containing 2% antibiotics and then vortexed. All of the samples were stored at –20°C until used.

**Nucleic acid extractions.** To determine viral RNA, lung and liver samples of the first dead animal, a lung sample of the second dead animal, and 15 leucocytes and swab samples that were taken from the diseased heifers were extracted by using a RNeasy Mini Kit (Qiagen, Spain) according to the manufacturer’s instructions.

**Antigen ELISA.** In order to investigate BVDV antigens together with other important viral pathogens including BHV-1, BPIV-3 and BRSV, homogenized lung samples of the two dead animals were tested by using Multiscreen Antigen Elisa Kit (Bio X diagnostic, Belgium, Cat Nr:Bio K 3402) according to the manufacturer’s instructions.

**RT PCR tests.** All RNA extracts were performed by using One-step RT-PCR kit (Qiagen, Spain) with specific primers (for) 5’-GCAGATTTTGAAGAAAGACACTA-3’; (rev) 5’-TTGGTGTG TGTAAGCCCA-3’ to amplify the expected size of 402 bp for ncp-BVD and 680 bp for cp-BVD (13).

All of the RNA extracts were also tested using specific primers, (for) 5’-TCGTGTTGAGATCCCTGAG-3’; (rev) 5’-GCAGAGATTTTTATACTAGCCTATRC-3’ to amplify the expected size of 225 bp amplicons for BDV (16). At the end of amplifications, 10 µl of each PCR product were loaded on a 1.5% agarose gel stained with ethidium bromide and were visualized by using Quantum gel imaging and documentation system (Vilber, Germany) after running on 100 V for 40 minutes. Positive controls for BDV were provided from the Virology Department at the Faculty of Veterinary Medicine, Ondokuz Mayis University. The PCR products were purified by using a gel-extraction kit (Qiagen, Spain) and were then sequenced by RefGen company, Turkey (http://www.refgen.com).

**Sequencing and phylogenetic analysis.** The raw sequence data were initially assembled using Contig Express (Vector NTI module, Invitrogen) (3). Consensus sequence of Samsun TR (KX428495) was aligned with other published sequences of BVDV in the Genbank database using ClustalW. A total of 14 strains from different geographical areas (Table 1) were used to create the phylogenetic tree by using Neighbor-Joining method in (Mega 1.02) and the tree was assessed by 1000 bootstrap samplings.

**Antibody detection.** The serum samples of 15 animals were tested by using commercial BVDV p80 antibody ELISA test kit (Idexx, the Netherlands, Cat No: P00645-5) in order to detect IgG antibodies against BVDV according to the manufacturer’s instruction. All serum samples were also tested in order to detect IgG antibodies against BHV-1, BRV, BPI-3, BAV and also BVDV using Multiscreen antibody ELISA bovine respiratory kit (Bio-X diagnostic, Belgium, Cat Nr: Bio K 028).

| Tab. 1. Pestivirus strains which were used to create the phylogenetic tree |
|------------------------|------------------|------------------|
| Strain Name | Country | Genbank ID | Origine |
| XJ-04/FJS27854/2009 | China | FJ527854 | Cattle (Bos taurus) |
| Alfort/Tuebingen/JJ04358/2005 | Germany | J04358 | Pig (Sus suis) |
| X881/AF37405/1998 | Germany | AF37405 | Sheep (Ovis aries) |
| NADL/AJ133738/2005 | USA | AJ133738 | Cattle (Bos taurus) |
| NADL/AJ133739/2005 | USA | AJ133739 | Cattle (Bos taurus) |
| USMARC-60780/KT832823/2016 | USA | KT832823 | Cattle (Bos taurus) |
| Singer_Arjp/DOO88995/2006 | Argentina | DOO88995 | Cattle (Bos taurus) |
| Camel-6/KC695810/2014 | China | KC695810 | Camel (Camelus bacterianus) |
| ZM-95/AF526381/2006 | China | AF526381 | Pig (Sus suis) |
| Egy/Ismailia/KR029825/2014 | Egypt | KR029825 | Cattle (Bos taurus) |
| 1-CP7/BVU63479/2005 | Germany | BVU63479 | Cattle (Bos taurus) |
| IS26/01ncp/LC089875/2016 | Japan | LC089875 | Cattle (Bos taurus) |
| Suanw NPC/KCB53440/2013 | Switzerland | KC853440 | Cattle (Bos taurus) |
| USMARC-55524/KP941590/2015 | USA | KP941590 | Cattle (Bos taurus) |
| Samsun-TR/KX428495_2016 | Turkey | KX428495 | Cattle (Bos taurus) |
Results and discussion

Antigen ELISA test. The homogenized lung samples of the first dead animal were found as BVDV antigen positive using Multiscreen ELISA. BVDV antigens were not detected in the organ sample of second dead animal. The organ samples of both dead animals were BoHV-1, BRSV and BPI-3 antigens negative with Multiscreen ELISA.

Antibody ELISA test. Seropositivity rate for BVDV were found 40% (6 out of 15) with both ELISA kits. No antibody found against BHV-, BRSV, BPIV-3 and BAV-3 using Multiscreen antibody ELISA kit.

RT-PC tests. Figure 1 indicates that 402 bp fragment, indicative for ncp BVDV, was observed by the PCR amplicons that were extracted from the lung and liver samples of first dead animal. No amplicons could be detected from second dead animal. Only 1 of 15 leucocyte samples, taken from diseased heifers, was ncp BVDV RNA positive.

No amplicons were observed by 15 swab samples for BVDV. Likewise, no BDV RNA could be detected in all samples.

Sequencing and phylogenetic analysis. BVDV RNA was detected in the lung and liver sample of the first dead heifer and in one of the leucocyte samples which were collected from the diseased animals. These isolates were named as Samsun TR (lung), Samsun TR1 (liver) and Samsun TR2 (leucocyte). These isolates were sequenced at NS 2-3 region. The sequences of Samsun TR1 and Samsun TR2 bore resemblance to Samsun TR, so they were not submitted to Genbank. The sequence of Samsun TR NS2-3 protein was submitted to Genbank (Accession number: KX428495) and was used for phylogenetic analysis. According to the phylogenetic tree analysis, KX428495 related to BVDV-1 in a cluster contains LC089875 (Japan), AF526381 (China), KP941590 (USA), KR029825 (Egypt), SDQ088995 (Argentina), KC853440 (Switzerland), KC695810 (China) and BVU63479 (Germany). The percentage similarities of

Fig. 1. RT-PCR results of BVDV
Explanations: Lane 1 and 9: 100 bp ladder; Lane 2: lung specimen of 1st dead animal; Lane 3: liver specimen of 1st dead animal; Lane 4: leucocyte sample that was antigen ELISA positive; Lane 5: lung specimen of 2nd dead animal; Lane 6: positive control for cp biotype of BVDV that indicated 680 bp; Lane 7: negative control; Lane 8: positive control for ncp biotype of BVDV that indicated 402 bp

Fig. 2. Phylogenetic comparison of NS 2-3 sequences of BVDV
Explanations: Our ncp BVDV isolate, Samsun-TR (KX428495), was compared with BVDV-1, BVDV-2, BDV and CSFV strains obtained from Europe, Asia, Africa, South and North America. Phylogenetic tree was created by using Neighbor-Joining method (Mega, version 1.02) with the sequences provided from GenBank and subjected to 1000 bootstrap samplings. A genetic distance was indicated by the bar.
nucleotide (nt) identity for KX428495 were determined as 83% for LC089875, AF526381 and KP941590, 82% for KR029825 and DQ088995, and 81% for KC853440, KC695810 and BVU63479.

Turkey is a country with a great number of small scale enterprises where extensive animal breeding is practiced. These enterprises contain between 1 to 20 animals and the rate of these enterprises to total animal breeding enterprises is around 94% (7). In the vast majority of these farms cattle, sheep and goats have been kept together in the same barn as well as grazed in the same pasture. Infections with BVDV that cause multi-systemic disorders may create a huge problem cost-wise for these enterprises.

Historically, several serological and virological studies repeatedly reported the existence of BVDV in Turkey (1, 2, 5, 18, 21). There are four important studies, illegal or uncontrolled animal movement cannot mend measures that include prevention of uncontrolled animal movements and smuggling, strict control of livestock markets, regular screening of herds to identify and eliminate PI animals and establishing a national control and eradication program.

In conclusion, BVDV circulation in Turkey still continues. This situation poses a huge risk for Turkey because the industry of animal-origin products is one of the sectors which play an important role in the country’s development. For that matter, we recommend measures that include prevention of uncontrolled animal movements and smuggling, strict control of livestock markets, regular screening of herds to identify and eliminate PI animals and establishing a national control and eradication program.

References


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