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

ZAFER YAZICI, SEMRA GUMUSOVA, CUNEYT TAMER, ABDURRAHMAN A. CAGIRGAN*,
AYHAN AKMAN*, EMRE OZAN*, HAMZA KADI*, HASAN S. PALANCI, HARUN ALBAYRAK

Department of Virology, Faculty of Veterinary Medicine, University of Ondokuz Mayıs, Samsun, Turkey

*Virology Laboratory, Samsun Veterinary Control Institute, Samsun, Turkey

Received 08.11.2016

Accepted 10.02.2017

Yazici Z., Gumusova S., Tamer C., Cagircan A. A., Akman A., Ozan E., Kadi H., Palanci H. S., Albayrak H.
Molecular identification and phylogenetic analysis of non-cytopathic bovine viral diarrhea virus isolate obtained from a local respiratory outbreak in Northern Turkey

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 syncytial 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 retake serum and blood samples from infected animals 28 days after the first sampling for retesting the presence of virus and/or seroconversion to confirm the diagnosis of PI but none of the animals could be found due to the fact that they had been slaughtered. All animals, including the dead ones, did not have an ear tag which was used for the animal identification system of the Ministry of Food, Agriculture and Livestock of Turkey.

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 $9000 \times \text{rpm}$ 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 $2500 \times \text{rpm}$ for 15 min and separated into sterile tubes. The peripheral blood samples in the EDTA tubes were centrifuged at $2000 \times \text{rpm}$ for 20 min and leucocyte fractions were collected with Pasteur pipettes and resuspended

in 5 ml PBS. Having been centrifuged, 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'-TCGTGGTGAGATCCCTGAG-3'; (rev) 5'-GCAGAGATTTTTTATACTAGCCTATRC-3' for 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 BVDV were provided from the Virology Department at the Faculty of Veterinary Medicine, Ondokuz Mayıs 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, BRSV, 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/FJ527854/2009	China	FJ527854	Cattle (<i>Bos taurus</i>)
Alfort/Tuebingen/J04358/2005	Germany	J04358	Pig (<i>Sus suis</i>)
X818/AF037405/1998	Germany	AF037405	Sheep (<i>Ovis aries</i>)
NADL/AJ133738/2005	USA	AJ133738	Cattle (<i>Bos taurus</i>)
NADL/AJ133739/2005	USA	AJ133739	Cattle (<i>Bos taurus</i>)
USMARC-60780/KT832823/2016	USA	KT832823	Cattle (<i>Bos taurus</i>)
Singer_Arg/DQ088995/2006	Argentina	DQ088995	Cattle (<i>Bos taurus</i>)
Camel-6/KC695810/2014	China	KC695810	Camel (<i>Camelus bactrianus</i>)
ZM-95/AF526381/2006	China	AF526381	Pig (<i>Sus suis</i>)
Egy/Ismailia/KR029825/2014	Egypt	KR029825	Cattle (<i>Bos taurus</i>)
1-CP7/BVU63479/2005	Germany	BVU63479	Cattle (<i>Bos taurus</i>)
IS26/01ncp/LC089875/2016	Japan	LC089875	Cattle (<i>Bos taurus</i>)
SuwaNcp/KC853440/2013	Switzerland	KC853440	Cattle (<i>Bos taurus</i>)
USMARC-55924/KP941590/2015	USA	KP941590	Calf (<i>Bos taurus</i>)
Samsun-TR/KX428495_2016	Turkey	KX428495	Cattle (<i>Bos taurus</i>)

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 on genetic and molecular characterization on the field isolates of BVDV in Turkey revealing the existence of BVDV-1 and BVDV-2 genotypes and subgenotypes using nt sequences and phylogenetic analysis of E2, N^{pro} and 5'UTR coding regions of BVDV genome (10, 11, 19, 20). However, there is no known data on NS2/3 gene sequences of Turkish BVDV strains in Genbank. Generally, the NS3 protein, responsible for the cleavage of NS2/3 gene, is continuously detected upon infection with cp BVDV as well as in the early phase of ncp BVDV infection and is accepted as a marker for the cp biotypes (6, 10). In the current study, the sequence and phylogenetic analysis data of KX428495 (Samsun TR) was compared with other published sequences in Genbank including BVDV, BDV and CSFV strains from Europe, Asia, Africa, South and North America. The results revealed that KX428495 related to BVDV-1 in a cluster contained LC089875, AF526381 and KP941590 with % 83 nt identity (14).

In this study 15 animals which had respiratory tract symptoms were also tested for BVDV, BHV-1, BRSV and BPI-3, but not bacteria. According to the obtained results, BVDV seropositivity was found as 40% whereas antibodies against BHV-1, BRSV, BPI-3 and BAV-3 were not determined. These results have been interpreted that a 40% seropositivity rate can indicate only the animals which infected by BVDV as a viral pathogen. Moreover the presence of ongoing respiratory symptoms can depend on secondary bacterial factors.

There are many factors which allow exposure to different BVDV strains in the country. Illegal or uncontrolled animal movement is one of them. Turkey serves as a land bridge between Europe and Asia and therefore has great geographical importance. It also shares approximately 1850 km of borderland with Iran, Iraq and Syria and animal smuggling or trafficking from these parts poses a risk for Turkey. Despite all punitive sanctions, illegal or uncontrolled animal movement cannot be prevented and ultimately these animals have been

brought to livestock markets to be sold (2, 15, 18). The importation of live animals and biological products, in particular fetal bovine serum (FBS) for *in vitro* studies, is another important route for BVDV to spread (11, 21). A great number of live animals has been carried out by Turkey in recent years and this is an ongoing issue. From this standpoint, the results of the current study may indicate that KX428495, which is involved in the same cluster of BVDV-1 together with Japan and China isolates, may be brought via the importation of live animals or uncontrolled animal movement because the Samsun province is an important port for imported animals to be landed and has also got several livestock markets (14).

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

1. Alkan F., Ozkul A., Bilge-Dagalp S., Yesilbag K., Oğuzoglu T. C., Akca Y., Burgu I.: Virological and serological studies on the role of PI-3 virus, BRSV, BVDV and BHV-1 on respiratory infections of cattle. The detection of etiological agents by direct immunofluorescence technique. Dtsch. Tierarztl. Wschr. 2000, 107, 193-195.
2. Albayrak H., Gumusova S. O., Ozan E., Yazici Z.: molecular detection of pestiviruses in aborted fetuses from provinces in Northern Turkey. Trop. Anim. Health. Prod. 2012, 44, 677-680.
3. Booth R. E., Thomas C. J., El-Attar L. M., Gunn G., Brownlie J.: A phylogenetic analysis of bovine viral diarrhoea virus (BVDV) isolates from six different regions of the UK and links to animal movement data. Vet. Res. 2013, 44, (1):43. Doi: 10.1186/1297-9716-44-43.
4. Fray M. D., Paton D. J., Alenius S.: The effects of bovine viral diarrhoea virus on cattle reproduction in relation to disease control. Anim. Reprod. Sci. 2000, 60-61, 617-625.
5. Gumusova S. O., Yazici Z., Albayrak H.: Pestivirus Seroprevalence in sheep populations from inland and coastal Zones in Turkey. Rev. Med. Vet. 2006, 157, 22-25.
6. Kameyama K. I., Sakoda Y., Matsuno K., Asako I., Tajima M., Nakamura S., Kida H.: Cleavage of the NS2-3 protein in the cells of cattle persistently infected with non-cytopathogenic bovine viral diarrhoea virus. Microbiol. Immunol. 2008, 52, 277-282.
7. Koseman A., Seker I.: Current status of cattle, sheep and goat breeding in Turkey. Van. Vet. J. 2015, 26, 111-117 [in Turkish with English abstract].
8. Passler T., Ditchkoff S. S., Daniel Givens M., Brock K. V., DeYoung R. W., Walz P. H.: Transmission of bovine viral diarrhoea virus among white-tailed deer (*Odocoileus virginianus*). Vet. Res. 2010 Mar-Apr; 41(2): 20:Doi: 10.1051/vetres/2009068
9. Peterhans E., Bachofen C., Stalder H., Schweizer M.: Cytopathic bovine viral diarrhoea viruses (BVDV): emerging pestiviruses doomed to extinction. Vet. Res. 2010, 41, (6):44: Doi:10.1051/Vetres/2010016.
10. Oğuzoglu T. C., Muz D., Yilmaz V., Alkan F., Akca Y., Burgu I.: Molecular characterization of bovine virus diarrhoea viruses species 2 (BVDV-2) from cattle in Turkey. Trop. Anim. Health Prod. 2010, 42, 1175-1180.
11. Oğuzoglu T. C., Muz D., Yilmaz V., Timurkan M. O., Alkan F., Akca Y., Burgu I.: Molecular characteristics of bovine virus diarrhoea virus 1 isolates from Turkey: approaches for an eradication programme. Transbound. Emerg. Dis. 2012, 59, 303-310.
12. OIE: Border disease, [in:] Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2008, chapter 2.7.1. Available: <http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals> Accessed 2011 Dec 25.

13. Ozkul A., Yesilbag K., Burgu I.: Comparison of four diagnostic techniques for detecting bovine virus diarrhoea virus (BVDV) in buffy coat samples after long-term storage. *Turk J. Vet. Anim. Sci.* 2002, 26, 1043-1048.
14. Sato A., Tateishi K., Shinohara M., Naoi Y., Shiokawa M., Aoki H., Ohmori K., Mizutani T., Shirai J., Nagai M.: Complete genome sequencing of bovine viral diarrhoea virus 1, subgenotypes 1n and 1o. *Genome Announc.* 2016, 4 (1): E01744-15. Doi:10.1128/Genomea.01744-15.
15. Tutuncu H., Yazici Z.: Screening for persistently infected cattle with bovine viral diarrhoea virus in small-scale family farms located in Samsun Province, Northern Turkey. *JAPS* 2016, 26, 291-293.
16. Vilcek S., Paton D. J.: A RT-PCR assay for the rapid recognition of border disease virus. *Vet. Res.* 2000, 31, 437-445.
17. Wilson D. J., Baldwin T. J., Kelly J. E., Wettore A. V., Hullinger G., Bunnell J.: Prevalence of bovine viral diarrhoea virus in bovine samples from the intermountain West of the USA: Comparison between age, sex, breed and diagnostic methods. *J. Vet. Sci. Tech.* 2016, 7, 326. Doi:10.4172/2157-7579.1000326.
18. Yazici Z., Serdar M. S., Gumusova S. O., Albayrak H.: Molecular diagnosis and seroepidemiology of pestiviruses in sheep diagnosis and seroepidemiology. *Vet. Arhiv.* 2012, 82, 35-45.
19. Yesilbag K., Forster C., Barbara B. W., Yilmaz Z., Alkan F., Ozkul A., Burgu I., Cedillo R. S., Jurgen T. H., Konig M.: Genetic heterogeneity of bovine viral diarrhoea virus (BVDV) isolates from Turkey: identification of a new subgroup in BVDV-1. *Vet. Microbiol.* 2008, 130, 258-267.
20. Yesilbag K., Forster C., Ozyigit M. O., Alpaz G., Tuncer P., Thiel H. J., Konig M.: Characterisation of bovine viral diarrhoea virus (BVDV) isolates from an outbreak with haemorrhagic enteritis and severe pneumonia. *Vet. Microbiol.* 2014, 21, 169, 42-49. Doi: 10.1016/J.Vetmic.2013.12.005. Epub 2013 Dec 18.
21. Yesilbag K., Gungor B.: Seroprevalence of Bovine Respiratory Viruses in North-Western Turkey. *Trop. Anim. Health Prod.* 2008, 40, 55-60.

Corresponding author: Professor Zafer YAZICI, Department of Virology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, 55139, Samsun, Turkey; e-mail: zyazici@omu.edu.tr