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# Co-existence of bovine viral diarrhea and border disease viruses in a sheep flock suffering from abortus and diarrhea

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**Abstract:** Pestivirus infections have a huge economic impact on livestock production. In 2014, an aborted fetus from a sheep flock suffering from abortus and diarrhea was submitted for virological diagnosis. Due to the positive result of the sample for pestivirus and continuing clinical symptoms in the flock, all of the animals were sampled individually. Blood samples for serum and peripheral blood mononuclear cells were collected from 93 animals (5 rams, 26 lambs, and 62 sheep). During sampling 1 ocular and 4 rectal swab samples were obtained from lambs that had a clinical eye problem and diarrhea, respectively. Additionally 5 aborted fetuses were submitted after the initial sampling. Thirteen of the 93 blood samples tested positive for pestivirus by antigen-detection ELISA. Propagation of noncytopathogenic virus was detected in blood samples from 6 lambs and in 1 aborted fetus sample by using the indirect immunoperoxidase monolayer assay. Pestivirus RNA was detected in 10 of 13 samples by RT-PCR employing pan-pestivirus primers. Border disease virus (BDV) RNA was identified with PBD1/PBD2 specific primers in all 10 samples that tested positive for pan-pestivirus primers. Differentiating RT-PCR further identified BVDV-1 sequences in 3 of the 10 samples. The Sequenced BDV strain (KY-57) was located in the cluster of BDV-7 (Aydin-like) while the BVDV strain was close close to BVDV-1c. The results of this study highlight the possibility of dual infection in sheep with BDV and BVDV-1.

**Key words:** BDV, BVDV-1, coexistence, sheep

## 1. Introduction

Antigenically related bovine viral diarrhea virus (BVDV), border disease virus (BDV), and classical swine fever virus (CSF) are classified in the genus *Pestivirus* of *Flaviviridae* (1). Newly discovered viruses such as HoBi-like virus, Giraffe virus, Pronghorn virus, and Bungowannah virus have been recently added to the genus (2). These viruses have a linear and positive-sense RNA genome, which encodes a single polyprotein that is posttranslationally cleaved into eleven proteins (5'- $N^{pro}$, C, $E^{rns}$, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A, and NS5B-3'). Pestiviruses exist in noncytopathogenic (ncp) and cytopathogenic (cp) biotypes based on their proliferation features in vitro. BVDV has two genotypes, named BVDV-1 and BVDV-2, while there are at least seven clusters (BDV 1–7) described for BDV (3).

Virus transmission can occur by direct contact (oro-nasal route) or by vertical transmission via the placenta. The effect of pestiviruses during gestation depends on the stages of pregnancy and virus strain. Immunocompetence develops at approximately the 125th day of gestation for calves and the 60th day of gestation for sheep and goats. Fetuses infected with an ncp strain before that period can be immunotolerant persistently infected (PI) and become lifelong shedders of live virus. Other fetuses may be born with arthrogryposis–hydranencephaly (AH) syndrome, can survive despite being low birth weight, or can be completely healthy with antibodies against the virus. Superinfection of the PI animals with a cp strain can cause mucosal disease (4).

BVDV infects not only cattle but also sheep, goats, deer, buffaloes, alpacas, and giraffes (5,6). The host range of BDV comprises cattle, pigs, and small ruminants (6). Virus isolation, antigen capture ELISA, and reverse transcriptase polymerase chain reaction (RT-PCR) are the preferred methods for detecting pestivirus infection in domestic and wild cloven-hoofed animals all over the world (5). The presence of pestivirus infection in small ruminant flocks has also previously been reported (7–9). Molecular characterization of Turkish field isolates of BDV has been established (10) and the existence of BVDV-2 in small ruminants has also been demonstrated (11).

The aim of this study was to evaluate the role of pestiviruses in a sheep flock suffering from abortus and diarrhea. We also describe the coexistence of BDV and BVDV-1.

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## 2. Materials and methods

### 2.1. Samples

The owner of a sheep flock in Balıkesir Province, in the South Marmara region of Turkey, requested the virological examination of abortion material in February 2014. The flock consisted of 93 animals with clinical symptoms such as abortus, loss of consciousness, grogginess, diarrhea, and death of newborn lambs within 1–2 days of the emergence of clinical illness. Because the submitted sample was detected positive for pestivirus by RT-PCR and antigen ELISA, the flock was further examined and blood samples (serum and peripheral blood mononuclear cells (PBMCs)) were collected from all 93 animals (5 rams, 26 lambs, and 62 sheep) on the farm. During the sampling, 1 ocular and 4 rectal swab samples were obtained from lambs that had clinical eye problems and diarrhea, respectively. Additionally, 5 aborted fetuses from the flock were submitted after initial sampling. The samples were stored at –20 °C until testing.

The lambs demonstrated no characteristic signs of border disease such as de-pigmentation or congenital disorders. In the neighboring flocks, there were no reported illnesses. The examined flock was closely managed and there were no other ruminants sheltered on the farm either transiently or permanently. The animals had been vaccinated against the sheeppox virus during that season, and no medication was administered during the disease outbreak. There was no record of vaccination against pestiviruses for the flock.

### 2.2. Cell line and viruses

Madin Darby bovine kidney (MDBK) and sheep fetal thymus (SFT) cell lines were used for virus isolation and indirect immunoperoxidase assays. Cells were maintained with Dulbecco's modified essential medium (DMEM; Biological Industries, 11-050-1G) that was supplemented with 10% of heat-inactivated fetal bovine serum (FBS; PAA Cell Culture Company, A11-151). The cell lines and FBS were tested for the absence of pestivirus contamination throughout the study by ELISA and indirect immunoperoxidase monolayer assay (IIPMA).

Reference strains BDVx818, an ncp strain of BDV, and BVDV TR-19, an ncp strain of BVDV, were used as control viruses in IIPMA, while BVDV-NADL was used as positive control in the pan-pestivirus RT-PCR method.

### 2.3. Detection of pestivirus antigens

Fetus blood and swab samples were tested with a commercially available pestivirus antigen ELISA kit (Herdcheck, Switzerland) against the structural protein $E^{rns}$ of pestiviruses for the detection of viral antigens. The test was conducted according to the manufacturer's instructions. There is no commercial ELISA kit specific to BDV and so for confirmatory analysis all the samples were further tested by RT-PCR with BDV specific primers.

### 2.4. Virus isolation and indirect immunoperoxidase monolayer assay (IIPMA)

All of the PBMC, swab, and fetal samples were inoculated in MDBK and SFT cell cultures (100,000 cells/mL). At the 3rd blind passage in cell culture IIPMA was performed as previously described (12).

Briefly, on the 3rd day of the last blind passage, 24-well plates were placed into an incubator and heated to 80 °C for 3 h for fixation. Next, 200 µL of O-D-glucopyranoside (Sigma, 03757) was added to each well to increase the permeability of the cells, and then the cells were incubated at room temperature for 10 min. The primary anti-mouse monoclonal antibody 1/4/7 (kindly supplied by Justus-Liebig University) specific to E2 protein of pestiviruses, which determines both BVDV and BDV strains (13), was diluted 1:40 in Tween-20 W-PBS, added to the wells, and incubated for 90 min at 37 °C. Biotinylated anti-mouse antibody (Pierce, 31800) was added as a secondary antibody and incubated for 90 min under the same conditions. Incubation with peroxidase-labeled streptavidin-biotin complex (Pierce, 21124) was conducted under the same conditions, and the test was terminated after 30 min by adding the substrate (3-amino-9 ethylcarbazole (Sigma, A5754), hydrogen peroxide, and sodium acetate) at room temperature. After each incubation step of the test protocol, the plates were rinsed 3 times with W-PBS. The results were evaluated by checking for reddish-brown intracellular aggregates with an inverted light microscope.

### 2.5. RNA isolation and RT-PCR

Total viral nucleic acid extraction was performed using a commercial kit (Axygen, Canada) for 6 serum, 5 aborted fetal tissue, and 2 rectal swab samples, which all tested positive for the pestivirus antigen by ELISA. cDNA synthesis was done with pan-pestivirus primer, p324 (5'-ATG CCC WTA GTA GGA CTA GCA-3'), using a cDNA synthesis kit (Biomatik, Canada) according to the manufacturer's instructions. For detecting pestivirus in the samples, RT-PCR was performed with primers p324 and p326 (5'-TCA ACT CCA TGT GCC ATG TAC-3') (14) using the following protocol: 35 cycles, denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min to amplify a 288-bp product.

The primers PBD1/PBD2 (5'-TCG TGG TGA GAT CCC TGA G-3'/5'-GCA GAG ATT TTT TAT ACT AGC CTA TRC-3') were used for BDV RNA detection, and the test was conducted using the following protocol: 36 cycles, denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min to amplify a 225-bp product (15).

Finally, RT-PCR was performed for BVDV type 1 RNA detection with primers B3/B4 (5'-GGT AGC AAC AGT GGT GAG-3'/5'-GTA GCA ATA CAG TGG GCC-3') and for BVDV type 2 RNA detection with primers B5/B6 (5'-

ACT AGC GGT AGC AGT GAG-3'/5'-CTA GCG GAA TAG CAG GTC-3') using the following protocol: 35 cycles, denaturation at 94 °C for 1 min, annealing at 51 °C for 1 min, and elongation at 72 °C for 1 min to amplify 221-bp products (16). The PCR products were visualized by ethidium bromide staining after separation by 1% agarose gel electrophoresis.

**2.6. DNA sequence analysis**

One isolate tentatively named KY-57 that was positive for both BDV and BVDV-1 (Sample 2 in Table 1) was selected among the three samples for DNA sequencing. Sequence analysis was performed by a commercial company (Dr. Zeydanli, Ankara, Turkey).

## 3. Results

The entire abortion sample was positive for pestivirus by ELISA and RT-PCR. At the end of the virological examinations applied to samples from all of the flock, a total of 13 (6 serum, 5 fetal tissue, and 2 swab samples) samples tested positive for pestivirus by the antigen ELISA (Table 1). No cytopathogenic effect was observed during the three blind passages of the isolated viruses. After IIPMA, successful virus isolation was performed from 6 out of 93 PBMC samples both in MDBK and SFT cell cultures. On the other hand, only 1 out of 5 fetus samples gave a successful result in virus isolation in the SFT cell line. Amplification of 288-bp products was performed successfully in 10 of 13 samples by pan-pestivirus RT-PCR. For the differential diagnosis of BDV, BVDV-1, and BVDV-2, RT-PCR was performed using these 10 samples. BDV was identified by amplification of 225-bp products in all samples that were also positive by pan-pestivirus RT-PCR. Out of the 10 BDV positive samples, 3 also tested BVDV-1 positive by amplification of 221-bp products (Figure 1).

Results of the nucleotide sequencing from amplicons of BVDV-1 and BDV PCR performed on serum sample No: 2 (Table 1) are submitted in Table 2. In BLAST analysis sequences obtained using BDV RT-PCR had an 82% compliance with BDV/Aydin/04-TR (GenBank: AM418427) while BVDV-1 RT-PCR product had a compliance note of 98% with the strain Kırıkkale-413 previously reported in Turkey (GenBank: KF425302.1). The phylogeny tree demonstrated clear differentiation of BVDV-1 (KY-59) and border disease virus (KY-57) from the same sample (Figure 2).

## 4. Discussion

Pestiviruses can infect cattle, sheep, and goats. In this study, the role of pestiviruses in a sheep flock suffering from abortus and diarrhea was investigated. Both BDV and BVDV-1 were detected in the same flock.

Pestivirus antigens were detected by antigen ELISA in 6 blood serum and 2 swab samples taken from 93 animals

**Table 1.** Virological results from pestivirus antigen-positive animals.

| Samples | Origin | Ag ELISA | Virus isolation-IIPMA* | RT-PCR | | | |
|---|---|---|---|---|---|---|---|
| | | | | Pan-pestivirus | BDV | BVDV-1 | BVDV-2 |
| 1 (serum) | Lamb | + | + | + | + | + | – |
| 2 (serum) | Lamb | + | + | + | + | + | – |
| 3 (serum) | Lamb | + | + | + | + | + | – |
| 4 (serum) | Lamb | + | + | + | + | – | – |
| 5 (serum) | Lamb | + | + | + | + | – | – |
| 6 (serum) | Lamb | + | + | + | + | – | – |
| 7 (fetus) | aborted | + | – | + | + | – | – |
| 8 (fetus) | aborted | + | – | + | + | – | – |
| 9 (fetus) | aborted | + | – | + | + | – | – |
| 10 (fetus) | aborted | + | – | – | – | – | – |
| 11 (fetus) | aborted | + | + | + | + | – | – |
| 12 (rectal swab) | Lamb | + | – | – | – | – | – |
| 13 (rectal swab) | Lamb | + | – | – | – | – | – |
| Total | | 13 | 7 | 10 | 10 | 3 | 0 |

*Virus isolation-IIPMA was applied to the PBMC samples instead of serum samples from the animals.
+: Positive, –: Negative

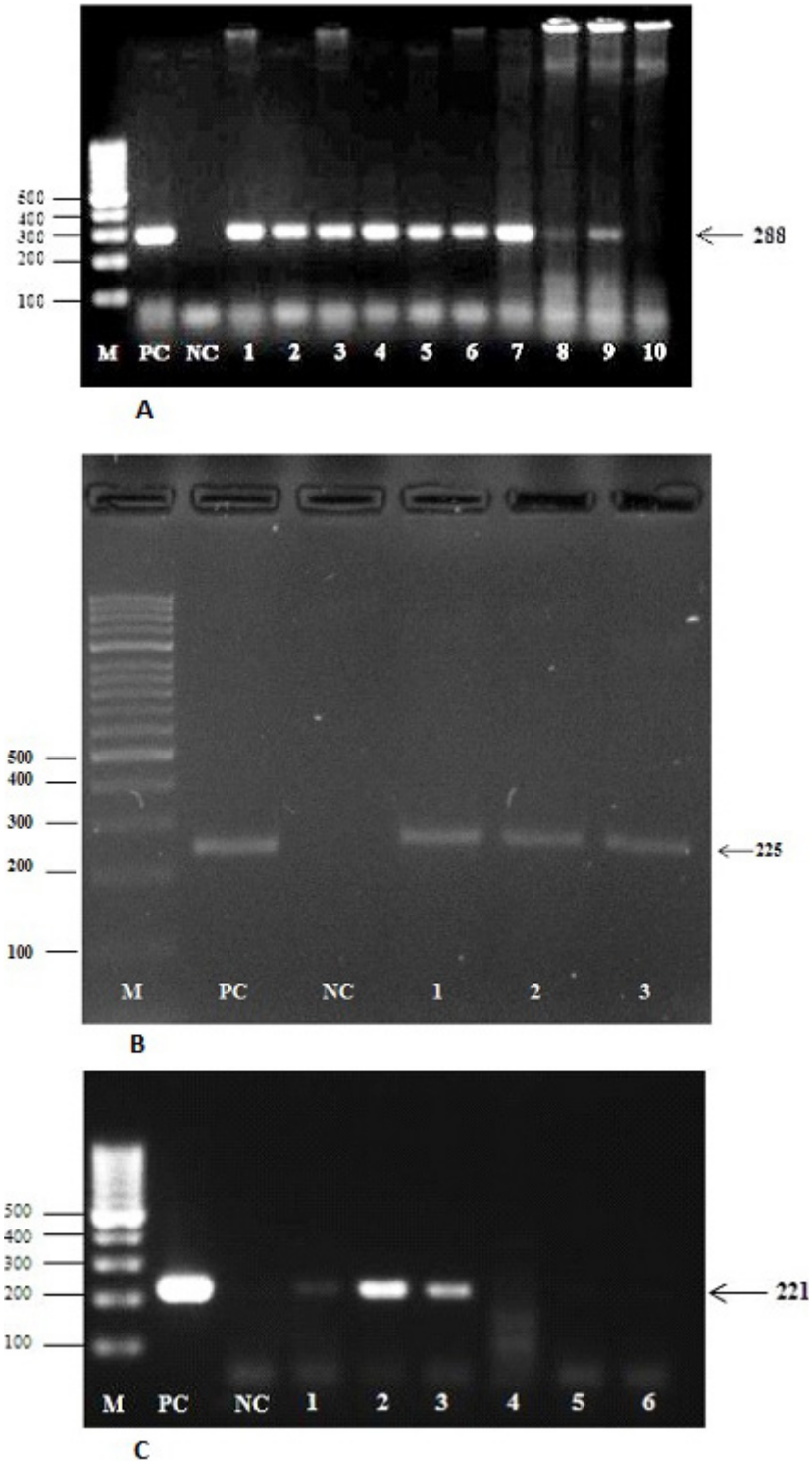


**Figure 1.** PCR products from different RT-PCR protocols A. A 288-bp product from the lamb samples amplified by pan-pestivirus RT-PCR* B. A 225-bp product from the lamb samples amplified by BDV RT-PCR C. A 221-bp product from the lamb samples amplified by BVDV-1 RT-PCR * M: Marker (Genesta 100-bp DNA Ladder — GeneAll), PC: Positive control, NC: Negative control, Lanes 1–6: serum samples, Lanes 7–10: aborted fetus (the order is in accordance with Table 1).

**Table 2.** Sequence analysis of the sample positive for both BDV and BVDV-1.

| Origin of sequenced product | Sequence of sample #2 | Level of similarity |
|---|---|---|
| BDV RT-PCR<br>BDV-KY-57 | CGTCGTCAGTAGTTCAACGCTGGTGGAGCCCAGCCTTGAGATGCTACGTGGACGAGGGTATGCCCTTAGT<br>AGGACTAGCACTGGGTGGTGTCGCCGGTAAATAAACACCCATTTTTCAAGGGTGTGGGGGGGTACAGCCTG<br>ATAGGGTGCTGCACAGGCCCACTATCAAGCTAGTATAAAAAACTCTGCTGTACATGGCACATGGAGTTAAA<br>ACCTTAACCTGGGCGGGGGTCGCCTGGGGGAAAGCACCCATTTTTCCCGGGTGAGGGGGGTACGGTCTGA<br>TAGGGGGCTGCAAAGGCCCTCTATCAA | 82% similarity with<br>BDV/Aydin/04-TR |
| BVDV-1 RT-PCR<br>BVDV-KY-59 | TACAGTCAGGTTAAACTGCTTTTACCTGGGCGACCCCCGCTCAGGTTAAGATGTGCTTTGGGCATGCCCTC<br>GTCCACGTGGCATCTCGAGACCTTTATTCCAAGGCGTCGAACCACTGACGACTACCCTGTACTCAGGGCTT<br>AAGCCATCCAACGAACTCACCACTGTTGCTACCCA | 98% similarity with<br>BVDV -1 isolate Kırıkkale413 5'UTR |

*From Table 1, sample no: 2 was selected for sequence analysis depending on the strong band luminescence for both BDV and BVDV-1 RT-PCR

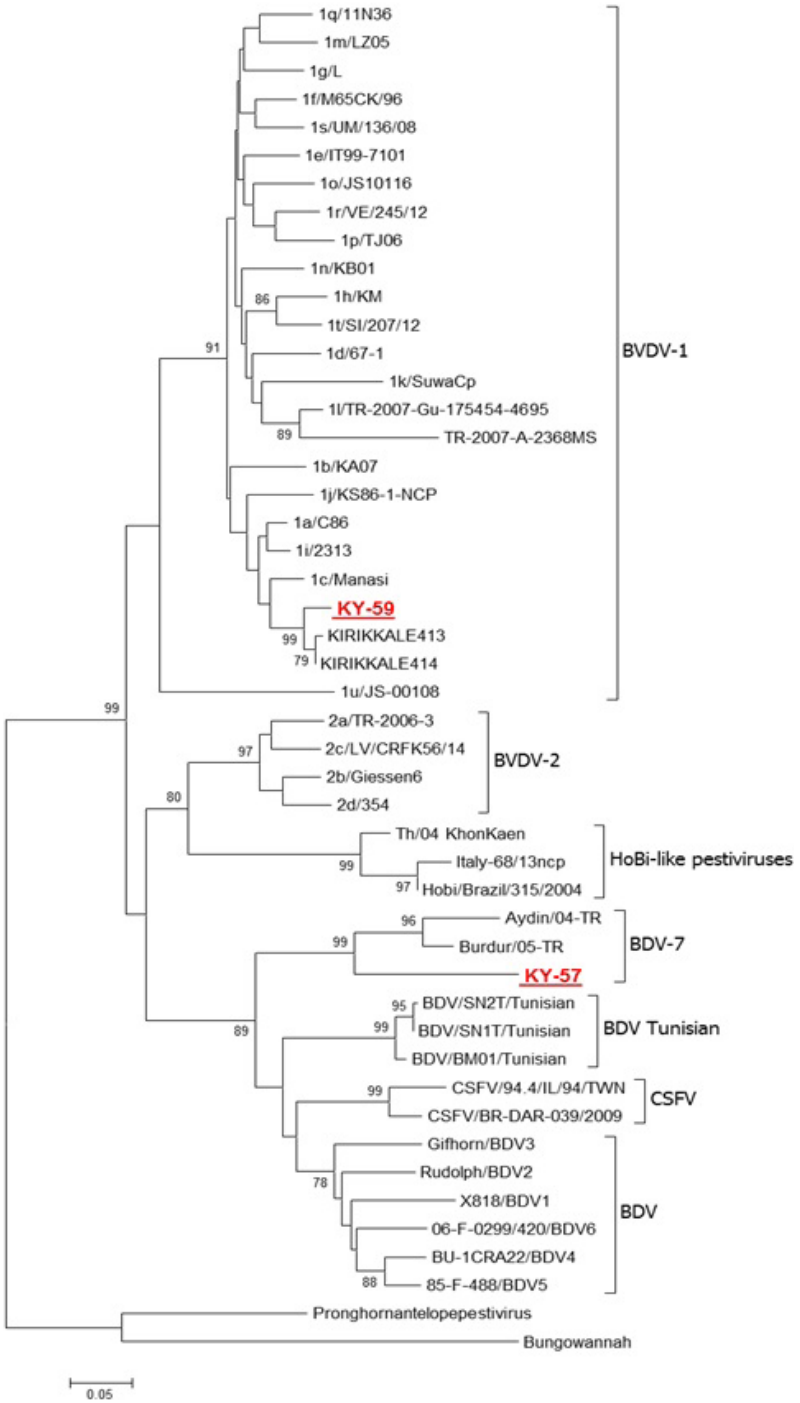


**Figure 2.** Phylogenetic tree is constituted based on the 5'-UTR of pestiviruses. Molecular analyses were performed with MEGA6 using the bootstrap method. Distances were computed using the LogDet (Tamura–Kumar) model. Bootstrap values >70% are shown. The GenBank accession numbers of the strains used for analysis were as follows: C86 (EU180026.1), KA07 (GQ495691.1), Manasi (EU159702.1), 67-1 (KF023366.1), IT99-7101 (AJ318618.1), M65CK/96 (U97456.1), L (AF298069.1), KM (AF298068.1), 2313 (JQ920162.1), TR-2007-Gu-175454-4695 (EU716150.1), TR-2007-A-2368MS (EU716148.1), KS86-1-NCP (AB042713.1), SuwaCp (AF117699.1), LZ05 (GU120241.1), KB01 (GQ495676.1), JS10116 (JN248734.1), TJ06 (GU120246.1), 11N36 (JX437156.1), VE/245/12 (LM994671.1), UM/136/08 (LM994673.1), SI/207/12 (LM994674.1), JS-00108 (KJ578848.1), KIRIKKALE413 (KF425302.1), KIRIKKALE414 (KF425301.1), TR-2006-3 (EU542423.1), Giessen 6 (AY379547.1), LV/CRFK56/14 (KP715134.1), 354 (AF244959.1), X818 (AF037405), Rudolph (AB122086.1), Gifhorn (KF925348.1), BU-1CRA22 (DQ275622), 85-F-488 (EF693985.1), 06-F-0299/420 (EF694002.1), Hobi/Brazil/315/2004 (EF683558.1), Th/04_KhonKaen (DQ897641.1), Italy-68/13ncp (KJ627179.1), BDV/Aydin/04-TR (AM418427), BDV/Burdur/05-TR (AM418428), BM01 (AY453630), SN1T (AF461997.1), SN2T (AF461996.1), CSFV-BR-DAR-039/2009 (KJ197334.1), 94.4/IL/94/TWN (AY646427.1), Bungowannah (DQ901402.1), Pronghorn antelope pestivirus (NC_024018.2).

and in 5 aborted fetuses (Table 1). Common surveys of the pestivirus infection in small ruminants have been reported worldwide in the last few years (9). The presence of small ruminant pestivirus infections in Turkey has already been reported (7,10,17–21). In the present study, virus isolation was performed successfully from the blood samples (n: 6) of lambs whose serum samples tested positive by antigen ELISA; meanwhile another virus isolation was done from a fetus (n: 1) only in the SFT cell line that is also detected positive by antigen ELISA (Table 1). By RT-PCR, pestivirus positivity was confirmed in each of these blood serum samples as well as 4 fetus samples (Figure 1). All of the samples positive for pestivirus RNA were consistent with BDV, while 3 animals were co-infected with BVDV-1. The selected sample (sample 2 in Table 1) was sequenced and the results were compared with the data in GenBank with a BLAST search (Table 2). The isolate was found to be in compliance with BDV/Aydin/04-TR at a rate of 82% and with BVDV-1 isolate Kırıkkale413 5'UTR at a rate of 98% (Table 2). The presence of both viruses in the same animal may be explained by the existence of a persistent infection by one of the viruses followed by an acute superinfection by the other viral species after birth. Unfortunately, there was no opportunity to obtain additional follow-up samples to demonstrate persistent infection in the lambs. The phylogeny tree allowed clear differentiation of BVDV-1 and BDV in sample 2. These mixed strains were tentatively named KY-57 (BDV) and KY-59 (BVDV-1). KY-57 has a 5'UTR sequence very close to that of previously described Turkish BDV strains Burdur/05-TR and Aydin/04-TR comprising the cluster of BDV-7. Both of the previous isolates were from distant locations where KY-57 was isolated, indicating possible endemic distribution of BDV-7 cluster among Turkish sheep populations. KY-59 located in the group of BVDV-1 created a possible new cluster with previous Turkish isolates Kırıkkale413 and Kırıkkale414 very close to subgroup of BVDV-1c.

Circulation of BVDV-1 in a sheep flock is compatible with the results of a study from Argentina (22). BVDV type-1 and -2 infections in small ruminants have also been previously reported (8,23–25).

The commercial ELISA kit (Herdcheck, Switzerland) is validated for detection of animals persistently infected with BVDV and it is speculated that such ELISAs can fail to detect BDV in the field (26). In contrast to that approach, our results show that the ELISA detected pestivirus presence in the 7 samples that were also positive for BDV RNA while negative for BVDV RNA (Table 1). In diagnostic samples, the detection rate of pestivirus with antigen ELISA is generally higher than with other methods. In the present study, of the 13 ELISA-positive samples, there were 3 negative samples by RT-PCR and 6 negative samples by virus isolation-IIPMA. These discrepancies between ELISA, RT-PCR, and virus isolation may be due to the rapid degradation of viral RNA and inactivation of infective viruses, especially in tissue samples.

There was no opportunity to screen other flocks or large animals in the same village. Thus, it was not possible to ascertain the source of the infection for the examined flock. One possible source is pestivirus contamination of the attenuated sheeppox virus vaccine that was given to the flock. However, we were unable to locate the same lot of vaccine for testing. Although all of the vaccine batches produced in Turkey are strictly tested by a government institution, small amounts of pestivirus particles may still lead to infection in susceptible host systems (27).

In conclusion, we have demonstrated a role of pestivirus infections in sheep populations suffering from abortus and diarrhea. Moreover, the possibility of dual infection of border disease virus with bovine viral diarrhea virus has also been presented.

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