Border Disease Virus (BDV) Prevalence And Genetic Typing In Ruminant Flocks In Turkey

Ayhan Akman* and Semra Okur Gumusova

Ondokuz Mayıs Üniversitesi, Samsun, Turkey.

**Corresponding author at: Ondokuz Mayıs Üniversitesi, Samsun, Turkey.*
E-mail: ayhanakman83@hotmail.com.

Veterinaria Italiana 2023, **59** (2), 197-206. doi: 10.12834/VetIt.2693.17780.2
Accepted: 22.06.2022 | Available on line: 30.06.2023

Keywords
Abort,
BDV,
Black Sea Region,
Cattle,
Goat,
Sheep.

Summary
This study aims to update current data regarding Border Disease in sheep and goats, determine the first prevalence of BDV in cattle and identify its circulated genotype in Turkey. For this purpose, 100 sheep, 20 goats and 193 cattle aborted fetuses sent for diagnosis to Samsun Veterinary Control Institute between 2015 and 2017 were analyzed in terms of pestivirus by Ag-ELISA, BDV by Real-Time test (RT-PCR) and Conventional RT-PCR test.
The rate of pestivirus positive animals was found at 50.26% (97/193) in cattle, 58% (58/100) in sheep and 55% (11/20) in goats by the pestivirus Ag-ELISA test. Total of 58 Ag-ELISA positive sheep were tested by Real-Time RT-PCR and conventional RT-PCR tests.
End of the tests, one sheep sample (1.72%) was found BDV positive by Real-Time RT-PCR test and three sheep (5.17%) and one cattle (1.03%) samples were detected as BDV positive by conventional RT-PCR test. BDV positivity was not detected in goats in this research.
All samples that were found positive by conventional RT-PCR test and Real-Time RT-PCR test were genotyped by phylogenetic sequence analysis, and obtained results showed that BDV-3 and BDV-7 genotypes of BDV in sheep and BVDV-1 genotype in cattle circulated in the investigated area.
The sequence analysis results revealed that conventional RT-PCR and Real-Time RT-PCR tests detected genotype BDV-3, while genotype BDV-7 was only detected by conventional RT-PCR test in sheep abortion materials.
Additionally, it was found that one bovine specimen was BDV positive by conventional PCR, but the same sample was identified as BVDV-1 at sequence analysis.
The obtained data of this study showed that new probes should be designed using our local strains for BDV diagnosis by Real-Time RT-PCR assay, and cattle must be sampled for BDV screening, and PCR tests results should always be confirmed by sequence analysis.

Introduction

Border disease virus (BDV) belongs to the *Pestivirus* genus that classified under the *Flaviviridae* family.
The original species would be re-designated as Pestivirus A (original designation, Bovine viral diarrhea virus 1), Pestivirus B (Bovine viral diarrhea virus 2), Pestivirus C (Classical swine fever virus) and Pestivirus D (Border disease virus) (Smith *et al*. 2017). This virus is considered a major cause of congenital infection in sheep and goats (OIE 2017). Several genotypes of BDV from cattle, sheep, goats and Pyrenees goats have been identified. Phylogenetic analysis using nucleotide sequence analysis showed that genetic diversity among BDV is more remarkable than each of the other pestivirus genotypes (Becher *et al*. 2003, Peletto *et al*. 2016, Peterhans *et al*. 2010, Oguzoglu *et al*. 2009, Vilcek *et al*. 2006, Vilcek *et al*. 2014). BDV has been identified in cattle populations alongside its known capability of posing a significant risk for sheep and goat populations. O'Neill *et al*. (2004) in Ireland and Lunden *et al*. (1992) in Sweden reported that

Please refer to the forthcoming article as: Akman *et al.* 2023. Border Disease Virus (BDV) Prevalence And Genetic Typing In Ruminant Flocks In Turkey. Vet Ital. doi: 10.12834/VetIt.2693.17780.2

transmission of pestivirus occurred essentially from cattle to sheep. Recent studies have shown that BDV can also be transmitted to cattle from sheep, causing abortion and persistent infections in pregnant animals and infertility in bulls, and even emphasize the necessity of determining the presence of BDV in cattle for eradication purposes of BVDV (McFadden *et al*. 2012, Elvira-Partida *et al*. 2017). Genotyping and phylogenetic analysis of pestiviruses are widely used in pestivirus grouping. Typing is very important for classifying the original viruses and their evolutionary history. Based on phylogenetic studies of sequences of the N^{pro} and/or 5'UTR (untranslated region) regions from GenBank, BDV isolates are divided into eight phylogenetic groups (Peterhans *et al*. 2010, Strong *et al*. 2010, Giammarioli *et al*. 2011).Phylogenetic studies related to BDV generally classify virus isolates based on the amplification of 5'UTR and N^{pro} regions (Strong *et al*. 2010). Becher *et al*. (1997) have demonstrated that fragments in the 5'UTR region may be too small for a detailed phylogenetic analysis, so the N^{pro} gene is more suitable for investigating genetic associations within the pestivirus genus and the statistical analysis of phylogenetic trees based on partial 5'UTR sequences revealed that clustering within the species and, in some cases, even classification of pestiviruses by species is uncertain. Identification of infected animals and effective genotypes in the field is required to prevent BDV infection, and vaccine development studies should be performed as well (Braun *et al*., 2015). This study aimed to determine the prevalence of BDV in aborted fetuses of cattle, sheep and goats by pestivirus Ag-ELISA, Real-time RT-PCR and conventional RT-PCR tests and to detect the circulating genotype in nine provinces located in the north of Turkey.

Materials and methods

Sampling procedure

A total of 313 (193 cattle, 100 sheep and 20 goats) aborted fetuses were sent to the Samsun Veterinary Control Institute, the responsible referenced regional lab, for diagnosis in nine provinces (Amasya, Giresun, Ordu, Rize, Samsun, Sinop, Sivas, Tokat and Trabzon) of northern Turkey (Table 1, Figure 1). Lung, liver, brain and spleen tissues of the aborted fetuses were sampled under aseptic conditions. The samples were mixed with 2 mL of PBS diluent (1/10) for virological diagnosis, homogenized for 3 minutes at 3000 g using beads in MagNa Lyser (Roche, Mannheim, Germany), and supernatants were stored in Eppendorf tubes at -20 ° C until use. The animal protocols used in this work were evaluated and approved by the Samsun Veterinary Control Institute Ethics Committee (Protocol:.2016/5/5).

Table I. *Species of abortion specimens and their locations.*

Province of Sample	Species		
	Cattle	Sheep	Goat
Amasya	10	11	-
Giresun	2	9	1
Ordu	7	2	-
Rize	1	-	-
Samsun	91	40	7
Sinop	3	5	-
Sivas	54	5	4
Tokat	17	24	8
Trabzon	8	4	-
TOTAL	**193**	**100**	**20**

Figure 1. *Display of cities where the samples collected on Turkey map in this study.*

Ag-ELISA Assay

All tissue samples were tested for pestivirus antigen detection by Ag-ELISA Assay Test, which was performed according to the manufacturer's instructions using the pestivirus Ag-ELISA kit (IDEXX, 06-43860-12 Westbrook, USA).

Real-Time RT-PCR Application

Real-Time RT-PCR application was made to detect BDV nucleic acids in the pestivirus Ag-ELISA positive samples. One-step RT-PCR kit (Qiagen, OneStep RT-PCR KIT, Cat No: 210212, Hilden, Germany) and BDV primer and probe (Table II) were used and the test was carried out according to previously reported by Willoughby *et al*.(2006).

Conventional RT-PCR Application

For the conventional RT-PCR product, the reaction concentration was prepared by using a one-step RT-PCR kit (Qiagen, OneStep RT-PCR KİT, Cat No: 210212, Hilden, Germany) with panpesti primers (324/326) and BDV specific primers (PBD1 / PBD2 primers) (Table II) and the test was carried out according to previously reported (Vilcek *et al*. 2000). Agarose gel was used to visualize the products obtained by conventional PCR, and the products were examined under UV.

Table II. *Primary and probe sequences, target regions and sizes used in RT-PCR tests.*

Primer and Probe	Index(5'-----3')	Target region	Size (bp)	Reference
BDV87F	CCGTGTTAACCATACACGTAGTAGGA	5' UTR	155	[17]
BDV237	GCCCTCGTCCACGTAGCA			
BDV136T (probe)	FAM-CTCAGGGATCTCACCACGA-TAMRA			
324	ATGCCCWTAGTAGGACTAGCA	5' UTR	288	[18]
326	TCAACTCCATGTGCCATGTAC			
PBD1	TCGTGGTGAGATCCCTGAG	5' UTR	225	[19]
PBD2	GCAGAGATTTTTTATACTAGCCTATRC			

Genotyping Determination of Obtained Strains

Positive BDV samples were sent to sequence analysis of 738 bp length sequence using BD1 and BD2 primers targeting the N^{pro} region (Vilcek *et al*. 1996).

Data Analysis

The N^{pro} gene region sequences amplified in the study and obtained DNA sequence analysis were edited with the BioEdit program and compared with other sequences in GenBank (Hall 1999). The sequences which were determined to be similar and obtained in the study were used in phylogenetic analysis. Sequences belonging to different BDV and BVD groups genotypes were also used for phylogenetic analysis.

Cluster analysis of the ClustalW program sequences in the BioEdit program was used. The data obtained were processed by neighbor-joining (NJ) analysis with the help of the MEGA 6.0 phylogenetic program (Tamura *et al*., 2011).

Alignment gaps were evaluated as missing data. The reliability of the generated dendrograms was tested for 1000 replications using bootstrap analysis using the MEGA 6.0 program.

Results

Pestivirus Ag-ELISA Results

As a result of Ag-ELISA, 97 (50.26%) of 193 cattle, 58 (58.0%) of 100 sheep, and 11 (55.0%) of 20 goats aborted materials were found to be positive. The distribution of the results by provinces and animal species is shown in Table III.

BDV Real-Time RT-PCR Results

Real-Time RT-PCR with specific BDV primers and probes (Table II) revealed that one aborted sheep material of Trabzon/Tonya was positive (1.72%) for BDV nucleic acid (Table IV).

Table IV. *The RT-PCR positive result of Ag ELISA positive samples.*

Species	Province	Sample Number	BDV	%
Sheep	TRABZON/ Tonya	44	Pozitif	1.72 (58/1)

Table III. *Pestivirus Ag-ELISA results by provinces.*

Province	Number of Materials			(+) Number of Materials			Prevalence (%)		
	Cattle	Sheep	Goat	Cattle	Sheep	Goat	Cattle	Sheep	Goat
Amasya	10	11	-	5	7	-	50	63,64	-
Giresun	2	9	1	2	7	0	100	77,78	0
Ordu	7	2	-	3	1	-	42,86	50	-
Rize	1	-	-	1	-	-	100	-	-
Samsun	91	40	7	40	21	4	43,96	52,50	57,14
Sinop	3	5	-	3	5	-	100	100	-
Sivas	54	5	4	31	1	3	57,41	20	75
Tokat	17	24	8	7	12	4	41,18	50	50
Trabzon	8	4	-	5	4	-	62,50	100	-
TOTAL	193	100	20	97	58	11	50.26	58.0	55.0

BDV Conventional RT-PCR Results

According to conventional RT-PCR, BDV nucleic acid was detected positive in three sheep (5.17%) and one cattle (1.03%) aborted materials with panpesti primers and BDV specific primers (Table V). Both Real-Time RT-PCR and conventional RT-PCR tests were found positive in one sample from Trabzon/ Tonya. DNA sequences were obtained using BD1 and BD2 primers from four samples with a positive N^{pro} gene region. Then they were compared with other sequences present in the NCBI Blast program of GenBank to determine percent similarities (Table VI).

Table V. *The Conventional RT-PCR results of Ag ELISA positive samples.*

Species	Province	Sample Number	BDV	PREVALANCE (%)
Sheep	TRABZON/ Tonya	44	Pozitif	
Sheep	SİNOP/ Merkez	78	Pozitif	**5.17 (58/3)**
Sheep	TOKAT/ Almus	186	Pozitif	
Cattle	AMASYA/ Merzifon	293	Pozitif	**1.03 (97/1)**

Phylogenetic Tree

In this study, phylogenetic similarities were determined to be closely related by sequence analysis in the positive samples. According to the obtained dendrogram, BDV positive three sheep abortion materials from Trabzon/Tonya, Sinop and Tokat/ Almus were detected as BDV. In addition, when the dendogram was examined, the virus obtained from Amasya/Merzifon in cattle aborted material was identified as BVDV (Table VI). The phylogenetic analysis of the N^{pro} region showed that two sheep abortion materials of Sinop and Tokat/Almus were possessed a nucleotide identity of 80.80-79.13 % and 80.75-79.74 % of grouped with Pestivirus Burdur/05-TR and Pestivirus Aydin/04-TR strains as BDV-7 genotypes isolated by Oguzoglu *et al*. (2009). On the other hand, sheep abortion material of Trabzon/Tonya shared 80.52-76.87 % nucleotide identity with other BDV-3 strains. Suquencing results of one cattle sample was closely grouped with AY323890, AY323888 and AY323886, BVDV-1 strains, as it shares a 90.10-89,90 % nucleotide identity (Figure 2, Table VI).

Table VI. *Percent similarity of the samples with N^{pro} region replication to other samples in GenBank.*

Sample No	N^{pro} gene region similar strain	Gen Bank No	Coverage (%)	Similarity (%)
44	Border disease virus strain JS12/04	KC537789	100	80,52
	Border disease virus strain AH12-01	JQ946320	100	79,45
	Border disease virus strain JSLS12-01	KC963426	100	76,87
78	Pestivirus Burdur/05-TR	KM408491	99	80,80
	Pestivirus strain Aydin/04-TR	JX428945	99	78,95
	Border disease virus BDV/Burdur/05-TR	EU930015	95	79,13
186	Pestivirus Burdur/05-TR	KM408491	100	80,75
	Pestivirus strain Aydin/04-TR	JX428945	100	80,26
	Border disease virus BDV/Burdur/05-TR	EU930015	99	79,74
293	Bovine viral diarrhea virus 1 isolate H-645/97	AY323888	100	90,10
	Bovine viral diarrhea virus 1 isolate M-573/99-15	AY323890	100	89,90
	ovine viral diarrhea virus 1 isolate V-804/98	AY323886	100	89,90

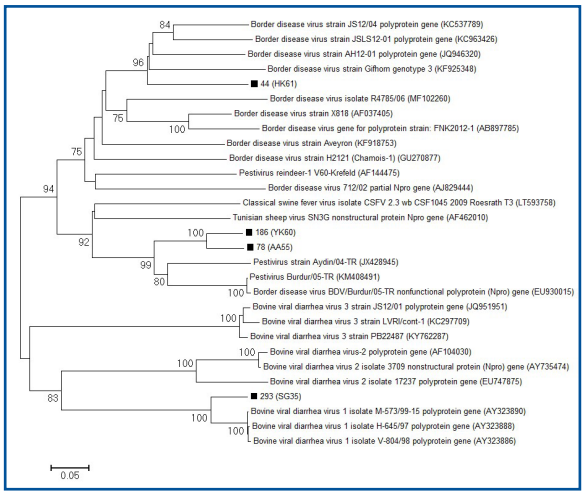

Figure 2. *Phylogenetic tree of samples. The dendogram was made by the neighbor-joining (NJ) method using the MEGA 6.0 phylogenetic program (Tamura* et al., *2011). The numbers next to the nodes indicate bootstrap values. The scale below the figure shows the degree of similarity. CSFV: Rosrath (LT593758); BDV-1: X818 (AF037405), FNK2012-1 (AB897785); BDV-2: V60 (AF144475); BDV-3: JS12 / 04 (KC537789), JS12-01 (KC963426), AH12-01 (JQ946320), Gifhorn (KF925348); BDV-4: H2121 (GU270877); BDV-5: Aveyron (KF918753); BDV-7: Aydin / 04-TR (JX428945), Burdur / 05-TR (KM408491), BDV / Burdur / 05-TR (EU930015); BDV-8: R4785 / 06 (MF102260); BDV-Italy: 712/02 (AJ829444); BDV-Tunisia: SN3G (AF462010); BVDV-1: M-573 / 99-15 (AY323890), H-645/97 (AY323888), V-804/98 (AY323886); BVDV-2: Giessen-1 (AF104030), 3709 (AY735474), 17237 (EU747875); BVDV-3: JS12 / 01 (JQ951951), LVRI / cont-1 (KC297709), PB22487 (KY762287). The Npro gene region sequence for BDV-6 could not be reached. In this study, Genbank accession numbers of viruses obtained from samples 44 (HK61), 78 (AA55), 186 (YK60), 293 (SG35), respectively: MN102085, MN102086, MN102087, MN102088.*

Discussion

Pestivirus prevelances of sheep, cattle and goat have been determined as 0.9-100% in Turkey and 5-88% in many areas of the World by antigen ELISA and RT-PCR tests (Albayrak *et al*. 2012, Ataseven *et al*. 2006, Burgu *et al*. 1987, Burgu *et al*. 2001, Çokçalışkan 2000, Garcia-Perez *et al*. 2010, Giangaspero *et al*. 2011, Gür 2009, Hasircioglu *et al*. 2009, Li *et al*. 2013b, Nettleton *et al*. 2010, Okur Gumusova *et al*. 2006, Silveira *et al*. 2018, Yazıcı *et al*. 2012). In the present study, pestivirus antigen prevalences were found at 50.26% in cattle, 58.0% in sheep and 55.0% in goats by pestivirus antigen ELISA test and 1.72% in sheep with RT-PCR test and 5.17% in sheep and 1.03% in cattle were detected as BDV positive by conventional RT-PCR from ruminant flocks in nine different provinces of Turkey.

Garcia-Perez *et al*. (2009) were to investigate the presence of pestivirus in 67 aborted fetus samples with 41.4% positivity with the Ag-ELISA test and 7.9% with the RT-PCR test. The authors emphasized the fact that any technique will be used to confirm the presence of pestivirus in a flock should be selected according to the type of samples available and that Ag-ELISA may be an alternative for fetuses or stillbirths with a certain degree of autolysis that may affect the stability of viral RNA. Göktuna *et al*. (2017) investigated Pestivirus and BDV prevalance by RT-PCR with specific BDV primers. Results were negative in one of the five Ag ELISA positive samples in sheep abortion materials in Turkey. In this study, we found pestivirus positivity at 50.26% with Ag ELISA and BDV positivity at 1.72% with RT-PCR test and 5.17% with conventional RT-PCR in sheep. Pestivirus and BDV positivity of cattle were found at 50.26% with Ag ELISA, 1.03% with conventional RT-PCR, respectively.

In different countries, the prevalences of BDV infection in sheep were determined between 5-50% by RT-PCR (Becher *et al*. 2003, Becher *et al*. 1994, Dubois *et al*. 2008, Kramette Froetscher *et al*. 2007, Sullivan *et al*. 1997, Vilcek *et al*. 1997, Vilcek *et al*. 1998).

In Turkey, Oguzoglu *et al*. (2009) reported 1.27% BDV prevalance in sheep in the Burdur region by RT-PCR. But BDV was not detected in goat abortion materials in this study. Yazıcı *et al*. (2012) found a 3% BDV prevalence in sheep, but no positivity was found in goat aborted fetus material in the Black Sea Region in Turkey by RT-PCR. In this study, sheep were detected positive for BDV at 1.72% with RT-PCR test, 5.17% with conventional RT-PCR test and goats were found BDV negative. According to these results, our findings were similar to previous studies in Turkey.

BDV in cattle has been identified in Austria and one of the isolates was identified as BDV-3 in sequence analysis from N^{pro} and 5'UTR regions (Hornberg *et al*., 2009). Gomez Romero *et al*. (2018) detected BDV-1 genotype with sequence analysis targeting the 5'UTR region in cattle samples. In this study, one cattle abortion material was detected as positive by conventional RT-PCR test with BDV specific PBD2 and PBD1 primers. According to the sequencing results of cattle abortion material identified from Amasya/Merzifon, the BVDV-1 was its affiliated genotype with a similarity index between 90.10-89,90 %. Our cattle sample was found to be very closely related (90%) to H-645/97 strain isolated from cattle herd in a study done by Toplak *et al*. (2004) in Slovenia; In addition, our isolate was similar (89.27%) to the TR28NEU isolate obtained from cattle (Yesilbag *et al*. 2008).

BDV field isolates have been genotyped in many countries. In particular, BDV-1 was isolated from sheep in the USA, England, Australia and New Zealand (Becher *et al*. 1994, Sullivan *et al*. 1997, Vilcek *et al*. 1997, Vilcek *et al*. 1998). BDV-2, BDV-3 and BDV-4 were isolated in Europe and China, whereas BDV-5 and BDV-6 were isolated in France (Becher *et al*. 2003, Dubois at al. 2008Krametter Froetscher *et al*. 2007, Li *et al*. 2013a, Li *et al*. 2013b). Additionally, Pestivirus isolated from sheep and

goats in Italy has been reported to be BDV-7 (Peletto *et al*. 2016, Giammarioli *et al*. 2015); similarly, BDV-7 was isolated from Turkey (Oguzoglu *et al*. 2009). In contrast, BDV-8 was isolated in Switzerland and Italy (Peterhans *et al*., 2010, Peletto *et al*., 2016). In our country, Oguzoglu *et al*. (2009) conducted the molecular characterization of BDV in sheep and goats in a study they have conducted for the first time in Turkey. They determined the isolates detected by RT-PCR in the provinces of Burdur and Aydın to be BDV-7 genotypes. BDV / Burdur / 05 / cp strain was defined as a result of sequence analysis targeting 5'UTR and N^{pro} gene regions. In the same study, BDV / Aydın / 04 / ncp strain was determined using the same gene regions. The researchers evaluated both strains as a new genotype (BDV-7). Azkur *et al*. (2011) reported that the virus was closely related to BDV-3 genotyping isolated from sheep. Toplu *et al*. (2012) also identified the BDV-3 genotype in small ruminants in Turkey. At the end of our study, molecular characterization of N^{pro} gene regions detected BDV-3 and BDV-7 genotypes similar to previous studies in Turkey. According to our results, one sheep abortion material (Trabzon / Tonya) was identified as BDV-3 with 80.52-76.87% similarity to the JS12 / 04 and AH12-01 strains isolated from a goat herd in China in 2013 (Li *et al*. 2013b), one sheep abortion material (Sinop/ Center) was identified as BDV-7 with similarity between 80.80-79.13% to Burdur / 05-TR and Aydin / 04-TR Pestivirus strains that were previously isolated (Oguzoglu *et al*. 2009) and sheep abortion material of Tokat / Almus were identified as BDV-7 genotype by sequence analysis. This genotype was closely related to Pestivirus Burdur / 05-TR and Pestivirus Aydin / 04-TR strains that were isolated by Oguzoglu *et al*. (2009), and it shares a similarity between 80.75-79.74 % with these strains. The presence of the N^{pro} region in both BDV and BVDV is closely related, it is essential to identify and perform more specific primer and probe designs in PCR studies. In addition, sequencing of the N^{pro} region of BDV isolates obtained from cattle in Genbank is limited. Positive samples from RT-PCR should be confirmed by sequence analysis because both the presence of BVDV in sheep and BDV in cattle can be determined by sequence analysis, which will be used for the exact differentiation of both viruses.

Conclusion

BDV causes a persistent infection that lower survival chances in infected sheep and goats and the importance of BDV has been increased for animal breeding in our country and globally. This study showed by Ag-ELISA test that 50.26% of cattle abortions, 58.0% of sheep abortions, and 55.0% of goat abortions were caused by a pestivirus in investigated area. When conventional PCR with pestivirus primer was applied to pestivirus Ag-ELISA positive samples, the results were shown that 1.03% of bovine abortions and 5.17% of sheep abortions were positive for BDV. Otherwise, RT-PCR with BDV probes and conventional PCR tests with specific BDV primers were applied to pestivirus Ag-ELISA positive samples. One bovine and three sheep abortions materials were found to be BDV positive, while in the sequence analysis, BDV was not detected in bovine abortion material. This result revealed that RT-PCR and conventional PCR tests that used specific BDV primer and probes are not always able to differentiate BDV from BVDV in cattle abortion materials, and sequence analysis is also important to confirm positive samples.

Another crucial data from this study was that the BDV-3 genotype was determined by conventional PCR and Real Time RT-PCR, while the BDV-7 genotype was determined by only conventional PCR application. Similarly, the BVDV-1 genotype was determined by only the conventional PCR technique. These data revealed that the used real-time PCR primers and probes were insufficient to detect the BDV-7 genotype; therefore, a new Real-Time RT-PCR panel designed according to the nucleotide sequences of our native isolates is necessary for accurate BDV diagnosis. In addition, it should be remembered that the BDV positivity detected in any studies must always be confirmed by sequence analysis; thus, integral data will be obtained to determine the circulating genotype.

As a result, a comprehensive eradication program is needed to combat BDV. Additionally, we suggest that more comprehensive studies about BDV infection be implemented in cattle.

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