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RESEARCH ARTICLE

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The Role of Pestiviruses (BDV and BVDV) in Ruminant Abortion Cases in the Afyonkarahisar Province

Murat ŞEVİK[1]*

[1]Department of Virology, Veterinary Faculty, Hatay Mustafa Kemal University, Alahan, 31001, Hatay, Turkey.

*Corresponding author e-mail: dr_muratank@hotmail.com

ABSTRACT

Pestiviruses are important viral agents that can cause abortion in ruminants. In this study, roles of Border Disease Virus (BDV) and Bovine Viral Diarrhoea Virus (BVDV) were investigated in ruminant abortion cases. Aborted foetal tissue samples were collected from 101 animals (74 sheep foetuses and 27 bovine foetuses), each from epidemiologically different farms, during the months of January 2016 and December 2017 in the Afyonkarahisar Province. One step real-time duplex RT-PCR was used for the detection of BDV and BVDV RNA. Genetic characterization of the field isolates of pestiviruses was conducted by sequencing 5' untranslated region (5' UTR). BDV RNA was detected in 9 (12.16%) of the 74 aborted sheep foetuses, whereas BVDV RNA was detected in 6 (22.2%) of the 27 bovine foetuses. Phylogenetic analysis based on the 5' UTR region indicated that BDV isolates in the present study belong to BDV-7 genotype whereas BVDV isolates belong to BVDV-1 genotype. The results of this study showed that pestivirus infections play important role in ruminant abortion cases in Afyonkarahisar province.
Keywords: Border disease virus, bovine viral diarrhoea virus, abortion, sheep, cattle

Afyonkarahisar İlinde Ruminant Abort Vakalarında Pestivirusların (BDV ve BVDV) Rolleri

ÖZ

Pestiviruslar ruminantlarda abortlara neden olan önemli viral ajanlardır. Bu çalışmada, ruminant abort vakalarında Border Disease Virus (BDV) ve Bovine Viral Diarrhoea Virus (BVDV)'un rolleri araştırılmıştır. Abort olmuş fötus doku örnekleri 101 hayvandan (74'ü koyun fötusu, 27'si sığır fötus), her biri epidemiyolojik olarak farklı çiftliklerden, Ocak 2016 ve Aralık 2017 ayları arasında Afyonkarahisar ilinden toplanmıştır. BDV ve BVDV RNA'sının tespiti için tek adımlı real-time dubleks RT-PCR yöntemi kullanılmıştır. Sahadan izole edilen pestivirus'ların genetik karakterizasyonu 5' translate olmayan bölge sonunun (5' UTR) sekansı ile gerçekleştirilmiştir. BDV RNA'sı, 74 aborte koyun fötusunun 9 (%12.16)'unda, BVDV RNA'sı ise 27 sığır fötusunun 6 (%22.2)'sında tespit edilmiştir. 5' UTR bölgesinin filogenetik analizi bu çalışmada izole edilen BDV izolatlarının BDV-7 genotipine, BVDV izolatlarının ise BVDV-1 genotipine ait olduğunu göstermiştir. Bu çalışmanın sonuçları, pestivirus enfeksiyonlarının, Afyonkarahisar ilindeki ruminant abort vakalarında önemli rol oynadığını göstermektedir.
Anahtar Kelimler: Border disease virus, bovine viral diarrhoea virus, abort, koyun, sığır

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INTRODUCTION

Border Disease Virus (BDV), Bovine Viral Diarrhoea Virus 1 (BVDV-1) and Bovine Viral Diarrhoea Virus 2 (BVDV-2) belong to the *Pestivirus* genus of the *Flaviviridae* family, together with Classical Swine Fever Virus (CSFV). Pestiviruses are enveloped, single-stranded, positive-sense RNA viruses genome of 12.5 kb in length. Based on the genetic analysis, BDV isolates have been segregated into seven clusters (BDV-1 to BDV-7) whereas BVDV has two genotypes: BVDV-1 and BVDV-2 (Simmonds et al. 2012). Pestivirus infections have been associated with abortions, mummified foetuses, infertility, diarrhoea, respiratory disease and persistent infection (PI) of the offspring (Nettleton et al. 1998; Munoz-Zanzi et al. 2004).

It has been reported that pestiviruses are not host specific. Both BDV and BVDV can infect sheep, goat, cattle and swine (Nettleton et al. 1998; Passler and Walz 2010). Main route of transmission of pestiviruses is horizontal via transiently infected and PI animals. Furthermore, vertical transmission occurs in all host species (Van Campen and Frolich 2001).

Pestivirus infection has a worldwide distribution. Previous studies of abortion cases in ruminants in different regions of Turkey identified pestiviruses as the cause of abortion (Hasircioglu et al. 2009; Azkur et al. 2011; Avci et al. 2013; Berber and Sozdutmaz 2013; Tuncer-Goktuna et al. 2016; Ural and Erol 2017; Bulut et al. 2018). Small ruminants and cattle are important livestock in Afyonkarahisar province. Abortion in ewes and heifers causes serious economic losses in the livestock industry. Therefore, the aim of the present study was to investigate the role of BDV and BVDV in abortion cases of ruminants in the Afyonkarahisar Province.

MATERIAL and METHOD

Sample collection

During January 2016 and December 2017, foetal tissue samples (lung, liver, spleen, kidney and brain) were collected from 74 aborted sheep foetuses and 27 aborted bovine foetuses from flocks and herds where abortion cases occurred in the Afyonkarahisar province. Details of the sampled flocks and herds given in Table 1. Farmers reported that animals were not vaccinated against pestivirus infection in sampled flocks and herds.

RNA extraction and one step real-time duplex RT-PCR

Foetal tissue samples were homogenised in PBS using the TissueRuptor (Qiagen, Hilden, Germany). Viral RNA extraction was carried out from tissue homogenates using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. One step real-time duplex RT-PCR was performed with primers and probes that targeting 92 bp and 103 bp conserved regions of the 5'-UTR of BDV and BVDV, respectively (Table 2). The protocol described by La Rocca and Sandvik (2009) was used for detection of pestiviruses. One step real-time duplex RT-PCR reaction was carried out with one step RT-PCR kit (Cat No. 210212, Qiagen, Hilden, Germany) in a final volume of 25 µl reaction mix which contained 5 µl 5 x RT-PCR buffer, 200 µM of each dNTP, 1 µL enzyme mix, 0.4 µM of forward primer, 0.6 µM of reverse primers, 0.5 µM of each probes and 2.5 µL of sample RNA. Amplification was performed using LightCycler 2.0 real time PCR machine (Roche Applied Science, Indianapolis, IN, USA) with the following conditions: reverse transcription step of 10 min at 50 °C and 5 min at 95 °C, followed by 45 cycles at 95 °C for 15 s and 60 °C for 30 s. The samples that had a Ct value <35 were considered positive.

One-step RT-PCR amplification and sequencing of 5' UTR region

Samples that were positive by real-time duplex RT-PCR were subjected to one-step RT-PCR amplification using primers 324 and 326 which amplify a 288 bp region of the 5' UTR region (Vilcek et al. 1994). The protocol described by Vilcek et al. (1994) was used for detection of pestiviruses. RT-PCR reaction was carried out with one step RT-PCR kit (Cat No. 210212, Qiagen, Hilden, Germany) in a final volume of 25 µl reaction mix which contained 5 µl 5 x RT-PCR buffer, 400 µM of each dNTP, 1 µL enzyme mix, 1 µM each primer, and 2.5 µL of sample RNA. Amplification was performed using MJ Research thermal cycler with the following conditions: reverse transcription step of 30 min at 50 °C and 15 min at 95 °C, followed by 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s and final extension step in 72ºC for 5 min. The PCR products were analysed on 1.5% agarose gel stained with Gelred (Biotium, USA) after electrophoresis at 90 V for 60 min (Fig. 2). Amplified PCR products were sequenced both the forward and reverse directions on the ABI 3500XL DNA Analyser (Applied Biosystems, USA) with the

BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) by Intron Saglik Urunleri (İzmir, Turkey). Primers 324 and 326 were used in sequence analysis. Phylogenetic tree was constructed, via the neighbour-joining method using MEGA software version 6, for the 5' UTR region of pestiviruses with additional sequences from GenBank. Kimura two-parameter model was used to describe the evolutionary distances between sequences.

Nucleotide sequence accession numbers

The 5' UTR region sequences reported in this paper are available in the GenBank under accession numbers MH395751 to MH395754.

Statistical analysis

The difference in the detected rate of BDV and BVDV was compared with Fisher's exact test. $P<0.05$ was considered to be statistically significant.

RESULTS

Detection of BDV and BVDV RNA by one step real-time duplex RT-PCR

BDV RNA was detected in 9 of the 74 aborted sheep foetuses whereas BVDV RNA was detected in 6 of the 27 aborted bovine foetuses (Table 1). Positive samples had Ct values between 20.17 and 34.06 (Fig. 1). There was no significant difference between the detected rate of BDV and BVDV ($P = 0.2193$). Furthermore, no significant differences were found between the districts where pestiviruses were detected ($P = 0.5294$).

Table 1. Districts where samples were collected
Tablo 1. Örneklerin toplandığı ilçeler

Districts	No. of examined flocks	No. of positive flocks	No. of examined herds	No. of positive herds
City Center	8	1	3	1
Çay	7	2	2	1
Çobanlar	5	-	3	1
Dazkırı	8	1	4	-
Dinar	7	-	3	1
Emirdağ	12	2	6	1
Hocalar	6	-	2	-
İhsaniye	7	-	1	-
Sinanpaşa	4	1	2	-
Sultandağı	10	2	1	1
Total	**74**	**9**	**27**	**6**

Table 2. Details of the primers and probes used for detecting pestiviruses by one step real-time duplex RT-PCR.
Tablo 2. Pestivirusların one step real-time dubleks RT-PCR ile saptanmasında kullanılan primerler ve problar

Primers and Probes	Sequence (5' - 3')	Target pestiviruses	Reference
106-F	CCATRCCCDTAGTAGGACTAGC	BDV-BVDV	La Rocca and Sandvik (2009)
190-R	GYGTCGAACCAYTGACGACT	BVDV	
179-R	GYGTYGAACTACTGACGACT	BDV	
Probe-162	FAM-TGGATGGCYKAABCCCTGAGTACAG-EDQ	BVDV	
Probe-128	YY-ACTAGCYDTCGTGGTGAGATCCCTG-EDQ	BDV	

Sequence and phylogenetic analyses of the 5' UTR region

Nucleotide sequences were obtained for two BDV and two BVDV field isolates. The analysis of the 5' UTR region sequences revealed that the homology between two BDV field isolates was 82.7% whereas the similarity with previously characterized BDV isolates ranged from 60.5% to 87%. The highest nucleotide homology was observed with

previous Turkish isolate (BDV-Aydin-04). The analysis of the 5' UTR region sequences revealed that the homology between two BVDV field isolates was 88.8% whereas the similarity with previously characterized BVDV isolates ranged from 70.2% to 96.5%. The highest nucleotide homology was observed with previous Germany isolate (BVDV CP7 strain).

The phylogenetic tree based on 5' UTR region sequences revealed that BDV field isolates in this study belonged to BDV-7 cluster whereas BVDV field isolates were typed as BVDV-1 (Fig. 3).

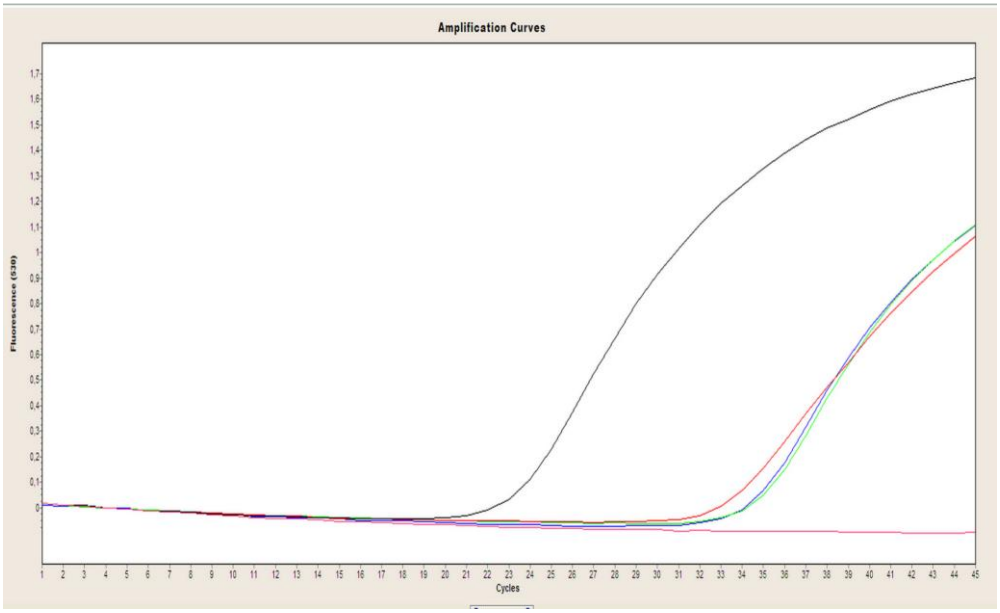

Figure 1. One step real-time duplex RT-PCR based on 5' UTR region of pestiviruses. Black line: positive control, pink line: negative control, other colourful amplification curves: positive pestivirus samples.

Şekil 1. Pestivirusların 5' UTR bölgesine dayalı one step real-time duplex RT-PCR. Siyah çizgi: pozitif kontrol, pembe çizgi: negatif kontrol, diğer renkli amplifikasyon eğirileri: pozitif pestivirus örnekleridir.

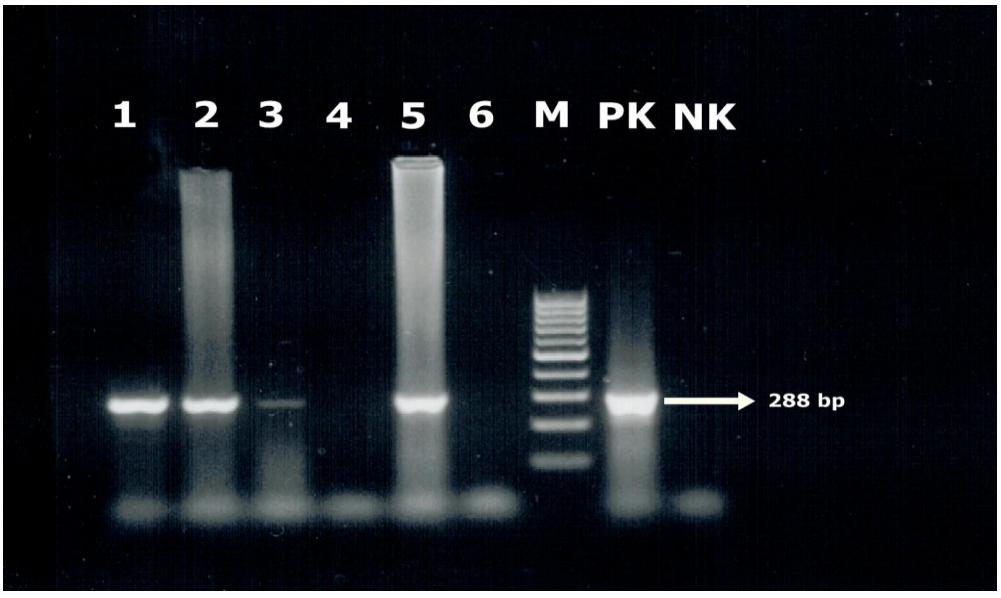

Figure 2. Agarose gel electrophoresis of RT-PCR product based on 5' UTR region of pestiviruses, M: Molecular marker of 100 bp, Lane 1-6: Samples, Lane PK: Positive control, Lane NK: Negative control.

Şekil 2. Pestivirusların 5' UTR bölgesine dayalı RT-PCR ürünlerinin agaroz jel elektroforezi, M: 100 bp moleküler marker, 1-6: Örnekler, PK: Pozitif kontrol, NK: Negatif kontrol.

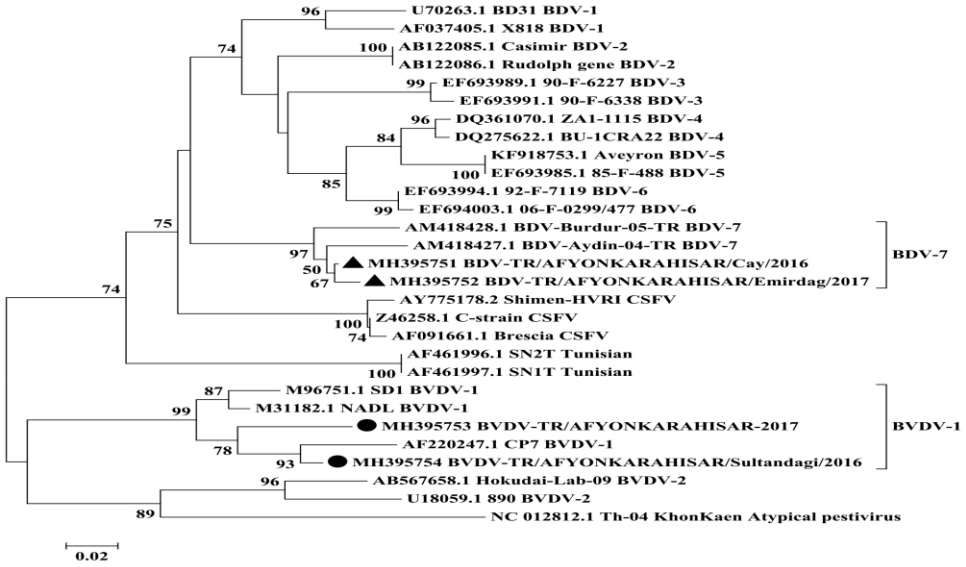

Figure 3. Phylogenetic tree constructed based on the 5' UTR region sequences using the Kimura two-parameter model. The BDV sequences obtained in this study are marked with black triangle (▲), and BVDV sequences are marked with round black spot (●).
Şekil 3. Kimura 2 parametre yöntemi kullanılarak oluşturulan 5' UTR bölgesi sekanslarının filogenetik ağacı. Bu çalışmada elde edilen BDV sekansları siyah üçgen ile (▲), BVDV sekansları ise siyah yuvarlak spotla (●) işaretlenmiştir.

DISCUSSION

Pestiviruses are distributed worldwide, and cause significant economic losses due to their impact on health and reproduction (Nettleton et al. 1998; Munoz-Zanzi et al. 2004). Pestiviruses are not highly host-specific (Nettleton et al. 1998; Passler and Walz 2010). Numerous studies have shown that both BDV and BVDV strains infect sheep, goat, cattle, swine and deer (Paton et al. 1995; Strong et al. 2010). However, in the study BDV RNA was only detected from aborted sheep foetuses, and BVDV RNA was from aborted bovine foetuses. Bulut et al. (2018) reported that prevalence of BVDV in sheep abortion cases in the Marmara and Eastern Anatolia regions in Turkey was 10.10% (40/396), and they suggested that the cause of BVDV infection in sheep may be pasture which contaminated with nasal drifts and saliva of persistently infected cattle. Furthermore, a previous study reported that close contact between small ruminants and cattle increases the risk of pestivirus transmission (Braun et al. 2013). In this study, BDV positive aborted sheep foetuses were from flocks which had only sheep for breeding, and according to farmers' report sheep and cattle were not use same pastures. Therefore there was no contact between sheep and cattle in BDV positive flocks. This could explain why BVDV RNA was not detected from aborted sheep foetuses.

The rate of pestiviruses in ruminant abortion cases in this study was 14.9% (15/101). This finding is in agreement with previous reports. Reported rates of pestiviruses in ruminant abortion cases in different regions of Turkey were between 0.93% and 66.6% (Cokcaliskan 2002; Hasircioglu et al. 2009; Albayrak et al. 2012; Avci et al. 2013; Tuncer-Goktuna et al. 2016; Bulut et al. 2018).

In this study, BDV RNA was found in 9 (12.16%) of the 74 aborted sheep foetuses. This result in agreement with previous report (Hasircioglu et al. 2009), but was lower than previous field studies that reported rates of the presence of pestiviruses in aborted sheep foetuses were 24.7%, 47.3% and 66.6% in the Marmara region, west part of Marmara region and Northern region of Turkey, respectively (Albayrak et al. 2012; Tuncer-Goktuna et al. 2016; Bulut et al. 2018). Possible explanations for this result may be the detection method, number of sampled animals and farm management. In this study, BVDV RNA was found in 6 (22.2%) of the 27 aborted bovine foetuses. This result in agreement with previous report (Albayrak et al. 2012), but was higher than previous study that detected BVDV antigen in 2.2% (2/92) of the aborted calves (Ozturk et al. 2012). Furthermore, Tuncer-Goktuna et al. (2016) detected pestivirus antigen in 31 (51.6%) of the 60 aborted calves in west part of Marmara region of Turkey. Possible explanations for these discrepancies may be the

number of sampled animals and number of sampled farms, and detection methods.
Serological and virological studies have been performed in the Afyonkarahisar province for pestiviruses (Gur 2009; Gur et al. 2009). However, molecular detection and genetic characterisation of pestiviruses in ruminant abortion cases in the Afyonkarahisar province has not been previously reported.

In previous studies, pestivirus isolates obtained from small ruminants in Turkey were classified into BDV-3, BDV-7 and BVDV-2 (Oguzoglu et al. 2009; Toplu et al. 2012; Yesilbag et al. 2014). Phylogenetic analysis of partial 5' UTR revealed that BDV field isolates in this study were of the BDV-7 genotype with the previous Turkish isolates (BDV-Burdur-05-TR and BDV-Aydin-04-TR). This result indicates that BDV-7 genotype is in circulation in the sheep population in Turkey.

The 5' UTR genetic analysis using sequences for pestiviruses revealed that BVDV field isolates in this study belonged to the BVDV-1 genotype (Fig. 3). The circulation of BVD-1 genotype in Turkey was also reported in previous studies (Yesilbag et al. 2008; Aslan et al. 2015). Furthermore, BVDV-2 genotype was detected from cattle in Turkey (Oguzoglu et al. 2010; Sarikaya et al. 2012; Yilmaz et al. 2012). It seems that both BVDV-1 and BVDV-2 are in circulation in cattle in Turkey.

In conclusion, a control programme for pestiviruses has not been applied in Turkey. Therefore, pestivirus infections are still animal welfare problem. Infection with pestiviruses causes serious economic losses in the livestock industry due to abortion problems, death and reduced reproductive performance. The results of this study showed that pestivirus infection play important role in ruminant abortion cases in Afyonkarahisar Province. A control programme for pestivirus infection will be beneficial to prevent economic losses.

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