

Molecular and Serological Characterization of Pestivirus Infection Among Sheep in Kirikkale, Turkey ^{[1][2]}

Ahmet Kursat AZKUR *  Serkal GAZYAGCI ** Muhammet Eren ASLAN * Nilgun UNAL *

[1] This research was supported by The Scientific and Technology Research Council of Turkey (TUBİTAK) Grant Number 108 O 864 and University of Kirikkale BAP grant number 2008-49

[2] The study has been presented, in part, at the IX. National Congress of Veterinary Microbiology (with International Guest Speakers) in Turkish Republic of North Cyprus- Oct 05 to 07, 2010

* Department of Microbiology, Faculty of Veterinary Medicine, University of Kirikkale, TR-71450 Kirikkale - TURKEY

** Department of Internal Medicine, Faculty of Veterinary Medicine, University of Kirikkale, TR-71450 Kirikkale - TURKEY

Makale Kodu (Article Code): KVFD-2010-3497

Summary

Border diseases virus causes economical loss in the sheep flocks and have high seroprevalence in the world. In the study 1075 samples from 25 sheep flocks obtained from Kirikkale and all district in the province of Kirikkale (Turkey) were used. Antibodies against pestivirus detected in 74.51% of 1075 sera samples using ELISA. However, range of seropositivity varied between 8.4% and 100% in the each sheep flocks. The correlation between seropositivity and race, age, and gender were investigated. While no relationships between seropositivity and gender and age were detected, a statistically significant correlation was noticed between the race of sheep and positive antibody response to pestivirus ($P<0.05$). Virus detection studies demonstrated that rate of viremic animals which did not show antibody response against to pestivirus in the sampling time was 4.37%. 5'UTR region of the infecting viruses were amplified through reverse transcription and nested PCR. PCR experiments after which sequencing and phylogenetic analyses were carried out. Accordingly, the viruses infecting the sheep flocks were different than BDV from previously reported from Turkey but very close to related to pestivirus type 3.

Keywords: Antigenic diversity, Border Disease virus, Pestivirus, Prevalence, Subgenotype

Türkiye Kırkkale Yöresinde Koyunlar Arasında Pestivirüs Enfeksiyonlarının Serolojik ve Moleküler Karakterizasyonu

Özet

Border hastalığı virüsü koyun sürülerinde ekonomik kayıplara neden olur ve dünyada yüksek seroprevalansa sahiptir. Bu çalışmada Kırkkale ili ve tüm ilçelerinde bulunan 25 koyun sürüsünden elde edilen 1075 örneği kullanılmıştır. Pestivirüse karşı oluşan antikorlar 1075 serumun %74.51'inde ELISA kullanılarak tespit edilmiştir. Bununla birlikte, seropozitiflik oranlarının her bir koyun sürüsünde %8.4 ve %100 arasında değişmektedir. Seropozitiflik ve ırk, yaş ve cinsiyet arasındaki ilişki araştırıldı. Seropozitiflik, cinsiyet ve yaş arasında bir bağlantı tespit edilemezken, koyun ırkları ve pestivirüse karşı oluşan pozitif antikor cevabı arasında istatistiksel olarak anlamlı bir ilişki tespit edildi ($P<0.05$). Virüs tespiti çalışmaları, virüs enfekte pestivirüse karşı antikor oluşturmayan hayvanların oranının %4.37 olduğunu göstermiştir. Moleküler aşamada enfekte virüsün 5'UTR bölgesi tersine transkripsiyon ve nested PZR ile çoğaltıldı. PZR sonrası sekans analizi ve filogenetik analiz yapıldı. Buna göre koyun sürülerini enfekte eden virüs Türkiye'de daha önceden bildirilen BDV suşlarından farklı fakat pestivirüs tip 3 ile çok yakın antijenik benzerliği olduğu belirlendi.

Anahtar sözcükler: Antijenik çeşitlilik, Border hastalığı virüsü, Pestivirüs, Prevalans, Subgenotip

INTRODUCTION

The genus *Pestivirus* is enveloped viruses containing single-stranded positive RNA molecule with a length of 12.3 kb which encodes a polyprotein of about 4.000 amino acids. These viruses belong to the family *Flaviviridae* together

with the genera *Hepacivirus* and *Flavivirus* ^{1,2}. Serologic survey have demonstrated prior infection with pestivirus in more than 40 species world-wide although pestiviruses infect a wide range of ungulate species like pigs, cattle,



İletişim (Correspondence)



+90 318 3573301/3158



azkurkursat@hotmail.com & kursatazkur@kku.edu.tr

sheep and other wild ruminant species. Pestivirus contains different viruses including bovine viral diarrhoea virus (BVDV), classical swine fever virus (CSFV) and ovine border disease virus (BDV). All four pestivirus species are closely related genetically and antigenically^{3,4}.

Border disease virus was detected throughout the world, causing endemic disease in sheep, goat and recently in chamois. BDV also cause significant economic losses in the small ruminants. In recent years, prevalence of BDV infection in small ruminant flocks have been reported for Americas, New Zealand, Australia and Spain, Italy, Switzerland, Sweden, Turkey, and Tunisia⁵⁻¹³. It was reported that prevalence rates of BDV could vary in sheep from 5% to 50% among countries and from region to region within countries^{14,15}. While pestivirus antibodies were found in 41% of the slaughterhouse sera in Turkey, seropositivity of BDV in west Anatolia and southeast Anatolia were 63.6% and 67.2% respectively¹⁶. Prevalence of infection with pestivirus might be influenced by other factors such as breed, age, gender. Moreover there are very few field studies describing specific antibody patterns in BDV-infected sheep from of flocks in various breed, age, gender. Although BDV is considered widely distributed in European countries, very few reports review of the literature revealed that few studies have been made of seroprevalence of BDV in the Turkey. Fewer studies of prevalence of BDV in sheep have been made compared with BVDV.

Border disease virus has two biotypes of the virus based on growth characteristic, and cytopathogenicity^{15,17}. Border disease virus infected animals may show clinical symptoms e.g. including abortions, stillbirths and small and weak lambs, sometimes with tremor, abnormal body conformation, hairy shaker and abnormal pigmentation. It may also cause a transient, and mild disease that could be exacerbated by superinfection with other pathogens and stimulates the production of long-lasting virus-neutralizing antibodies^{6,14-19}. Infection of a fetus before it reaches immunocompetence will cause its death or result in the birth of pestivirus-immunotolerant, antibody-negative and persistently infected (PI) weak or apparently normal animal, with poor growth and lower life expectancy. PI sheep can contaminate almost constantly through secretions and excretions^{14,15,18-21}. Furthermore PI animals are the single most important source of infection for other animals. Since there is no effective commercial BDV vaccine, identification and eliminating PI animals is the most effective way for BDV control. Therefore it is important to detect PI BDV sheep which are antibody negative and virus positive in precolostral blood sample. Several studies have reported prevalence of PI or viremia in sheep as ranging from 0.3% to 20% in flocks with clinical BDV²². Krametter-Froetscher et al. reported that prevalence of sheep PI with pestivirus was 0.32%²³. In Turkey, BVDV antigen was detected in 3% of samples from clinically healthy sheep and in 0.93% (1/108) of fetuses from slaughtered sheep^{5,16}. However

there is no comprehensive study to detect prevalence of PI BDV in Turkey.

Detection of pestivirus in clinical samples is based on virus isolation and immunoassay including antigen capture EIA, immunofluorescence or other immunohistochemical techniques^{15,24,25}. Detection of pestivirus in animal clinical samples has been reported as difficult but not impossible in many publications. Nested RT-PCR assay is preferred in detection and differentiation of ruminant pestiviruses. The judicious selection of appropriate primers pair(s) is required in detection of pestivirus by PCR techniques such as nested, multiplex or real time PCR require since a considerable variation exists among pestiviruses^{1,24,26,27}. In the virus-encoded polyprotein, the viral proteins are arranged in the following order C, N^{pro}, E1, E2, p7, NS2-3, (NS2, NS3), NS4A, NS4B, NS5A, NS5B¹. Different regions of pestivirus genomes such as the 5' UTR, N^{pro}, E1, have been used to study genetic diversity and detection of viruses in clinical samples^{28,29}. Recently it was announced that circulation BDV in sheep might be new subgroup and it was first study partial classification of BDV infection in small ruminants in Turkey. But there was no more and comprehensive any study in Turkey to determine genetic type of BDV.

Firstly we aimed to detect the seroprevalence and viro-prevalence of BDV infection in sheep population in the Kirikkale city located in the middle of Anatolia. Second aim of the study was to describe age, gender, breed-specific BDV antibody patterns and to indicate their relationship with the persistent infection, and transmission. Finally, BDV would be characterized at the genetic level using the 5' UTR gene region sequences together with published sequences of pestivirus reference strains to develop new control strategy and help investigate BDV distribution in the region.

MATERIAL and METHODS

Sample Collection

Blood samples from 1075 animals in 25 randomly selected sheep flocks were collected between November 2008 and March 2009. The one flock with a known recent history of BDV infection³⁰ and 8 flocks with a known history of abortion problem was included in the study. The animals belonged to the Merino, Kangal, and Whitekaraman races (*Table 1*). Numbers of flock 1 and 10 might be Kangal hybrid. Data collected at the time of blood sampling included the owner's name, address, phone number, flock location, eartag number or name of sheep, age of animal, gender and, breed description of animal and vial number. All blood samples were obtained from left or right jugular vein of sheep at ≥ 5 months old by using vacutainer tubes with or without anticoagulant and transported to the laboratory at ambient temperature. While whole blood samples

Table 1. Sample size as related to sheep flock size and age, breed and gender**Tablo 1.** Koyun ağıllarının yaş, cinsiyet ve ağıl büyüklüklerinin sayıları

Number of Sheep Flocks	1	2	3	4	5	6	7
Breed	Whitekaraman	Merino	Whitekaraman	Whitekaraman	Whitekaraman	Kangal	Whitekaraman
Age 1<	3	unavailable	3	3	unavailable	unavailable	unavailable
Age 1>	53	64	53	40	10	26	36
Ram	2	2	2	3	unavailable	unavailable	1
Female Sheep	54	62	54	40	10	26	35
Sample size	56	64	56	43	10	26	36
Sheep Flocks Size	150	100	120	250	30	85	91
Total Seropositivity	98.2%	81.2%	89.2%	90.6%	100%	96.1%	100%
Number of Sheep Flocks	8	9	10	11	12	13	14
Breed	Kangal	Kangal	Whitekaraman	Kangal	Kangal	Merino	Kangal
Age 1<	unavailable	unavailable	9	unavailable	unavailable	unavailable	unavailable
Age 1>	30	30	unavailable	76	27	52	77
Ram	1	1	unavailable	5	2	1	1
Female Sheep	29	29	9	71	25	51	76
Sample size	30	30	9	76	27	52	77
Sheep Flocks Size	120	100	120	130	142	80	100
Total Seropositivity	90%	93.3%	100%	77.6%	85.1%	9.6%	19.4%
Number of Sheep Flocks	15	16	17	18	19	20	21
Breed	Kangal	Whitekaraman	Kangal	Whitekaraman	Whitekaraman	Whitekaraman	Kangal
Age 1<	unavailable	22	unavailable	unavailable	unavailable	unavailable	unavailable
Age 1>	25	111	67	59	40	25	50
Ram	2	5	3	3	4	2	5
Female Sheep	23	128	64	56	36	23	45
Sample size	25	133	67	59	40	25	50
Sheep Flocks Size	110	146	150	125	90	250	236
Total Seropositivity	39.1%	86.4%	82%	8.4%	100%	84%	90%
Number of Sheep Flocks	22	23	24	25			
Breed	Whitekaraman	Whitekaraman	Whitekaraman	Whitekaraman			
Age 1<	unavailable	unavailable	unavailable	unavailable			
Age 1>	25	22	22	15			
Ram	2	4	unavailable	unavailable			
Female Sheep	23	18	22	15			
Sample size	25	22	22	15			
Sheep Flocks Size	25	80	100	110			
Total Seropositivity	92%	90.9%	90.9%	100%			

were collected in EDTA coated tubes (Vacutest, K3 EDTA; Arzergrande, Italy), blood samples were collected into tubes (Vacutest, Arzergrande, Italy). Blood samples allowed to clot at room temperature and centrifuged at 1.200 rpm for 10 min on the same day and sera were collected and kept at -20°C until analyzed ¹¹.

Enzyme Linked Immunosorbent Assay (ELISA)

An antibody screening ELISA from a commercial source (Pourquier ELISA BVD/MD/BD P80, France) was used to detect BDV antibodies in sheep according to the

manufacturer's instructions. Briefly, 25 µl serum samples, and controls were added to each well. Following overnight incubation at +4°C, all wells were washed three times and peroxidase labeled conjugates was added in all well as a 100 µl. Washings were performed again after 30 min incubation at 25°C. In the final step, 100 µl substrate solution TMB was added to each well and incubated for 20 min at 25°C and reaction was stopped by adding 100 µl 0.5M H₂SO₄. A commercial ELISA kit (Institute Pourquier, France) detecting NSP2-3 and E0 of BVDV or BD virus in sera, whole blood, and plasma was used in the

study according to the manufacturer's instructions. Briefly, samples incubated with antisera were dispensed into each well. Following ninety minutes incubation at room temperature, plate was washed three times. Afterwards, peroxidase labeled conjugate was added and incubated for 30 min at room temperature. After washing, the substrate TMB was added and the reaction was developed. Both ELISA results were analyzed and calculated with an automated ELISA reader at 450 nm (SIRIO S ELISA reader, Jakarta Raya, Indonesia).

Reverse Transcription and Nested-PCR

Viral RNA was extracted from whole blood and sera samples using a spin column system (QIAamp Viral RNA Mini Kit, Qiagen, Germany) according to the manufacturer's instructions. RT-PCR for detection of Border disease virus was performed. In addition, RNA samples were treated with RNase-free DNase (Qiagen, Germany). Random primer (1.25 mM random primer; Promega, Madison, WI, USA) was used in the first step of cDNA synthesis. For this purpose, 10 µl total RNA was combined with 1 µl (0.5 µg) random primer and preheated at 70°C for 10 min to denature secondary structures. The mixture was cooled rapidly, and 2 µl (100 mM) dNTPs, 5 µl 5X RT buffer, 1 µl I MMLV RT (200 IU/µl Promega, USA), and 8 µl H₂O were added reaching to the total volume of 25 µl. The RT mix was incubated at 37°C for 120 min and stopped by heating at 95°C for 5 min. The yield of cDNA was checked by PCR signal generated from the internal standard housekeeping gene, *Ovis aries* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GenBank accession number: DQ386891). The GAPDH forward primer was 5'-AGCTCGTCATCAATGGAAAGGC-3'; and the GAPDH reverse primer was 5'- ATGGCGTGGACAGTGG TCATAA-3'. The cDNA stock was stored at -20°C. After confirming RT reaction using GAPDH primers, first round of nested-PCR was carried out in a total volume of 50 µl. The reaction mixes contained 5 µl of the generated cDNA, 2 U *Taq* DNA polymerase (Promega, USA), 10 pmol of primer 324 (5'-ATGCCC WTAGTAGGACTAGCA-3') and 326 (5'-TCAACTCCATGTGCCATGTAC-3'), which are directed at the 5' UTR of the pestivirus genome to amplify all pestiviruses (Vilcek et al.²⁷), in 200 mM dNTPs (Promega, USA) and 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂ (Promega, USA). The cycling conditions were 95°C for 2 min; 34 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. Second round nested-PCR was performed with PBD1 primer (5'-TCGTG GTGAGATCCCTGAG-3') and PBD2 (5'-GCAGAGATTTTTAT ACTAGCCTATRC-3') and BDVR modified from PBD2 primer (5'-GCAGAGATTTTTATACTAGCCTAT-3'). First round PCR amplicons were diluted 1:10 in distilled water; 5 µl was used as template in a 50 µl nested PCR. Reaction conditions of second round nested PCR reactions were as follows; 95°C for 5 min, followed by 28 cycles of 95°C for 5 min, followed by 28 cycles of 95°C for 20 s, 54°C for 20 s, 72°C for 30 s and finally a cycle of 72°C for 10 min. All of the reverse

transcription and nested-PCR reactions were carried out in BO-PCR-5 thermal cycler (Hamburg, Germany). The InGenius LHR (Syngene, Cambridge, UK) was used to see PCR amplicons by using ethidium bromide staining after 1.5% agarose gel electrophoresis^{24,27,31,32}.

DNA Sequence Comparisons and Phylogenetic Analysis

Second round PCR amplicons was purified from 0.75% agarose gel by using commercial DNA purification kit GENE CLEAN® (GeneClean III, Q-BIOgene, Heidelberg, Germany) according to the manufacturer's instructions. Two of three PCR amplicons were sequenced using BDVR primer, and the other PCR amplicons was sequenced using PBD1 primer. Gene sequences were compared to Mega 4.1 beta and Clustal W analyzing software. Each PCR amplicons were sequenced at least three times. The phylogenetic tree generated by a neighborjoining method with Kimura two-parameter distances by using MEGA software (version 3.1 beta) showed a geographic clustering of the sequences of BDV^{33,34}.

Statistical Analysis

Statistical analysis was carried out with Statistical Package for Social Sciences software (SPSS Release 11,5, SPSS inc., Chicago, IL, USA). Significant differences between groups were evaluated using the chi-square (χ^2). A *P* value ≤ 0.05 was regarded as a significant difference between groups³⁵.

Nucleotide Sequence Accession Numbers

The nucleotide sequences were deposited in the GenBank databases under the accession numbers GU979818, GU979819 and, GU979820.

RESULTS

It was collected samples from 25 sheep flocks in the Kirikkale province in Turkey. While 40 of 1075 sheep were younger than 1 year old, 51 of 1075 sheep was male. All the animals belonged to the Merino, Kangal, and Whitekaraman sheep races. A total of 3040 animals were housed in 25 sheep flocks (Table 1). Although rate of antibody response to BDV were varied among 77.6% and 100% in 21 out of 25 flocks, 4 of 25 were showed 8.4% and 39.1% seropositivity value. Seropositivity proportions were varied among 8.4% and 100% in flock basis (Table 1). We determined the antibody responses to P80 antigen were determined in 801 of 1075 (74.51%) animals (Table 2). 1024 females and 51 ram out of 1075, 759 (74.12%) and 42 (82.35%) were positive, respectively. The differences in seropositivity between genders were not statistically different (*P*=0.188) and neither was the differences in seropositivity of sheep at the age of <1 and at the age of >1 (Table 2). In 458 of 551 (83.12%) whitekaraman, 286 of 408 (70.1%) kangal and, 57 of 116 (49.1%) merino sheep,

Table 2. Pestivirus seropositivity in Kirikkale province with regard to age, gender**Table 2.** Kirikkale ilindeki Pestivirüs seropozitifliğinin yaş ve cinsiyet ilişkisi

Age and Gender	Number of Sheep	Number of Seropositive Sheep	Percentage of Seropositivity
Age 1<	40	26	65%
Age 1>	1035	775	74.9%
Ram	51	42	82.35%
Female Sheep	1024	759	74.12%
Total Sheep	1075	801	74.51%

seropositivity for antibody against to P80 were noticed (Table 3). ELISA positive percentage of in overall of sheep was detected whitekaraman, kangal and, merino sheep were 42.6%, 26.6% and, 5.3%, respectively (Table 3). Surprisingly it was found that there were relationships between race and seropositive outcome meaningfully ($P<0.005$). To determine persistently infected sheep, serum samples with no antibody response against to p80 protein were tested for viral antigen. For this purpose, 274

seronegative samples tested for NSP2-3 and E0 of BVDV or BDV (Table 4). 47 of 274 (17.1%) whole blood samples were antigen positive. Rate of virus infected animals that did not show antibody response against to pestivirus in the sampling time was calculated to be 4.37% (Table 4).

To determine BDV antigen, 47 whole blood samples submitted for BVDV diagnosis were tested by RT-nested PCR. Firstly, we checked whether RT-PCR assay was working. Therefore *Ovis aries* GAPDH gene was targeted as an internal control. All samples demonstrated a very strong GAPDH signal (Fig. 1A). After confirming RT reaction by using GAPDH primers, first round of nested-PCR was carried out by using 324 and 326 primers which could be amplified at the 5' UTR of the pestivirus genome and are considered to amplify all pestiviruses. While PCR amplicon of 5' UTR of the pestivirus genome was not demonstrable in first round nested PCR except positive control for PCR, three clinical samples showed bands in the expected PCR product size (Fig. 1B). Firstly when second round of PCR was carried out PBD1 and PBD2 for the BDV specific primers,

Table 3. Pestivirus seropositivity in Kirikkale province with regard to breed**Table 3.** Kirikkale ilindeki pestivirüs seropozitifliğinin ırk ile ilişkisi

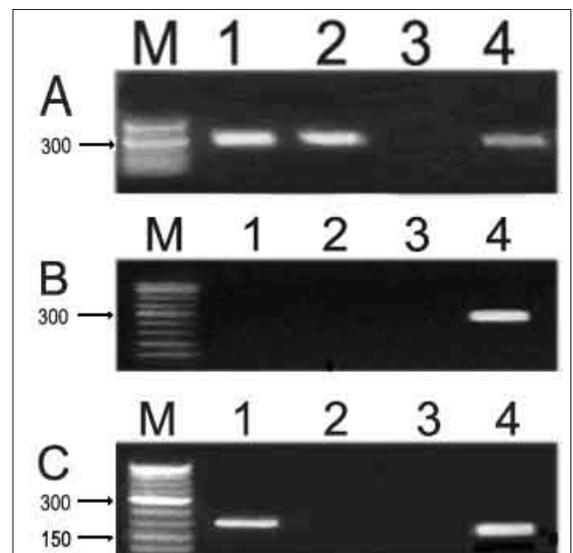
Breed of Sheep	Number of Sheep	Number of Seropositive Sheep	Percentage of Seropositivity	Total Seropositivity
Whitekaraman	551	458	83.12%	42.6%
Kangal	408	286	70.1%	26.6%
Merino	116	57	49.1%	5.3%

Table 4. Detection of BDV-specific native antigens NSP2-3 and E0 of BVDV or BDV in randomly selected whole blood samples from sheep which were no antibody response against to BVD/MD/BD P80**Table 4.** BVD/MD/BD P80 antikor cevap neagatif koyunların rastgele seçilen tam kan örneklerinde BVDV veya BDV NSP2-3 ve E0 antijenlerinin belirlenmesi

ELISA	Number of Whole Blood Samples	Number of Positive Samples in the Antibody ELISA Negative Samples	Percentage of Seropositivity	Total Percentage Results
Antigen ELISA Positive + Antibody ELISA Negative -	274 out of 1075	47 out of 274	17.1%	4.37%

Fig 1. RT- nested PCR with RNA extracted from clinical samples (whole blood). A: RT-PCR control, RT-PCR was carried out *Ovis aries* GAPDH primers. Whole blood samples lane 1 (positive), lane 2 (negative), lane 3 (dH₂O) and BDV positive control (lanes 4) Lanes M: molecular length marker. B: First round RT-nested PCR was carried out 324 primer and 326 primer. Whole blood samples lane 1 (positive), 2 (negative), lane 3 (dH₂O) and BDV positive control (lanes 4) Lanes M: molecular length marker C: Second round RT-nested PCR was carried out PBD1 primer and BDVR primer. Whole blood samples lane 1 (positive), 2 (negative), lane 3 (dH₂O) and BDV positive control (lanes 4) Lanes M: molecular length marker

Şekil 1. Klinik örneklerin (tam kan) RNA'larından RT-nestsed PCR. A: RT-PCR kontrol, RT-PCR *Ovis aries* GAPDH primerleri ile kuruldu. Tam kan örnekleri hat 1 (pozitif), hat 2 (negatif), hat 3 (dH₂O) and hat 4 BDV pozitif kontrol, hat M: moleküler ağırlık markeri. B: İlk round nested RT-PCR 324 ve 326 primerleri ile kuruldu. Tam kan örnekleri hat 1 (pozitif), hat 2 (negatif), hat 3 (dH₂O) and hat 4 BDV pozitif kontrol, hat M: moleküler ağırlık markeri. C: İkinci round nested RT-PCR PBD1 ve BDVR primerleri ile kuruldu. Tam kan örnekleri hat 1 (pozitif), hat 2 (negatif), hat 3 (dH₂O) and hat 4 BDV pozitif kontrol, hat M: moleküler ağırlık markeri



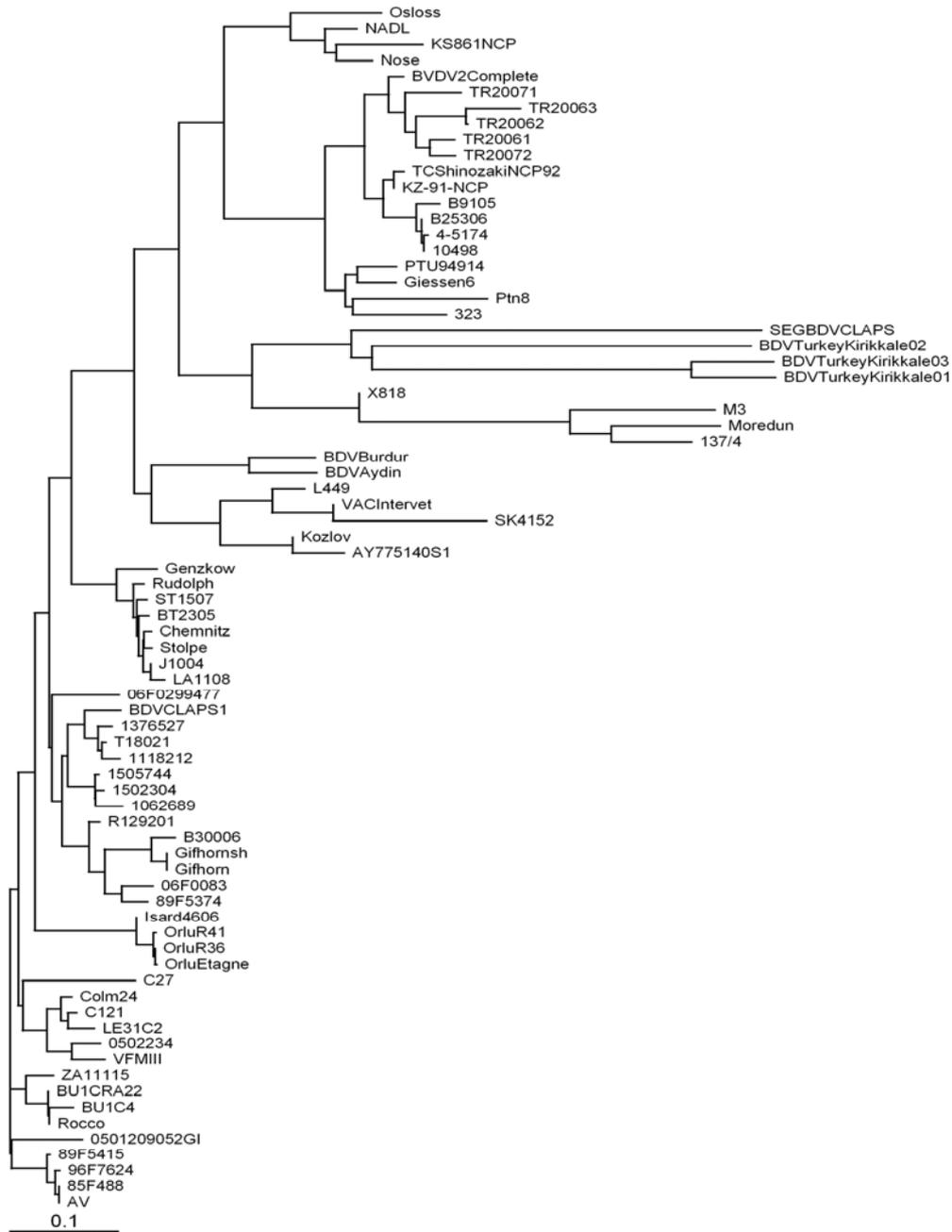


Fig 2. The Phylogram tree for 5' UTR of the pestivirus genome was inferred using Clustal W software. Sequences not generated by this study are derived from genbank sequences except for (GU979818, GU979819, GU979820):

Şekil 2. Pestivirüs genomunun 5' UTR bölgesinin Clustal W bilgisayar programı kullanılarak filogram ağacı. Bu gen numaraları hariç GU979818, GU979819, GU979820, aşağıdaki dizilimler bu çalışmadan elde edilmedi.

BDV; BDV/Burdur/05-TR (AM418428), BDV/Aydin/04-TR (AM418427), X818 (AF037405), T1802/1 (U65046), SEGBDVCLAPS (AH005065), BDVCLAPS1 (U17142), 1505744 (EU887956), 1502304 (EU637005), 1376527 (EU887954), 1062689 (EU887952), C121 (DQ275625), C27 (DQ275623), BU-1CRA22 (DQ275622), Moredun (U65022), Gifhorn-sh (EU637007), chemnitz (EU637006), isard4606 (EU637005), BT2305 (EU637004), ST1507 (EU637003), J1004 (EU637001), LA1108 (EU637000), Genzkow 701 (EU636999), Stolpe (EU636998), Gifhorn (EU636997), B300/06 (EU224227), Orlu-R41 (DQ898295), Orlu-R36 (DQ898294), Orlu-Etagne (DQ898291), M3 (FJ493488), 137/4 (FJ493487), 0502234 (EU711348), 06-F-0299/477 (EF694003), 06-F-0083 (EF693999), 96-F-7624 (EF693998), 89-F-5415 (EF693988), 89-F-5374 (EF693987), 85-F-488 (EF693985), AV (EF693984), Colm24 (DQ361073), LE31C2 (DQ361072), VFMIII (DQ361071), ZA1-1115 (DQ361070), BU1-C4 (DQ361069), Rocco (DQ361067), Rudolph (AB122086), R1292/01 (AY081182), KIRIKKALE BDV_Turkey_Kirikkale_03 (GU979820), 0501209-052GI (DQ679902), BDV_Turkey_Kirikkale_01 (GU979818), BDV_Turkey_Kirikkale_02 (GU979819), BVDV-1;NADL (M31182), Nose (AB019670), KS86-1-NCP (AB042713), Osloss (M96687), CD87 (L32887), BVDV-2; TC Shinozaki NCP/92 (AB042676), KZ-91-NCP (AB003620), 4-5174 (AF298063), PTU94914 (U94914), Giessen (AY379547), 104/98 (AJ304381), BVDV-2 complete genome (NC_002032), B91/05 (EU224242), B253/06 (EU224225), Ptn8 (EU034175), strain 323 (EF683554), TR-2007/1 (EU051826), TR-2007/2 (EU051825), TR-2006-3 (EU542423), TR-2006-2 (EU542422), TR-2006-1 (EU542421) CSFV; VAC-Intervet (AJ605590), Kozlov (AY972477), SK4/152 (EU117550), L449 (AY761194), AY775140S1 (AY775140)

we did not find any positive results (data not shown). After that BDVR primer designed to delete last two nucleotides from PBD2 and checked using nucleotide blast programme to homologous pestiviruses sequences. Consequently, second round of nested-PCR was performed with PBD1 and BDVR primers. In the second round, 38 of 47 whole blood samples were detected as a positive (Fig. 1C).

To study the genetic relatedness of border disease viruses studied in this project, we focused on the 5' UTR region. Because there is a financial problem, PCR products from three samples were randomly sequenced. Other PCR positives samples also will use further more investigation in the future. The sequences (GU979818, GU979819 and, GU979820) were aligned with the known sequences of pestiviruses published in the literature. Hence a phylogenetic analysis was carried out. According to sequence analysis three PCR positive sequences were randomly selected were closely related to pestivirus type 3 published previously SEGBDVCLAPS (AH005065) (Fig. 2).

DISCUSSION

In this study, samples from 801 (74.51%) sheep were found seropositive against to pestivirus. The range of seropositivity varied between 8.4% and 100% in different flocks. We did not find any relationships between seropositivity and gender, and age of the animals. But we found that a significant correlation between race of the animals and seropositivity of sheep was noticed ($P < 0.05$). 274 seronegative samples were used in testing for NSP2-3 and E0 of BVDV or BDV pestivirus antigen. We noticed that 47 of antibody response to pestivirus 274 whole blood contained pestivirus antigen (17.1%). The results indicated that 4.37% of pestivirus infected animals were in the sampling time. After determine pestivirus antigen, we found the partial sequences of 5'UTR region of circulation BDV virus and compared to previously published isolates. These results demonstrated that circulating virus of Kirikkale province in Turkey represented a group of BDV different from previous study in Turkey.

This study is the first wide-range study to determine the seroprevalence of pestivirus in randomly sampled sheep in Turkey. There was no study to show any relationship between the seroprevalence and the age of animals. The location of the study was chosen because of the fact that Kirikkale is located on the middle of animal transit route between eastern and western Anatolia which functions as a bridge between Asia and Europe. There are approximately 60.000 sheep in the province of Kirikkale in Turkey. A thousand seventy five blood samples were collected from 25 sheep flocks which contain a total of 3040 sheep. These sheep usually use common pasture. BVDV seroprevalence reported to be varied between 25% and 70% in this region in a previous study in Turkey³⁶. While researcher determined BDV seropositivity as a varied among 82% and

90% in 7 flocks³⁶, it was found in this study that range of seropositive prevalence varied between 9.6% and 100% in 25 flocks. It was found that percentage seropositivity of results obtained from 25 sheep flocks is 74.51% in 1075 sheep (Table 1). This data is also supported by the antibody prevalence found in slaughterhouse sera tested for BDV neutralizing antibodies in Turkey^{5,16}. While it was found in the 5 sheep flocks 100% pestivirus seroprevalence in the flock numbers 5, 7, 10, 19 and 25, pestivirus seroprevalence was determined 9.6%, 19.4% and 8.4% in the flock numbers 13, 14, 18 respectively. Maybe low seroprevalence among sheep flock 13, 14, 18 may also be related to stage of infection (Table 1). To assess of potential to differentiate infected and vaccinated animals, different BVDV ELISA kits was used to determine antibody responses to inactivated vaccines and natural infection in cattle. While some of ELISA kits were used plates coated with whole BVDV-1 particles, some of them were used to plates coated with p80 protein. Researchers found that plates coated with whole BVDV-1 particles could give better results more than plates coated with p80 protein ELISA kits³⁷. Pestiviruses in sheep seroprevalance of Turkey data obtained from this study also might be higher in order to it was used ELISA kit that have plates coated p80 protein.

BDV studies performed in Turkey did not address the details such as race, gender, and age of sheep. Thus, the comparison of the present study was done with the reports from other countries but there is no study in Turkey. In other countries such as Spain, Italy and Austria lower prevalences have been reported^{12,13,23}. Berriatua et al.^{38,39} investigated age-specific BDV-antibody patterns in the sheep flocks and identified specific age groups most susceptible to infection in presence or absence of PI. They found that seroprevalence of BDV can be influenced by the presence of PI animals. If sheep flock did not have any PI infection, a-year-old sheep had lower seroprevalence than in older sheep. On the contrary if there was PI infection in the flocks, a-year-old sheep showed higher seroprevalence as well as older sheep³⁹. In this study we firstly wanted to see whether there was any relationship between gender, race and age-specific pestivirus antibodies. We found that there was a correlation between merino sheep and seropositivity at a statistically significantly level ($P < 0.05$). Age-specific seroprevalence pattern in the flocks noticed in this study resembled those in dairy-cattle herds in the same phase of BVDV infection⁴⁰. There is no report about resistance of merino or other sheep species to BDV infection. Drögemüller et al.⁴¹ reported PrPARR/PrPARR sheep were resistance for scrapie. Similarly, it might be speculated that merino sheep might be resistant this infection or transmission. However, no evidence indication such resistance is at hand. Persistent infections are initiated when an ncp biotype of BVDV invades the fetus *in utero* early in its development. Infected fetuses may develop normally, but the infection persists for life^{30,42}. Krametter-Froetscher et al.²³ reported prevalence of sheep pestivirus

PI with as 0.32% and prevalence of goat pestivirus PI as 0.08%. Valdazo-Gonzalez et al.¹¹ found a prevalence of 0.24% PI sheep in Spanish lambs and Berriatua et al.³⁸ found 9% virus positive flocks among milk sheep in Basque. Rate of PI in this study was higher than those reported from Austria and Spain. Our results indicate that rate of virus infected sheep as 4.37% in Kirikkale province in Turkey (*Table 4*). Pestivirus antigen positives animals that did not show antibody response against to pestivirus in the sampling time were detected in the numbered flocks 11, 12, 13, 14, 15, 16, 17. Number of pestivirus antigen positive animals was to be detected as 4, 3, 3, 25, 4, 6, 1 and, 1 in the numbered flocks 11, 12, 13, 14, 15, 16, 17, respectively. Interestingly eventhough flock 14 showed the highest viremia rate, pestivirus seroprevalence was determined 19.46% and all sheep were higher than a year (*Table 1*). It was suggested that this flock was face to with pestivirus recently. Although sheep owner of flock 12 did not mention in abortion problems, we could not detect any viremia (*Table 1*). Nine flocks with a known history of abortion problem were included in the study (Numbered flocks 11, 13, 14, 16, 21, 22, 23). It was previously reported that flock 11 contained BDV infection and four animals in the flock 11 have still pestivirus in their blood³⁰. However number flock 11 showed 77.6% seropositivity all sheep were higher than a year (*Table 1*). Sheep owner of flock 13 mentioned in abortion problem too. Flock 13 contained merino sheep and three out of fifty-two viremic animals. Eventhough these viremic animals were higher than a year, flock 13 has showed 9.6% seropositivity. It was suggested that fetus abortions problem might attributed to BDV infection as well other pathogens⁴³. While flock 15 included four viremic sheep and showed 39.1% seropositivity, flock 16 and 17 had have only one viremic animal and showed 86.4% and 82% seropositivity respectively. A ram in the flock 17 was detected also viremic. Eventhough flocks 17 did not have any sheep more than a year ages, flock 16 had twenty-two sheep less than a year. But all viremic animals a year than age were as determined as a viremic. Burgu et al.⁴⁴ detected pestivirus in the in some precolostral blood samples from lambs but not blood samples by using immunoperoxidase techniques and immunoplaque assay. However present study showed pestivirus antigen could detect in the blood samples because RT-PCR and antigen ELISA was more sensitive than immunoperoxidase techniques and immunoplaque assay. In Turkey, sheep breeders do not survey their sheep for any pestivirus infection and no efforts are exerted to eliminate sheep with pestivirus PI. Consequently, there is no concrete data for rate of frequency PI sheep with pestivirus in this country.

Because pestiviruses can infect farm animals as well as wild animals, several authors have proposed for classifications based on genomic and antigenic characteristics rather than virus isolation^{7,15}. Serum samples, plasma and tissues could be suitable to detect pestivirus antigen, but that serum is superior to heparinised

plasma due to RT-PCR inhibition. We did not find BDV antigen in the all of the positive samples for antigen ELISA. We agree with Silvak observation that antigen ELISA test occasionally might produce false positive results²⁰. To confirm ELISA positive result we carried out RT-PCR using panpestivirus primers. Potential presence of inhibitory factors in clinical samples requires the incorporation of a positive internal control²⁴. To eliminate negative effect of inhibitory factors in clinical we used GAPDH internal control. Generally genetic diversity of the 5' UTR, N^{pro} and E2 genomic regions was used genetic typing of pestiviruses⁴⁵⁻⁵⁰. Because, Vilcek et al.⁵¹ demonstrated that 5' UTR genomic region give meaningful phylogenetic inferences and has the highest degree of sequence conservation, and 5' UTR region was amplified by RT- nested PCR in this study⁵¹. We used three different primers which are called PBD1, PBD2 and BDVR in the RT- nested PCR in this study. However we did not find any PCR band using PBD1 with PBD2 in the second round of nested PCR. But we determined PCR positive expected band size using PBD1 with BDVR primer pairs in the second round of nested PCR. After that we identified pestiviruses in sheep circulating in Kirikkale province in Turkey. For this purpose, the 5' UTR was partially amplified by RT- nested PCR using total RNA directly extracted from ELISA antibody response negative spleen, lymph nodes and/or blood of animals^{50,51}. Recently Oguzoglu et al.³⁶ reported occurrence of BDV infection from two Pestivirus isolates from a sheep and a goat in Turkey by using the 5'-UTR and N^{pro} gene region. They formed a cluster clearly separated from the known clusters BDV-1 to BDV-6. They also speculated these isolates might represent a new subgroup (BDV-7). Although it was found that sequences were closely related to X818 (AF037405), SEGBDVCLAPS (AH005065), Moredun (U65022), M3 (FJ493488), 137/4 (FJ493487) in the study, Oguzoglu et al.³⁶ found that BDV isolates from Aydin and Burdur cities where are located in the west of Turkey were close to CSFV^{36,52}. It is also known that there are some swine farms to supply meat for western world tourist. The presence of genetically homogenous pestiviruses may also have implications for potential prophylactic measures or diagnosis aiming at reducing clinical losses due to BDV. A successful BDV control program was required to develop rapid diagnostic kit for surveillance and identifying PI animals and as a result PI animals could be removed from sheep flocks. We concluded that high seroprevalence of BDV was observed in sheep and according to results it was speculated that merino sheep might be resistance against to BDV. To confirm to some sheep race might be resistant BDV infection need to be further investigation in future. We also found that circulation BDV in Turkey is close to SEGBDVCLAPS (pestivirus type3) (*Fig. 2*). However, there is no data of BDV transmission in the sheep. Although how to prevent and control this disease remains a serious problem, determine and eliminate PI animals from sheep flocks will provide a temporarily solution when one discover efficient any vaccine against to BDV. Molecular

characterization of BVDV-1 from naturally infected sheep has indicated that viruses could spread host species from cattle to sheep within the Turkey. We also observed that these sheep flocks had possibility to contact with cows. Therefore the results of relationships between race of sheep and seropositive and some mutation might come from frequency in contacting with cows infected with BVDV. As a conclusion pestiviruses seroprevalance of pestiviruses is very high in sheep in the Turkey. Circulations of pestiviruses have homology close to pestiviruses type 3.

ACKNOWLEDGEMENTS

We thank Dr. Mehmet Ziya DOYMAZ for valuable suggestions and critical reading of the manuscript. We thanks also Oya CINAR and Sinasi ASKAR for preparation sera for excellent technical assistance.

REFERENCES

1. **Becher P, Avalos Ramirez R, Orlich M, Cedillo Rosales S, Konig M, Schweizer, M, Stalder H, Schirrmeyer H, Thiel HJ:** Genetic and antigenic characterization of novel pestivirus genotypes: Implications for classification. *Virology*, 311, 96-104, 2003.
2. **Becher P, Orlich M, Thiel HJ:** Complete genomic sequence of border disease virus, a pestivirus from sheep. *J Virol*, 72, 5165-5173, 2003.
3. **Reimann I, Depner K, Trapp S, Beer M:** An avirulent chimeric Pestivirus with altered cell tropism protects pigs against lethal infection with classical swine fever virus. *Virology*, 322, 143-157, 2004
4. **Schirrmeyer H, Strebelow G, Depner K, Hoffmann B, Beer M:** Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. *J Gen Virol*, 85, 3647-3652, 2004.
5. **Oguzoglu TC, Tan MT, Toplu N, Demir AB, Bilge-Dagalp S, Karaoglu T, Ozkul A, Alkan F, Burgu I, Haas L, Greiser-Wilke I:** Border disease virus (BDV) infections of small ruminants in Turkey: A new BDV subgroup? *Vet Microbiol*, 135, 374-379, 2009.
6. **Thabti F, Fronzaroli L, Dliissi E, Guibert JM, Hammami S, Pepin M, Russo P:** Experimental model of Border Disease Virus infection in lambs: comparative pathogenicity of pestiviruses isolated in France and Tunisia. *Vet Res*, 33, 35-45, 2002.
7. **Marco I, Rosell R, Cabezon O, Mentaberre G, Casas E, Velarde R, Lopez-Olvera JR, Hurtado A, Lavin S:** Epidemiological study of border disease virus infection in Southern chamois (*Rupicapra pyrenaica*) after an outbreak of disease in the Pyrenees (NE Spain). *Vet Microbiol*, 127, 29-38, 2008.
8. **Sullivan DG, Chang GJ, Akkina RK:** Genetic characterization of ruminant pestiviruses: sequence analysis of viral genotypes isolated from sheep. *Virus Res*, 47, 19-29, 1997.
9. **Stadejek T, Vilcek S, Lowings JP, Ballagi-Pordany A, Paton DJ, Belak S:** Genetic heterogeneity of classical swine fever virus in Central Europe. *Virus Res*, 52, 195-204, 1997.
10. **Vilcek S, Nettleton PF, Paton DJ, Belak S:** Molecular characterization of ovine pestiviruses. *J Gen Virol*, 78, 725-735, 1997.
11. **Valdazo-Gonzalez B, Alvarez M, Sandvik T:** Prevalence of border disease virus in Spanish lambs. *Vet Microbiol*, 128, 269-278, 2008.
12. **Danuser R, Vogt HR, Kaufmann T, Peterhans E, Zanoni R:** Seroprevalence and characterization of pestivirus infections in small ruminants and new world camelids in Switzerland. *Schweiz Arch Tierheilkd*, 151, 109-117, 2009.
13. **Buonavoglia C, Marsilio F, Tempesta M, Buonavoglia D, Cavalli A:** Persistent pestivirus infection in sheep in Apulia (southern Italy). *New Microbiol*, 17, 163-165, 1994.
14. **Nettleton PF, Gilmour JS, Herring JA, Sinclair JA:** The production and survival of lambs persistently infected with border disease virus. *Comp Immunol Microbiol Infect Dis*, 15, 179-188, 1992.
15. **Nettleton PF, Gilray JA, Russo P, Dliissi E:** Border disease of sheep and goats. *Vet Res*, 29, 327-340, 1998.
16. **Gur S:** A investigation of border disease virus in sheep in Western Turkey. *Trop Anim Health Prod*, 41, 1409-1412, 2009.
17. **Becher P, Meyers G, Shannon AD, Thiel HJ:** Cytopathogenicity of border disease virus is correlated with integration of cellular sequences into the viral genome. *J Virol*, 70, 2992-2998, 1996.
18. **Garcia-Perez AL, Minguijon E, Estevez L, Barandika JF, Aduriz G, Juste RA, Hurtado A:** Clinical and laboratorial findings in pregnant ewes and their progeny infected with Border disease virus (BDV-4 genotype). *Res Vet Sci*, 86, 345-352, 2009.
19. **Garcia-Perez AL, Minguijon E, Barandika JF, Aduriz G, Povedano I, Juste RA, Hurtado A:** Detection of Border disease virus in fetuses, stillbirths, and newborn lambs from natural and experimental infections. *J Vet Diagn Invest*, 21, 331-337, 2009.
20. **Sandvik T:** Selection and use of laboratory diagnostic assays in BVD control programmes. *Prev Vet Med*, 72, 3-16; Discussion 215-219, 2005.
21. **Hurtado A, Sanchez I, Bastida F, Minguijon E, Juste RA, Garcia-Perez AL:** Detection and quantification of pestivirus in experimentally infected pregnant ewes and their progeny. *Virology*, 6, 189, 2009.
22. **Strong R, La Rocca SA, Ibata G, Sandvik T:** Antigenic and genetic characterisation of border disease viruses isolated from UK cattle. *Vet Microbiol*, 141, 208-215, 2009.
23. **Krametter-Froetscher R, Duenser M, Preyler B, Theiner A, Benetka V, Moestl K, Baumgartner W:** Pestivirus infection in sheep and goats in West Austria. *Vet J*, 186, 342-346, 2009.
24. **Willoughby K, Valdazo-Gonzalez B, Maley M, Gilray J, Nettleton PF:** Development of a real time RT-PCR to detect and type ovine pestiviruses. *J Virol Methods*, 132, 187-194, 2006.
25. **Vilcek S, Nettleton PF:** Pestiviruses in wild animals. *Vet Microbiol*, 116, 1-12, 2006.
26. **Fenton A, Entrican G, Herring JA, Nettleton PF:** An ELISA for detecting pestivirus antigen in the blood of sheep persistently infected with border disease virus. *J Virol Methods*, 27, 253-260, 1990.
27. **Vilcek S, Herring AJ, Herring JA, Nettleton PF, Lowings JP, Paton DJ:** Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Arch Virol*, 136, 309-323, 1994.
28. **Vilcek S:** Secondary structure of the 5'-noncoding region of border disease virus genome RNAs. *Vet Med (Praha)*, 42, 125-128, 1997.
29. **Sullivan DG, Chang GJ, Trent DW, Akkina RK:** Nucleotide sequence analysis of the structural gene coding region of the pestivirus border disease virus. *Virus Res*, 33, 219-228, 1994.
30. **Kul O, Kabakci N, Ozkul A, Kalender H, Atmaca HT:** Concurrent peste des petits ruminants virus and pestivirus infection in stillborn twin lambs. *Vet Pathol*. 45, 191-196, 2008.
31. **Vilcek S, Greiser-Wilke I, Nettleton P, Paton DJ:** Cellular insertions in the NS2-3 genome region of cytopathic bovine viral diarrhoea virus (BVDV) isolates. *Vet Microbiol*, 77, 129-236, 2000.
32. **Vilcek S, Paton DJ, Rowe LW, Anderson EC:** Typing of pestiviruses from eland in Zimbabwe. *J Wildl Dis*, 36, 165-168, 2000.
33. **Tamura K, Dudley J, Nei M, Kumar S:** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol*, 24, 1596-1599, 2007.
34. **Kumar S, Nei M, Dudley J, Tamura K:** MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform*, 9, 299-306, 2008.
35. **Ergün G, Aktas S:** ANOVA modellerinde kareler toplamı yöntemlerinin karşılaştırılması. *Kafkas Univ Vet Fak Derg*, 15 (3): 481-484, 2009.
36. **Oguzoglu TC:** Border Disease. *Ankara Univ Vet Fak Dergisi*, 55, 69-74, 2008.

- 37. Raue R, Harmeyer SS, Nanjiani IA:** Antibody responses to inactivated vaccines and natural infection in cattle using bovine viral diarrhoea virus ELISA kits: Assessment of potential to differentiate infected and vaccinated animals. *Vet J*, (In Press).
- 38. Berriatua E, Barandika JF, Aduriz G, Hurtado A, Estevez L, Atxaerandio R, Garcia-Perez AL:** Flock-prevalence of border disease virus infection in Basque dairy-sheep estimated by bulk-tank milk analysis. *Vet Microbiol*, 118, 37-46, 2006.
- 39. Berriatua E, Barandika J, Aduriz G, Atxaerandio R, Garrido J, Garcia-Perez AL:** Age-specific seroprevalence of Border disease virus and presence of persistently infected sheep in Basque dairy-sheep flocks. *Vet J*, 168, 336-342, 2004.
- 40. Houe H:** Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Vet Microbiol*, 64, 89-107, 1999.
- 41. Drogemuller C, Leeb T, Distl O:** PrP genotype frequencies in German breeding sheep and the potential to breed for resistance to scrapie. *Vet Rec*, 149, 349-352, 2001.
- 42. Kalaycıoğlu AT:** Control of Bovine Viral Diarrhoea Infections Through Vaccination. *Kafkas Univ Vet Fak Derg*, 13: 103-108, 2007.
- 43. Hasırcıoğlu S, Kale M, Acar A:** Investigation of Pestivirus Infections in Aborted Sheep and Goats in Burdur Region. *Kafkas Univ Vet Fak Derg*, 15, 163-167, 2009.
- 44. Burgu I, Akca Y, Alkan F, Ozkul A, Karaoglu T, Dagalp SB, Oguzoglu C, Yesilbag K:** The serological and virological investigations and pathogenesis of BVDV Infection in sheep during pre- and post-partum periods. *Turk J Vet Anim Sci* 25, 551-557, 2001.
- 45. Schweizer M, Matzener P, Pfaffen G, Stalder H, Peterhans E:** "Self" and "nonself" manipulation of interferon defense during persistent infection: Bovine viral diarrhoea virus resists alpha/beta interferon without blocking antiviral activity against unrelated viruses replicating in its host cells. *J Virol*, 80, 6926-6935, 2006.
- 46. Vilcek S, Durkovic B, Kolesarova M, Greiser-Wilke I, Paton D:** Genetic diversity of international bovine viral diarrhoea virus (BVDV) isolates: Identification of a new BVDV-1 genetic group. *Vet Res*, 35, 609-615, 2004.
- 47. Rasmussen TB, Reimann I, Hoffmann B, Depner K, Uttenthal A, Beer M:** Direct recovery of infectious pestivirus from a full-length RT-PCR amplicon. *J Virol Methods*, 149, 330-333, 2008.
- 48. Sullivan DG, Akkina RK:** A nested polymerase chain reaction assay to differentiate pestiviruses. *Virus Res*, 38, 231-239, 1995.
- 49. La Rocca SA, Sandvik T:** A short target real-time RT-PCR assay for detection of pestiviruses infecting cattle. *J Virol Methods*, 161, 122-127, 2009.
- 50. Hyndman L, Vilcek S, Conner J, Nettleton PF:** A novel nested reverse transcription PCR detects bovine viral diarrhoea virus in fluids from aborted bovine fetuses. *J Virol Methods*, 71, 69-76, 1998.
- 51. Vilcek S:** Identification of pestiviruses contaminating cell lines and fetal calf sera. *Acta Virol*, 45, 81-86, 2001.
- 52. Barros SC, Ramos F, Pauperio S, Thompson G, Fevereiro M:** Phylogenetic analysis of Portuguese bovine viral diarrhoea virus. *Virus Res*, 118, 192-195, 2006.