TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2018) 42: 205-210

doi:10.3906/vet-1711-83

Seroepidemiology and molecular investigation of pestiviruses among sheep and goats in Northwest Anatolia

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Received: 27.11.2017 • **Accepted/Published Online:** 07.05.2018 • **Final Version:** 08.06.2018

Abstract: Pestiviruses, which are distributed worldwide, may cause losses in sheep and goat populations. The current seroprevalence and circulation of pestiviruses in small ruminants of the South Marmara Region of Turkey were investigated. A total of 607 blood samples were collected from 22 different flocks in 4 different provinces, and 24 necropsy materials were investigated. Pestivirus seroprevalence in sheep and goats was determined by virus neutralization test against the NADL strain of bovine viral diarrhea virus. The mean antibody titer was 3.5, while the overall seropositivity rate was 32.78% (199/607). Seronegativity was markedly more prevalent in goats. The mean detected antibody titer was 6.8 in sheep and 1.1 in goats. All blood samples were negative as assessed by Ag-ELISA and RT-PCR. Fourteen out of 24 fetal samples were detected as antigen-positive by Ag-ELISA. The amplification was observed using pan-pesti primers in four tissue samples. All four samples were identified as border disease virus by discriminative RT-PCR. In conclusion, the moderate seroprevalence but low antibody titers detected in this study indicate that small ruminants in the South Marmara Region may be at risk of extensive new circulations of pestivirus infections.

Key words: Pestivirus, small ruminant, prevalence, RT-PCR, Ag-ELISA, virus neutralization

1. Introduction

Pestiviruses are economically important pathogens that not only cause clinical syndromes but are also subclinically shown in wild and domestic ruminants, pigs, and camelids worldwide. The genus *Pestivirus* belongs to the family *Flaviviridae* and contains four species, bovine viral diarrhea virus (BVDV-1 and BVDV-2), classical swine fever (CSF) virus, and border disease virus (BDV), which are closely related based on genetic and antigenic similarities between each other (1). Pestivirus-infected animals may have subclinical infections or may show clinical signs such as coughing, poor performance, an increase in the frequency of other infections, diarrhea, infertility, growth retardation and wasting, abortions, stillbirths, abnormal body conformations, persistent infection of fetuses, and mortality with or without the development of mucosal disease (2).

Pestiviruses are not host-specific pathogens. Although classification at early stages is based on host origin, BVDV species have been isolated from diverse domestic and wildlife animal species (3). Sheep and goats play a role in the transmission of this infection among ruminants (3,4). Moreover, natural infection of cattle with BDV and pigs with ruminant pestivirus species has also been shown (3,5,6). In addition to reported natural infections, there are a growing number of recommended pestivirus species subdivisions (7). Ruminant pestiviruses still lead to significant economic losses in cattle and sheep populations.

The detection of ruminant pestiviruses is based on virus isolation, immunoassays, and PCR techniques (8–10). The molecular analyses of partial or complete genomes of pestiviruses have been recently preferred by a majority of workers, because this method makes it possible to rapidly detect and discriminate the species of pestiviruses and even exhibit genomic differences in the genome of new strains. The different regions of the pestivirus genome, such as 5'UTR, N^{pro}, and E2, have been used for molecular detection (7). Although some limitations may occur, analysis of the 5'UTR sequence is favored and usually allows correct allocation of established isolates (7). In addition to molecular analyses, seroprevalence studies enable us to exhibit the prevalence of pestiviruses in a region.

Several European countries have organized BVDV eradication programs (10), but eradication of BDV has not been accomplished yet. Additionally, high seroprevalence rates and the possibility of persistently infected sheep may play an important role in the spread (11). Hence, demonstrating the epidemiological status of

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small ruminants in the field is important for the success of control application. The seroprevalence rates of pestiviruses in small ruminants before 2012 ranged between 0.0% and 78.5% (12,13) in Turkey. In this study, the molecular diagnosis and seroepidemiology of ruminant pestiviruses were investigated in sheep and goats in the South Marmara Region of Turkey, and current seroprevalence rates are presented.

2. Materials and methods

2.1. Animals and sampling

A total of 607 blood samples were collected from 386 sheep and 221 goats in 2015. The animals were handled in 22 different flocks located in four different provinces (Bursa, Balıkesir, Çanakkale, and Bilecik) in the South Marmara Region of Northwest Anatolia (Figure 1). In two of the 22 flocks, goats and sheep were kept mixed; all other flocks contained either sheep or goats. The sampling was performed randomly, but sex, age, and clinical health conditions were recorded. None of the flocks were vaccinated against pestiviruses, and none of the animals had clinical signs at sampling time. Of the 22 flocks sampled, 16 had a known history of abortion, while there were no abortion cases recorded in 6 flocks. All 607 blood samples were collected from the jugular vein into vacutainer tubes with and without anticoagulant. In addition to blood samples, 20 sheep and 4 goat tissue samples from Bursa and Balıkesir submitted for routine diagnosis were also investigated. Most of the fetal materials (19 lambs and 2 kids) were collected and tested between 2010 and 2014 (14).

2.2. Viruses and cell lines

Madin-Darby bovine kidney (MDBK) and sheep fetal thymus (SFT-R) cell lines were used for virus neutralization assays and for virus isolation. Cell lines were maintained in Dulbecco's modified essential medium (DMEM; Biological Industries, 11-050-1G) supplemented with 10% 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 before use in this study.

A noncytopathogenic strain of BDV (x-818 strain) as well as reference cytopathogenic strains of BVDV-1 (NADL) and BVDV-2 (Gi-2), which were propagated with the MDBK cell line, were used as control viruses.

2.3. Virus isolation and indirect immunoperoxidase monolayer assay (IIPMA)

MDBK and SFT-R cell cultures were used for virus isolation as described previously (14). For this purpose, after inoculation of 100 µL of sample, cell cultures (2×10^5 cell/mL) were cultivated at 37 °C in an atmosphere with 5% CO_2 for 7 days. The cells were harvested by freezing (–80 °C) and thawing (37 °C). Three blind passages were applied in both cell cultures. The cells that were negative for a cytopathogenic effect under microscopic evaluation were tested by immunoperoxidase assay as described previously (14). The culture fluids were used to infect MDBK cells in 24-well plates and incubated at 37 °C in a 5% CO_2 atmosphere for 72 h. After heat fixation at 80 °C for 3 h, O-D-glucopyranoside (0.5%, Sigma, USA) solution was added to the wells. In the next steps, monoclonal anti-BVDV mouse antibody (pool 1/4/7, kindly supplied

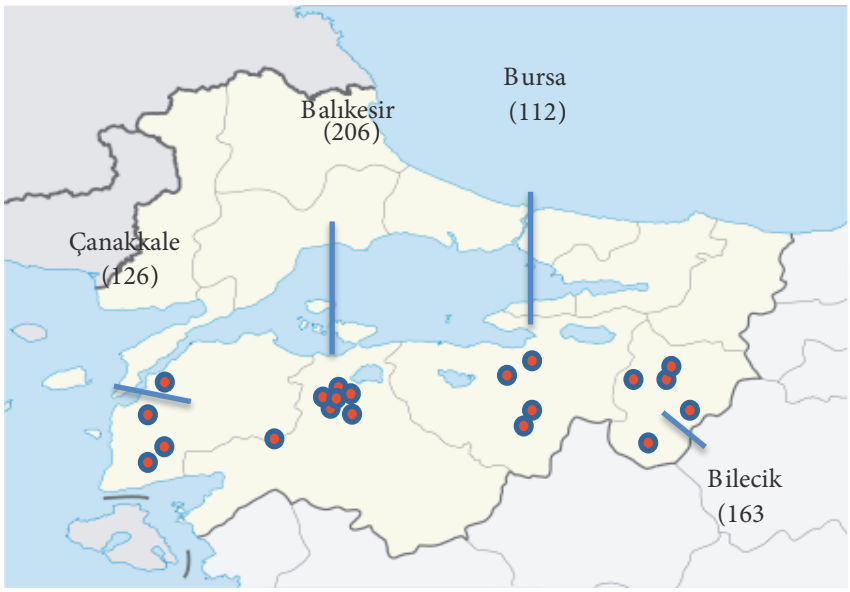

Figure 1. Distribution of the samples in the studied area.

by Justus Liebig University), biotin-labeled antimouse antibody (Pierce, 31800), and streptavidin-biotinylated-HRPO conjugates (Pierce, 21124) were added. At each step, 90 min of incubation and rinsing with Tween-PBS (0.05% Tween 20 in PBS) were performed. After adding substrate solution (2 mg of AEC in 0.3 mL of DMF, 4.7 mL of Na-acetate buffer [pH 5.5], and 0.05% H_2O_2), cells with intracellular reddish-brown staining were evaluated as positive.

2.4. Antibody detection

The serum neutralization 50 (SN_{50}) method was applied using the BVDV-NADL strain to investigate the antipestivirus antibody titers in sera samples as described previously (15). Briefly, heat-inactivated serum samples were diluted twofold, beginning from 1:8, placed in 2 parallel columns in 96-well microplates. Fifty microliters of each of the diluted serum and virus suspensions (100 $TCID_{50}$) were mixed. After incubation for 1 h in a 5% CO_2 atmosphere at 37 °C, MDBK cells (3×10^5 cells/well) were added. After 4 days of incubation in the same conditions, the highest serum dilution inhibited virus propagation and was evaluated as the antibody titer for the tested serum.

2.5. Detection of pestivirus antigens by ELISA

A commercial pestivirus antigen ELISA kit (Herdchek BVDV Ag test kit, IDEXX) was used to detect E^{rns} protein of pestiviruses in each individual sample. The tests were performed on plasma samples and fetal materials according to the recommendations of the manufacturer. Due to the absence of a specific ELISA kit for BDV, as confirmatory analysis, all the samples were also tested by RT-PCR using pan-pesti virus primers.

2.6. Viral RNA extraction and RT-PCR amplification

Nucleic acids were extracted from whole-blood samples from all the animals and from fetal tissues. Livers, spleens, kidneys, and lungs of fetal tissues were homogenized with a tissue homogenizer in 5 mL of phosphate-buffered saline (PBS) and centrifuged at 3000 rpm 4 °C for 10 min. Viral RNA was extracted using a NucleoSpin Virus DNA/RNA/protein purification kit (Macherey-Nagel, Germany). The precipitated RNA, dissolved in 30 µL of RNase-free water, was used for cDNA synthesis with the IScriptTM cDNA Synthesis Kit (Bio-Rad, USA) by using random hexamers. First, all the samples were evaluated using the pan-pestivirus primer pair 324 and 326 (P324: 5'-ATG CCC WTA GTA GGA CTA GCA-3' / P326: 5'-TCA ACT CCA TGT GCC ATG TAC-3') (16). The thermal protocol profile was 94 °C for 2 min, 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min with final incubation at 72 °C for 7 min. The 288-bp PCR products were visualized on a 2% agarose gel including Safe View Classic (Applied Biological Materials, Canada) for staining.

After pan-pestivirus RT-PCR was performed, the positive samples were subjected to BVDV-1, BVDV-2, and BDV RT-PCR analyses. Amplification of 221 bp of BVDV-1 and BVDV-2 products was generated using the primers B3 / B4 (5'-GGT AGC AAC AGT GGT GAG-3', 5'-GTA GCA ATA CAG TGG GCC-3') and B5 / B6 (5'-ACT AGC GGT AGC AGT GAG-3', 5'-CTA GCG GAA TAG CAG GTC-3'), respectively (17). The protocol was applied as follows: 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 51 °C for 1 min, and 72 °C for 1 min and finally 72 °C for 10 min. For the molecular detection of BDV, PBD1 / PBD2 primers were selected (PBD1 5'-TCG TGG TGA GAT CCC TGA G-3', PBD2 5'-GCA GAG ATT TTT TAT ACT AGC CTA TRC-3') (18), and the 225-bp product was amplified as follows: 95 °C for 5 min followed by 36 cycles at 94 °C for 1 min, 51 °C for 1 min, and 72 °C for 1 min and finally 72 °C for 7 min.

2.7. Statistical analysis

Statistical significance of the differences in seroprevalence values was determined using SPSS 23.0 (IBM Corp., Armonk, NY, USA). Pearson's chi-square test was performed, and differences were considered significant at values of $P < 0.05$.

3. Results

The seropositivity rate in the tested population was 32.78% (199/607); the rates in sheep and goats were 49.74% (192/386) and 3.17% (7/221), respectively (Figure 2). The rates of seropositivity in the examined herds ranged between 12.70% (Çanakkale) and 53.57% (Bursa) (Table 1). Among the seropositive animals, antibody levels ranged between titers of 8 and 1024 (Table 2). The geometric mean value of antibody titers was 6.8 for sheep and 1.1 for goats, and the average value was 3.5. The total number of seropositive animals housed in the flocks with a history of abortion was 143 out of 441 (32.43%), while 55 animals out of 166 (33.13%) housed in clinically healthy flocks were seropositive.

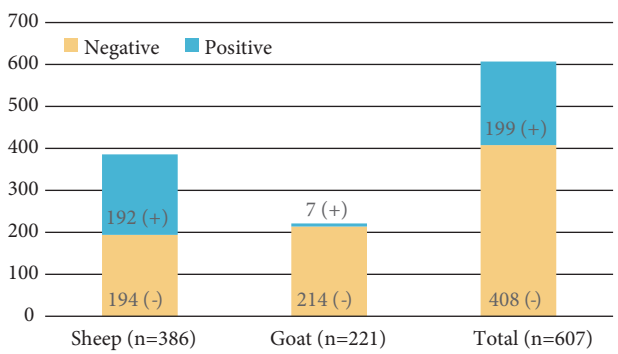

Figure 2. Comparative distribution of animal numbers according to serological status.

Table 1. Results from serological and virological analysis of tested blood samples.

Provinces	SN positive (%)	Ag-ELISA positive (%)	RT-PCR positive (%)
Bursa (n = 112)	60 (53.57%)	0 (0%)	0 (0%)
Balıkesir (n = 206)	63 (30.58%)	0 (0%)	0 (0%)
Bilecik (n = 163)	47 (28.83%)	0 (0%)	0 (0%)
Çanakkale (n = 126)	16 (12.70%)	0 (0%)	0 (0%)
Total (n = 607)	199 (32.78%)	0 (0%)	0 (0%)

Table 2. Distribution of antibody titers in sheep and goat samples.

Numbers	Antibody titers / percentage								
	8↓	8	16	32	64	128	256	512	1024
Sheep (n = 386)	194 / 50.3%	23 / 6.0%	27 / 7.0%	42 / 10.9%	46 / 11.9%	33 / 8.5%	16 / 4.1%	4 / 1.0%	1 / 0.3%
Goats (n = 221)	214 / 96.8%	2 / 0.9%	0 / 0.0%	0 / 0.0%	2 / 0.9%	1 / 0.5%	1 / 0.5%	1 / 0.5%	0 / 0.0%
Total (n = 607)	408 / 67.2%	25 / 4.1%	27 / 4.5%	42 / 6.9%	48 / 7.9%	34 / 5.6%	17 / 2.8%	5 / 0.8%	1 / 0.2%

Analyzing the 607 blood samples to demonstrate the rate of viremia in adult animals by antigen ELISA and RT-PCR produced no positive results. However, 14 positive results were obtained by Ag-ELISA in 24 tissue samples submitted for routine diagnosis. Using both the RT-PCR analyses applied with pan-pesti virus primers 324–326 and the discriminative molecular analyses with BVDV-1, BVDV-2, and BDV primers, only 4 tissue samples showed positive results (19). All 4 pan-pestivirus-positive tissue samples were detected as BDV (20).

Regarding the molecular results, all tissue samples were inoculated onto the MDBK and SFT-R cell lines. After 3 blind passages, no cytopathogenic virus propagation was detected, while noncytopathogenic virus growth was detected in only one sample by IIPMA (14).

4. Discussion

In this study, we investigated the frequency of pestiviruses and obtained current prevalence rates in the South Marmara Region. Although the mean antibody titers detected in this study are low, the percentages of seropositive animals are high in some provinces.

Seroprevalence in goats and sheep varies between 0.0% and 75.9% in different parts of Turkey (12,21,22). However, there was 30.2% prevalence in other sampled goats (23); 65.75% (24) and 75.9% (22) prevalence rates were found in sheep in the eastern part of the country. In inland areas, a prevalence rate of 69.3% was found in goats (25) and rates of 70.3% (26), 74.51% (27), and 78.5% (13) were found in sheep. These values decrease to 18.94% (21) and 42.8% (28) in northern Anatolia, respectively. A similar decrease in the seroprevalence rates is found in western Anatolia, where rates of 32.2% (29) and 45.87% (30) were obtained. Detected seroprevalence rates are higher in eastern Turkey than in western Turkey. The results of this study (seropositivity of 32.78%) also support this dynamic.

The neutralizing antibody responses in Bursa Province (53.57%) were higher than the rates from all of the other provinces in this study, and current data show an increase in seropositivity compared with previous reports (29,31). However, there is a decrease in the other provinces investigated in this study when the same reports are considered. This finding may be due to differences in the type of breeding, herd density, and distance between the herds. Further investigations are needed to confirm this.

On the other hand, seronegative animal numbers in randomly sampled goats were markedly higher than in randomly sampled sheep, and seropositivity numbers between sheep and goats were significantly different ($P < 0.05$). This result was similar to other published data (15,26,29). However, based on the neutralization test results, no significant difference was exhibited at the levels of serological responses between flocks with and without abortion history ($P = 0.869$).

Even though the antigen ELISA method has high sensitivity and specificity, incompatibility can be seen between this method and PCR (19). Molecular analyses were completed on all blood samples using primers 324–326 that could amplify all the pestiviruses (16). Although

the results of blood samples were similar with both ELISA and RT-PCR, the results of tissue samples with the same methods were incompatible.

Seroprevalences in the South Marmara Region of Turkey were not detected at extreme levels. Low levels of persistently viremic animals can be the reason for comparatively low (32.78%) seropositivity in the study area.

In conclusion, moderate rates of seropositivity detected in the region demonstrate that small ruminant populations in the region may be at high risk of circulation of virulent pestivirus strains.

Acknowledgment
This study was supported by the Uludağ University Research Fund (Project No: OUAP(V)-2014/19).

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