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Molecular diagnosis and seroepidemiology of pestiviruses in sheep

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ABSTRACT

In this research, the virological and serological presence of pestiviruses, such as border disease virus (BDV) was investigated in a sheep in the Central and Eastern Black Sea Regions of Turkey. The study material consisted of 40 organ materials collected from 13 aborted lambs, which were suspected to have pestiviruses, BDV and Bovine Viral Diarrhoea Virus (BVDV). Viral nucleic acids were investigated by using the reverse transcription polymerase chain reaction (RT-PCR). Nine of 13 aborted lambs (69.2%) and 24 of 40 organ samples (60%) obtained from those sheep were BDV RNA positive, whilst all the animals and organ samples were BVDV-RNA negative. Serum samples collected from 401 randomly selected sheep were investigated for pestivirus antibodies using competitive-ELISA (cELISA) and the serum microneutralisation test (SNT) using BVDV-NADL strain. Seropositivity was found in between 7.22 and 74.38% with cELISA and 4.81 and 67.76% with SNT. Seropositivity rates in Amasya and Tokat provinces were higher than in Samsun and Giresun. The obtained data indicated that pestivirus infection in sheep is widespread in the Central and Eastern Black Sea Region.

Key words: ovine, pestiviruses, border disease virus, polymerase chain reaction, ELISA

Introduction

Pestivirus genus is a member of the family *Flaviviridae* and contains a single stranded RNA genom (LINDENBACH and RICE, 2001; LIU et al., 2009). The viruses of this genus are divided into four well-defined species: Bovine viral diarrhea virus type 1 and 2 (BVDV-1 and 2), border disease virus (BDV), and classical swine fever virus (CSFV) (HEINZ et al., 2000; KRAMETTER-FROETSCHER et al., 2007). In addition, phylogenetic analyses have shown that BDV may be divided into at least three different genotypes, such as BDV-1, 2 and 3 (MUDRY et al., 2010). Pestiviruses, apart from CSFV, can cross the host species barrier. BVDV can infect sheeps, goats, cattle, pigs and wild animals, such as deer and wild boars, while BDV may be an important source of infection for calves

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(PATON et al., 1997; KRAMETTER-FROETSCHER et al., 2007; MARCO et al., 2008; LIU et al., 2009; KRAMETTER-FROETSCHER et al., 2010). Pestiviruses can cause economic losses by affecting the reproductive system of sheep and cattle in the animal industry. Vertical transmission and persistently infected (PI) animals are a major source of pestivirus infections in flocks and herds. The use of common pasture with PI infected animals in the summer season is the most important risk factor for the transmission of pestiviruses within a population. PI sheep may also be a potiential source of pestivirus transmitter for the cattle.

BDV is distributed worldwide and causes abortions, still birth, blind lambs, abnormal body configurations and congenital anomalies in sheep and goats during early or mid gestation (GARCIA-PEREZ et al., 2009; ORSEL et al., 2009). The presence of pestiviruses has been investigated previously in many studies in Turkey (BURGU et al., 1987; BURGU et al., 2001; GUMUSOVA et al., 2006; GÜR, 2009) and seropositivity rates of pestiviruses have been found between 3 and 78.5%. The aim of this study was to investigate the pestivirus infection in aborted fetuses and to update the serolocigal status of pestivirus in sheep in the Central Black Sea Region of Turkey.

Materials and methods

Cell cultures and viruses. The Madin Darby Bovine Kidney cell culture (MDBK) was used for the neutralization tests. Cells were grown in Dulbecco's minimum essential medium (DMEM, Biological Industries, Israel) with 10% fetal calf serum (FCS, Biological Industries, Israel) and 1% penicillin/streptomycin (Biochrome, KG).

Due to the antigenic relationship and BVDV presence, sheep may be infected; a cytopathogenic NADL (National Animal Disease Laboratory) strain of BVDV was used in the microneutralisation tests (BURGU et al., 1987; GUMUSOVA-OKUR et al., 2006; SALEM et al., 2010). The virus was propagated in MDBK cell line. Before the experiments, all growing cell line, mediums and FCS were controlled for non-cytopatchic BVDV

Preparation of organ materials. Fourty organ specimens [13 lung (L), 13 liver (Lv), 12 brain (B) and 2 spleen (S)] obtained from 13 aborted fetuses from different provinces, were analysed to detect BDV and/or BVDV. These materials were homogenised in PBS with homogenisator and centrifuged at 1500 g at 4 °C for 15 min. The supernatants were filtered in a 0.45 μm filter and 2 mL of each supernatant was put into sterile cryovials, and stored at -80 °C until the RT-PCR studies.

Serum specimens. For serological screening, blood samples were collected from a total of 401 randomly selected sheep from eight herds (two herds for each province) located in four different provinces of the Central Black Sea Region, Turkey, and transported to the laboratory at 4 °C. On arrival at the laboratory, the blood samples were centifuged at 2500

rpm for 15 mins at 4 °C. The obtained sera were separated into vials, heat inactivated at 56 °C and stored at -20 °C until tested.

RNA extraction and cDNA synthesis. Organ samples were extracted using the protocol described by CHOMCYNSZKI and SACCHI (1987) for BVDV and BDV-RNA. Briefly, 400 µL supernatant from the organ homogenizat was mixed in denaturing solution of 4 M guanidinium-isothiocyanate, 0.5% laurosylsarcosine (Sarcosyl), and 0.1M β-mercaptoethanol in 25 mM sodium citrate (pH 7.0). Then, RNA was precipitated twice with isopropanol and washed in 70% ethanol. The RNA pellet was air dried, resuspended in 20 µL RNAse free water and was used for cDNA synthesis. cDNA synthesis was carried out using a first strand cDNA synthesis kit (Fermentas, Lithunia) as described in the manufacturer's protocol, using random primers. The reaction mixture was incubated first at 25 °C for 10 mins, followed by a second incubation at 42 °C for anhour and then 70 °C for 10 min, for inactivation on Moloney murine leukaemia virus reverse transcriptase (MMLV-RT).

Reverse transcriptase PCR (RT-PCR) assay. The polymerase chain reaction amplification was performed as described elsewhere (VILCEK and PATON, 2000) using PBD1 (5´- TCG TGG TGA GAT CCC TGA G -3´) and PBD2 (5´- GCA GAG ATT TTT TAT ACT AGC CTA TRC -3´) primers flank a 225 bp DNA fragment for BDV and using (5´- GCA GAT TTT GAA GAA AGA CAC TA- 3´) and (5´- TTG GTG TGT GTA AGC CCA -3´) primers flank a 402 or 680 bp DNA fragments (to determine ncp or cp strain of fields viruses) for BVDV (GREISER-WILKE et al., 1993). PCR was carried out in a total volume 30 µL containing 3 µL of cDNA to the master mix, 3 µL of 10 X reaction buffer, 2.4 µL of $MgCl_2$, 10 pmole from of each of the primers, 0.5 µL of dNTP mix, and 0.25 µL of Taq DNA polymerase (MBI, Fermentas, Lithuania), 19.85 µL of RNAse free distiled water. The steps of amplification on the thermal cycler (Thermo, USA) were set up as follows: reverse-transcription at 94 °C for 6 min, 35 cycles of denaturation at 94 °C for 45s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. Amplification was terminated by the final step at 72 °C for 10 min. The PCR products were analysed in 2% agarose gel, containing 0.5 µg/mL ethidium bromide stain at 80 V for 30 min and were visualised under ultraviolet light.

Competitive-ELISA (cELISA). A commercial cELISA (ELISA BVD/MD/BD p80 Antibody Screening, Institute, Pourquier P00645/03, France) test kit was used for pestivirus antibodies in sheep. The test kit is based on the principle of competitive enzymatic immunoassay between pestivirus antibodies in serum samples and peroxidase coupled monoclonal anti-P80 antibody. WB112 Tests were performed according to the manufacturer's recommendations. For the test validation, OD 450 of the negative control is a minimum 0.800 and PI% of the positive control is lower than 20%. For

the interpretation, the percentage inhibition (I%) for each serum sample was calculated according to the formula: I% = OD of serum samples/OD of negative control ×100.

If I% of serum samples is higher than or equal to 50% they were considered seronegative. If I% of serum samples is between 40% and 50% they were accepted doubtful and with I% of serum samples lower than or equal to 50% they were considered seropositive. According to literature provided by manufacturer, the sensitivity and specifity of ELISA was 97.6-97.2% respectively, compared to the SNT.

Serum neutralisation test. Antibodies against pestiviruses were investigated by the microneutralisation test protocol described by FREY and LIESS (1971) using MDBK cells in 96 well microplates (Grainer, Germany). Briefly, 50 µL of each serum were added to wells, mixed with equal well volume of 100 $TCID_{50}$ dilution of the virus strain and incubated for 1 h at 37 °C. Afterwards, MDBK cells were distrubuted into the wells. Test plates were incubated at 37 °C, with 5% CO2 atmosphere for 3 days until CPE was detected in the positive control.

Results

RT-PCR. Thirteen aborted lamb and 40 organ materials taken from them were analysed by RT-PCR. BDV-RNA was detected in 69.23% (9/13) of the lamb and 60% (24/40) of the organ materials (Table 2). BVDV-RNA was found to be negative in all specimens. BDV RNA was detected in 69.23% (9/13) of lung specimens, 66.66% (8/12) of brain specimens and 61.53% (8/13) of liver specimens while all spleen specimens were found to be negative (Table 1). The amplicon size 225 bp DNA product of BDV was observed in all the positive samples (Fig. 1) while DNA product of BVDV was not detected (Fig. 2).

Fig. 1. RT- PCR test on samples for BDV. Lane M: 100 bp DNA ladder, Lane 1: Positive control, Lanes 2-7: BDV positive specimens, Lane 8: Negative control.

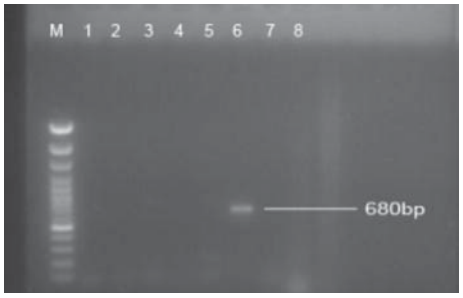

Fig. 2. RT- PCR test on samples for BVDV. Lane M: 100 bp DNA ladder, Lane 6: Positive control, Lanes 1-5 and 8: BDV positive specimens, Lane 7: Negative control.

Table 1. Geographical distirbution of tested animals and results of RT PCR according to organ specimens

Provinces	Animal	Organ specimens	RT-PCR	
			BDV	BVDV
Sinop	Lamb	B, Lv, L	+ + +	- - -
	Lamb	B Lv, L	+ + +	- - -
Samsun	Lamb	B, Lv, L, Sp	+ + +-	- - - -
	Lamb	B, Lv, L, Sp	+ + + -	- - - -
Amasya	Lamb	B, Lv, L	+ - +	- - -
	Lamb	B, Lv, L	- + +	- - -
	Lamb	B, Lv, L	+ + +	- - -
Trabzon	Lamb	B, Lv, L	+ + +	- - -
	Lamb	B, Lv, L	+ + +	- - -
Rize	Lamb	B, Lv, L	- - -	- - -
Corum	Lamb	Lv, L	- -	- -
Tokat	Lamb	B, Lv, L	- - -	- - -
Sivas	Lamb	B, Lv, L	- - -	- - -

B: Brain, L: Lung, Lv: Liver, Sp: Spleen

Table 2. BDV-RNA positivity rate according to organ specimens

Organs	Tested	Positive (%)
Lung	13	9 (69,23)
Liver	13	9 (61,53)
Brain	12	8 (66,66)
Spleen	2	-
Total	40	24 (60.00)

Table 3. The prevalence of pestivirus antibodies in sheep in the Central Black Sea Region of Turkey: comparison of ELISA and SNT results

Provinces	cELISA		SNT*	
	Tested	Positive (%)	Tested	Positive (%)
Samsun	83	11 (7.22)	83	4 (4.81)
Tokat	121	90 (74.38)	121	82 (67.76)
Amasya	123	86 (69.91)	123	78 (63.41)
Giresun	74	11 (14.86)	74	8 (10.81)
Total	401	193 (48.12)	401	172 (42.89)

* BVDV-NADL strain was used in SNT

ELISA and neutralisation. A total of 401 sera were examined for pestivirus antibodies using cELISA, and the microneutralisation test with BVDV-NADL as detailed in Table 2. The overall seroprevalance of pestivirus in sheep was 48.12% (193/401) with cELISA and 42.89% (172/401) with SNT. All cELISA positive specimens were also found to be positive for SNT, except eleven specimens which had positive results by cELISA. Seropositivity rates were between 7.22%-74.38% with cELISA and 4.81-67.76% with SNT (Table 3). Pestivirus seropositivity was found to be 7.22% in Samsun, 14.86% in Giresun, 69.91% in Amasya and 74.38% in Tokat with cELISA, while it was 4.81%, 10.81%, 63.41% and 67.76% with SNT, respectively.

Discussion

In recent years abortions, mumified fetuses, stillbirths, the birth of blind lambs, weak lambs with congenital anomalies and central nervous system disorders have increased in the Central Black Sea Region of Turkey, and pestiviruses, especially BDV, are one of the main causes of these symptoms. Different materials such as blood, tissue, and organ specimens may be used for laboratory diagnosis of pestivirus infection in sheep. RT-PCR has been widely used for organ materials and blood specimens for pestivirus detection (GARCIA-PEREZ et al., 2009).

In this study, virological diagnosis was targeted at organ materials of aborted sheep fetuses using RT-PCR. For this purpose a total of 40 organ materials from the 13 aborted fetuses were collected. The suitability of these materials has been verified by many researchers (VILCEK et al., 1997; WILLOUGHBY et al., 2006; DUBOIS et al., 2008) and BDV-RNA was identified in 9 (69.23%) of the 13 aborted lambs and 24 (60%) of the 40 organ materials collected from them, by the RT-PCR test, while BVDV-RNA was found negative in all specimens. These data were similar to the results of many other researchers (VILCEK and PATON, 2000; WILLOUGHBY et al., 2006; DUBOIS et al., 2008; OGUZOGLU et al., 2009). According to the test results obtained in our study, the rate of BDV RNA positivity in the organ materials tested was 69.23% in the lungs, 61.53% (8/13) in the livers, and 66.66% (8/12) in brain specimens while all spleen samples were negative. 225 bps long DNA amplicons detected in the specimen were identified as BDV RNA positive. Similar results were reported by OGUZOGLU et al. (2009).

Seroprevalance studies are important to identify the pestivirus infections (MISRA et al., 2009). cELISA and neutralization tests are reliable for the detection of antibodies against pestiviruses. Especially, cELISA is the more sensitive and specific test in comparison to SNT and it has been used in several countries implementing control and eradication programmes (MISRA et al., 2009). During recent years, there have been many serological studies about pestivirus infections in lambs and sheep in Turkey (BURGU et al., 1987; BURGU et al., 2001; GUMUSOVA-OKUR et al., 2006; GÜR, 2009) and several countries like Norway (LÖKEN et al., 1991), Spain (HURDATO et al., 2003; VALDAZO-GONZALEZ et al.,

2008), Italy (DE MIA et al., 2005), Switzerland (STALDER et al., 2005), Tunisia (THABTI et al., 2005) and Austria (KRAMETTER-FROETSCHER et al., 2008). In our study, the regional seroprevalance of pestivirus was investigated in 401 randomly selected sheep from 8 herds and 4 provinces in the Central Black Sea Region using ELISA and SNT. Overall seroprevalence rate was identified as 48.12% (193/401) with cELISA and 42.89% (172/401) with SNT. BURGU et al. (2001) investigated serologically the existence of pestivirus using a BVDV/NADL reference strain with SNT in blood specimens collected from rams, lambs, and ewes in nine flocks in Turkey. That study showed that the seropositivity rate of rams was 20.6% (6/29), while it was 21.5% (142/661) in sampled pregnant sheep. GUMUSOVA et al. (2006) studied pestivirus seroprevalance using a BVDV-NADL strain with SNT in sheep from coastal and inland parts of the Black Sea region in Turkey and found 18.94% (463/2444) pestivirus seropositivity. In the Western Anatolia Region of Turkey, 78.5% (446/568) pestivirus seropositivity in sheep was reported by GÜR et al. (2009). In addition to these results, it was concluded that the cELISA technique used in the serodiagnosis of pestivirus infection is of high sensitivity and diagnostic value in this study, when compared to microneutralization.

In the present study, the overall 48.12% pestivirus seropositivity rate in sheep is higher than 21.5% reported by BURGU et al. (2001) and 18.94% reported by GUMUSOVA et al. (2006) in previous studies in Turkey. Our seropositivity results are also higher than the 18% reported in Norway (LÖKEN et al., 1991), 46% reported in Ireland (O'NEILL et al., 2004), 17.6% reported in Spain (VALDAZO-GONZALEZ et al., 2008), 16.32% reported in Switzerland (DANUSER et al., 2009), and 23.4% reported in India (MISRA et al., 2009). There are many factors in the epidemiology of pestivirus infections. PI (persistence infected) animals, uncontrolled animal movement and interspecies transmission are important for the spread of infection. PI animals are a major cause of spreading the infection and therefore, they represent a risk to the flock (LÖKEN et al., 1991). Uncontrolled animal movements are less in coastal areas of the Black Sea Region compared to inland areas in the present study. The results of the seroprevalence study showed that the infection rate in inland provinces (Amasya and Tokat) was higher than in the costal areas (Samsun and Giresun).These results reveal that the number of lamb and animal movements are more intense in inland provinces with a higher infection rate. One of the other important factors in the spread of infection is that sheep and cattle are bred on the same farms and also share the same pasture.This situation may lead to interspecies transmission and may increase the incidence of pestivirus infections (GÜR, 2009).

In our study, the existence of BDV infection in the Middle and Eastern Black Sea Region was identified virologically using molecular techniques and it was also confirmed serologically. It also proved again that the cELISA and SNT techniques were sensitive methods when used in serological studies. All of these methods may be successfully applied to identify PI animals in a flock, which is a major cause of the spread of infection.

In conclusion, the results of this study show that pestivirus infection has an increasing seroprevalence in the Central and Eastern Black Sea region. Therefore, it appears to be inevitable that there will be very high rates of pestivirus infections in both small and large ruminants very soon. This situation may be very harmful for Turkey's economy. Previous studies and our study results indicate the need for a comprehensive eradication program to prevent pestivirus infections in Turkey.

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SAŽETAK

U radu su prikazana virološka i serološka istraživanja pestivirusa odnosno borderske bolesti u ovaca na središnjem i istočnom području Crnoga mora u Turskoj. Ukupno je bilo pretraženo 40 uzoraka tkiva različitih organa uzetih od 13 pobačenih plodova pod sumnjom da je uzročnik bio virus borderske bolesti ili virus virusnog proljeva goveda. Uzorci su bili pretraženi na prisutnost virusne nukleinske kiseline lančanom reakcijom polimerazom uz prethodnu reverznu transkripciju (RT-PCR). Devet od 13 pobačenih plodova (69,2%) i 24 od 40 uzoraka tkiva organa (60%) sadržavalo je RNA virusa borderske bolesti. Svi pretraženi uzorci bili su negativni na virus virusnoga proljeva. Uzorci seruma nasumce prikupljeni od 401 ovce bili su pretraženi na prisutnost specifičnih protutijela uporabom kompetitivnoga imunoenzimnoga testa (cELISA) i serum neutralizacijskoga testa (SNT) sa sojem NADL virusa virusnoga proljeva goveda. Ustanovljeno je da se

postotak serološki pozitivnih cELISA-om kretao od 7,22 do 74,38%, a SNT-om od 4,81 do 67,76%. Postotak serološki pozitivnih životinja u provincijama Amasya i Tokat bio je veći nego u provincijama Samsun i Giresun. Rezultati upućuju na zaključak da su pestivirusne zaraze rasprostranjene u ovaca na središnjem i istočnom području Crnoga mora.

Ključne riječi: ovce, pestivirusi, borderska bolest, lančana reakcija polimerazom, ELISA