Molecular and serological investigation of Bovine Viral Diarrhea Virus in dairy cattle in Konya province

Research Article

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ABSTRACT

Bovine viral diarrhea virus (BVDV) infection is a viral disease observed in many parts of the world and causing significant economic losses in dairy cattle enterprises. The present study was carried out to determine the seropositivity of BVDV and perform the molecular detection of the virus in cattle in dairy enterprises situated in Konya province and its surroundings in the Central Anatolia Region. In this cross-sectional study performed between March 2017 and April 2019, a total of 393 serum samples were collected from twenty-four dairy cattle enterprises by random sampling. The presence of antibodies in the obtained blood serum samples was investigated by the virus neutralization test using NADL, the BVDV reference strain. Samples were controlled for BVDV specific antibody (Ab) presence and titter values using serum neutralization test. The serum samples were examined in terms of the presence of BVDV-specific antigens and specific RNA using a commercial ELISA kit and the RT-PCR method, respectively. According to the results of the analysis, the animal and herd-level seropositivity was 55.72% (219/393) and 79.16% (19/24), respectively. It was revealed that seropositivity between age groups was statistically significant (χ2:11.81; p=0.002). Moreover, the samples were determined to be 45.13%, 60.53%, and 73.07% seropositive in the 6 months-2 years, 2-5 years, and above 5 years age ranges, respectively. It was revealed that all of the samples tested to detect persistently infected animals were negative for antigen and BVDV-specific RNA. As a result, it indicates the presence of BVDV infection in dairy cattle enterprises in Konya province. Therefore, it is essential for the country's economy to prevent the spread of the infection in question and implement voluntary eradication programs.

Keywords: Bovine viral diarrhoea virus, ELISA, RT-PCR, seropositivity, virus neutralization test, cattle

INTRODUCTION

Bovine viral diarrhea (BVD) is a viral disease that leads to poor reproductive performance, low milk yield, abortions, congenital anomalies, early embryo deaths, and births of persistently infected (PI) calves in adult cattle and causes considerable economic losses in cattle breeding worldwide (Handel et al., 2011; Ran et al., 2019; Şevik, 2021).

Bovine viral diarrhea virus (BVDV) belongs to the *pestivirus* subgroup of the *Flaviviridae* family. The viral genome is approximately 12.5 kb in size and has a large open reading frame (ORF) ending in the 5' and 3' non-coding end (Kokkonos et al., 2020). BVDV, which is in antigenic affinity with classical swine fever and border disease viruses, has two biotypes: cytopathic and non-cytopathic according to the proliferation status in cell cultures (Schweizer et al., 2006; Abe et al., 2016).

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The infection has three clinical forms: (a) acute transient form, (b) PI form, and (c) mucosal disease form characterized by severe lesions in the oral and intestinal mucosa, diarrhea, and death (Handel et al., 2011).

Fetal infections and births of PI calves are important in the epidemiology of BVDV. The causative agent is spread with secretions and extracts such as nasal discharge, semen, stool, tear discharge, blood, and milk (Passler et al., 2007; Yavru et al., 2013). The virus can be transmitted vertically to the fetus in pregnant animals. The timing of transmission of BVDV infection is of critical importance to the epidemiology of the disease. Infection occurring in the first 3 months of pregnancy may result in fetal death or the birth of a live PI calf permanently infected with BVDV due to the non-development of fetal immune competence. PI animals play the role of the most important source of transmission of BVDV since they carry the causative agent throughout their lives and spread it with their whole-body secretions (Houe, 1999; Scharnböck et al., 2018).

The complex pathogenesis of the disease caused by BVDV and sometimes the insidious nature of infections caused by BVDV in cattle represent a significant challenge for diagnosis (Horner et al., 1995). The virus neutralization test (VNT) is among the tests with high sensitivity and specificity in the detection of BVDV and is accepted as the gold standard (Kampa, 2006). Nowadays, ELISA tests are also employed to diagnose BVDV due to their advantages, such as obtaining faster results (Yavru et al., 2013). Moreover, it has become possible to detect the nucleic acids of the virus and diagnose the disease in a short time owing to technological developments (OIE, 2018).

For a voluntary BVDV eradication project, which is likely to be established on a national basis in the future, information on the epidemiological course of the disease on a regional basis is required to set priorities and raise the awareness of breeders. Thus, this study aimed to investigate BVDV molecularly and serologically in dairy cattle enterprises in Konya province and its surroundings in the Central Anatolia Region.

MATERIAL and METHOD

Sample collection and study design

This cross-sectional study was carried out on 393 animals aged six months and above in twenty-four dairy cattle enterprises in Konya province and its surroundings located in the Central Anatolia Region between 36°41' and 39°16' north latitudes and 31°14' and 34°26' east longitudes between March 2017-April 2019. In the sampled family enterprises with the number of animals in the range of 5 to 50 animals, the enterprise owners declared that cattle were not vaccinated against BVDV. The minimum sample size required was calculated as a total of 393 animals based on a mean expected BVDV prevalence of 50%, a confidence interval of 90%, a desired relative precision of 5%, and a design effect value of 1.45 by taking into account previously determined prevalence rates (McDermott and Schukken, 1994; Yavru et al., 2005, Avcı and Yavru, 2013; Dean et al., 2013). 10 ml of blood was drawn from the *Vena jugularis* of animals in accordance with animal welfare. Blood was centrifuged at 2500 rpm for 10 minutes to obtain serum. After blood serum were inactivated by being kept at 56 ^{0}C for 30 min, they were stored in a deep freezer at -20 ^{0}C until the test time. As part of the routine veterinary surgeon, the bloodletting process was performed with the consent of the animal owners to blood sampling and under the international recommendations (NRC, 2011; ADSA, 2020).

Laboratory analysis

Cell culture and test virus

Virus titration and VNT were performed with Madin Darby Bovine Kidney (MDBK) cells (ATCC CCL-22) and NADL strain of BVDV. Cells were cultured in Eagle's minimal essential medium supplemented with 2% penicillin/ streptomycin (Sigma-Aldrich, MO, USA) and 10% fetal calf serum. To apply the VNT test, tissue culture infective dose 50% ($TCID_{50}$) of the NADL strain was calculated by Spearman and Karber method (Hierholzer and Killington, 1996), and titer of strain was estimated as $10^{4.5}$ $TCID_{50}$/0.1ml. All positive serum samples were subsequently diluted in rates of 1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, and tested again for the determination of titter values. As a result of the test, 1/5 above dilutions were accepted as positive.

Virus neutralization test

VNT is a highly sensitive and reliable method for the detection of cytopathogenic viruses, in the controls of specific antibodies. To detect neutralizing antibodies specific to the BVDV, the micro-virus neutralization test was applied according to the method described by Frey and Lies (1971). First of all, serum samples (50 µl) were twofold diluted in 96-well plates and then 100 $TCID_{50}$ of the NADL strain (50 µl) was added to each well. Virus and serum mixtures were incubated in incubator (Heal Force, Lishen, Shanghai Lishen Scientific Equipment Co. Ltd., Chin. Model HF151UV) at 37 °C with 5% CO_2 for 2 hr. Following incubation period, 50 µl MDBK cell suspensions (300,000 cell/ml) were added to each well, and plates were incubated for 3-5 days. Results were evaluated based on the absence or presence of cytopathological changes in cells.

Test results were evaluated on the basis of micromorphology of cells using inverted microscope (Olympus IX71, Japan. Model IX71 S8F-3). Later on, all Ab positive serum were diluted in a series as 1/5, 1/10….1/320 for BVDV and test applied to determine Ab titer values (50% serum neutralization test (SN_{50})).

Results of the SN_{50} were calculated according to the method described by Reed and Muench (Lorenz and Bögel, 1973). The virus used in present study are in cytopathogenic nature, thus, inhibition of virus growth indicated by non-destructed monolayers of cell cultures was evaluated as indicator of virus neutralization. For scoring a sample as positive for the investigated antibodies, both wells used for the same sample were asked to be free of cytopathogenic effect.

Antigen ELISA

Serum samples were examined for the presence of BVDV antigen using a commercial antigen kit (IDEXX BVDV Ag/Serum Plus IDEXX Laboratories, Westbrook, Maine, USA). Analysis was done on serum samples according to the instructions provided by the manufacturer. Ag ELISA results were calculated for each sample

$$S\text{-}N = ODS_{450} - ODN_{450controlmean}$$

where OD450: optical density at 450 nm of the sample (S) and negative control mean (N). The samples with the values of 0.30 and below were evaluated as negative and above 0.30 as positive

RNA extraction and one-step RT-PCR

Viral RNA in the serum samples of animals was obtained using a commercial extraction kit (RNeasy Mini Kit, Qiagen, Germany). Extraction was performed following the manufacturer's instructions. One-step RT-PCR analysis was conducted using the primers shown in Table 1. In the analysis, nuclease-free water was used as a negative control, and the RNA of the BVDV NADL strain obtained from Selçuk University, Faculty of Veterinary Medicine, Department of Virology was used as a positive control. One-step RT-PCR reaction

was performed using a commercial kit (OneStep RT-PCR Kit, Qiagen, Germany). To this end, a 25 µl reaction mix was prepared, containing 5 µl of 5 x RT-PCR buffer, 1 µL of the enzyme, 1 µM of each primer, and 2.5 µL of the sample RNA. Amplification was performed in a Techne Prime thermal cycler (BIO-RAD Model, T100 Thermal Cycler, Singapure) under the following conditions: 30 minutes at 50 °C and 15 minutes at 95 °C (reverse transcription), followed by 40 cycles including 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min with a final extension of 10 min at 72 °C (Vilcek et al., 1994). PCR products were examined by electrophoresis on a 1.5% agarose gel stained with Gelred (Biotium, USA) at 90 Volts for 1 hour.

Table 1: Details of primers used to detect BVDV by One step RT-PCR

Primer	Primer sequence	Amplikon size	Reference
324F	5' ATGCCCTTAGTAGGACTAGCA 3'	288 bp	Vilcek et al. (1994)
326R	5' TCAACTCCATGTGCCATGTAC 3'		

Statistical analysis

In the present study, the statistical analysis of the data was conducted using the R program (V: 3.6.0). Chi-squared tests were employed in the analysis of differences in the age groups. A value of $p<0.05$ was considered statistically significant.

RESULTS

Serological findings

The serum neutralization test applied to the 393 cattle blood serum samples were found to be positive for pestivirus specific antibodies in 219 (55.72%) of them. At the herd level, the presence of pestivirus antibodies was detected in 19 (79.16%) of 24 dairy cattle enterprises.

As a result of the microneutralization test applied to two-fold dilutions of blood serums in which the presence of BVDV-specific antibodies was detected, it was revealed that the BVDV antibody titer had antibodies in titers ranging from 1:5 to 1:160 and the peak value was 1:5. SN_{50} values were found as 1/5 in 29.68% of the total 219 serum samples found seropositive, 1/10 in 27.85%, 1/20 in 21.92%, 1/40 in 8.22%, 1/80 in 6.85%, and 1/160 in 5.48% (Figure 1).

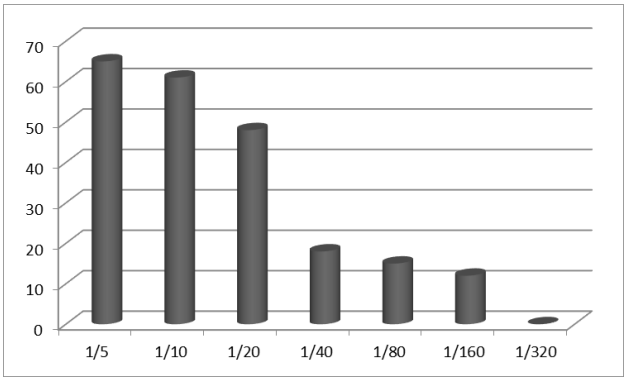

Figure 1: Ab titer distribution of BVDV seropositive samples (%)

Seropositivity was 45.13%, 60.53%, and 73.07% in the age ranges of 6 months-2 years, 2-5 years, and above 5 years, respectively. A statistically significant difference was revealed when the seropositivity rates in animals were compared based on age ($p<0.05$). (Table 2).

Antigen and BVDV-specific RNA detection

BVDV-specific RNA and antigen could not be detected in the 393 serum samples analyzed.

Table 2: Distribution of antibodies against BVDV in age groups of the tested animals

Age	No of tested	Pozitive	Negative	%
06 months -2 years	144	65	79	45.13
2-5 years	223	135	88	60.53
>5 years	26	19	7	73.07
Total	393	219	174	55.72
($\chi 2$:11.81, p=0.002)				

DISCUSSION

BVDV infection is among the viral infections that are quite common in Turkey, as in many countries of the world, and cause economic losses. The infection is usually subclinical in adult animals and is among the most important problems of cattle breeding as a herd problem due to its latent and persistent characteristics. In a study conducted using a survey and literature review on the prevalence of BVDV and covering the years 1960-2017, 88 countries confirmed the infection, and 107 countries reported infection reduction activities (Richter et al., 2019). The results of studies carried out worldwide in Brazil (48.8%) (Almeida et al., 2013), Bangladesh (51.1%) (Uddin et al., 2017), and Iran (54.3%) (Ghaemmaghami et al., 2013) and the results of this study are similar. However, studies performed in Uruguay (69%) (Guarino et al., 2008), Mexico (78.8%) (Milián-Suazo et al., 2016), and Ethiopia (69.8%) (Aragaw et al., 2018) revealed a high rate of seroprevalence.

In summary, BVDV seroprevalence studies carried out in various provinces and regions of Turkey reported seropositivity of 40.83% (İnce 2020), 74.9% (Gür, 2018), and 84.6% (Erol et al., 2014) in Afyonkarahisar, 68.51% in Antalya province and its surroundings (Demirsoy and Mamak, 2020, 86% in Aydın (Tan et al., 2006), 75.22% in Burdur (Bilgili and Mamak, 2019), 44.09% (Yavru et al., 2005), 46.22% (Avcı and Yavru, 2013), and 79.5% (Şimşek and Öztürk, 1997) in Konya and its surroundings, 20.19% in the provinces in the Black Sea region (Yazıcı et al., 2007), 96.8% in the Eastern and Southeastern regions (Çabalar and Karaoğlu, 1999), and 81.62% in the northeastern Anatolia region (Yıldırım and Burgu, 2005). The prevalence of BVDV infection in this study was determined as 55.72%. This rate is consistent with the rate obtained by Yavru et al., (2005), Avcı and Yavru (2013) in their study performed in Konya province and its surroundings. Upon evaluating these seropositivity rates, it is observed that BVDV infection is observed on the basis of provinces and regions with variable rates of prevalence in Turkey and in the world, and even studies conducted in the same region or province have determined different positivity values. These heterogeneities in seroprevalence studies in Turkey and in the world may originate from the different test methods used in the study, different regions of the country, time, and sample design.

In this study, in the distribution of the seropositivity of BVDV infection by age groups, 65 (45.33%) of the 144 animals in the age group of 6 months-2 years were seropositive and 79 (54.67%) animals were seronegative, 135 (60.53%) of the 223 animals in the age group of 2-5 years were seropositive and 88 (39.47%) were seronegative, and 19

(73.07%) of the 26 animals in the age group of 5 years and above were seropositive and 7 (26.93%) were seronegative. The lowest seropositivity rate among the age groups was 45.33% in the age group of 6 months-2 years, while the highest seropositivity was 73.07% in the group with the age of 6 years and above. The difference between the age groups was statistically significant ($p<0.05$). Moreover, the relationship between age and seropositivity and seronegativity indicated that seronegativity decreased as age increased. As the age increases, the disease may be observed more commonly in elderly animals, depending on the increase in the rate of infection in animals and the immunological status. The results of our study are consistent with the high incidence of infection in adult animals, as reported in other studies (Mockeliuniene et al., 2004; Bilgili and Mamak, 2019; Erfani et al., 2019; Hou et al., 2019).

Additionally, a high rate of seropositivity in a herd is regarded as an indicator of the presence of PI animals in that herd, and the prevalence of PI animals in the herd varies by 1-2% worldwide (Houe, 1999). The PI rate for BVDV infection in Turkey varies between 0.07-4.9% (Avcı and Yavru, 2013). Burgu et al. (2003) found the PI rate as 0.61-0.83% in their study from the samples collected from dairy cattle enterprises in different regions of Turkey, and Yılmaz et al. (2012) determined the PI rate as 0.5% in blood samples collected from four different regions. Moreover, Şimşek and Öztürk (1997) reported the PI rate as 0.7% and Bulut et al. (2006) as 0.1% in Konya province. This study detected no PI animals. This suggests differences in the number of samples tested, the rate of retesting, and differences in the regions studied, or the low number of persistently infected animals in the herds sampled. Although PI animals could not be detected in the study, it should not be ignored that there may be economic losses due to BVDV in the region and throughout Turkey. It is a known fact that acute and PI animals ensure the circulation of the disease in the field. Although the number of PI animals is usually low, it ensures the main persistence of the virus in the herd. Therefore, it is important for future eradication programs to control animals in the enterprise periodically in terms of virology and determine whether newborns are PI.

Furthermore, studies on the molecular epidemiology of BVDV in Turkey may help monitor virus isolates circulating on a national scale. Detailed information about the molecular typing of BVDV provides useful information for the establishment of disease control programs (Yeşilbağ et al., 2008). In the study carried out by Şevik (2018) in Afyonkarahisar province, the samples collected from enterprises where abortion problems were detected (fetus tissue samples of sheep and cattle) were evaluated for the presence of BVDV nucleic acid using the real-time RT-PCR method, and 22.2% positivity was determined. Another study performed in Ankara, Çorum, Kırıkkale, and Yozgat provinces examined the presence of BVDV in cows with reported abortion and infertility problems, and 3.55% positivity was detected in the study carried out with RT-PCR in seropositive whole blood samples (Aslan et al., 2015). This study did not detect BVDV RNA in the samples tested using RT-PCR. The fact that the prevalence of PI animals with regard to BVDV infection in cattle populations in the world is generally low in the range of 0.5-2% (Houe, 1999) and the PI rate in Turkey is low in the range of 0.07-4.9% (Burgu et al., 2003; Avcı and Yavru, 2013) may partially explain the reason why BVDV nucleic acid could not be detected in serum samples in our study.

CONCLUSION

As a result, there is currently no control and eradication program for BVDV infection in Turkey. Establishing eradication programs within the framework of principles, including

identifying and eliminating persistently infected animals, increasing immunity against BVDV infection with vaccination, and implementing biosafety strategies, is a known reality already. Considering dairy cattle breeding throughout Turkey, it is thought that the share of small-medium-sized enterprises is important, and it will be beneficial in designing future eradication programs on this basis.

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Ethical approval: In this study, animal owners gave their consent to blood sampling at the stage of collecting samples and epidemiological data. Furthermore, blood sampling was carried out under the supervision of a veterinarian in accordance with international ethical standards (Directive 2010/63/UE).

Conflict of interest: None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. The authors declare that they have no conflict of interest.

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