BVDV monitoring by pooling and real time RT-PCR as economical monitoring technique with low BVDV prevalence

ABSTRACT

The aim of this study was to determine whether the use of pooled blood samples and real-time RT-PCR are feasible for detecting BVDV in infected animals. For this purpose, blood samples obtained from 2701 cattle, brought from 62 different farms, were used to test for the presence of bovine viral diarrhoea virus (BVDV) at the Veterinary Virology Department of Aksaray University Faculty of Veterinary Medicine. The sampling was done from all geographical regions in Turkey. Blood samples were pooled in groups of eight, creating a total of 342 pools. Each pool was checked for BVDV with a real-time RT-PCR test. BVDV nucleic acid was detected in 18 (5.26%) of a total of 342 pools. BVDV was detected in 18 of 62 farms. The most important result obtained from this study is that BVDV monitoring by pooling and real time RT-PCR can be done very economically when the disease prevalence is low (<10%). A risk/benefit estimation can be done for breeders who want to start vaccination programs.

Keywords: BVDV, Pooling, Real Time RT-PCR

INTRODUCTION

Bovine viral diarrhoea virus (BVDV) infection was first described by researchers at Cornell University in the USA (Olafson et al., 1946). This infection is an important disease that affects the reproductive system in cattle all over the world (Grooms, 2004). The resulting infection creates clinical signs characterized by diarrhoea, thrombocytopenia, hemorrhage, respiratory tract diseases, gastrointestinal system ulcers, abortion, reproductive disorders, and growth retardation (McGowan et al., 1993; Blanchard et al., 2010). Infection-related reproductive disorders have an important economic impact on cattle breeding (Houe, 2003; Grooms, 2004; Richter et al., 2017). The resulting economic losses are mostly related to death and weight loss caused by the infection of the digestive system, and reduced reproductive performance as a result of a transplacental infection in the reproductive system. Births of persistently infected (PI) calves that spread the virus for life are important in transmission (Ames, 1986; Baker, 1995; Bowen, 2011; OIE, 2017). Therefore, the disease is one of the most important threats to sustainable cattle breeding (Gunn et al., 2005).

BVDV, which is the causative agent, is a prototype virus in the pestivirus genus in the Flaviviridae family (Bowen, 2011; Koonin et al., 2020).

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Research Article

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The agent, which is serologically uniform, exhibits two biological (biotype) different characters (cytopathogen-cp and non-cytopathogen-ncp). It has been reported that the relationship between biotypes plays a decisive role in the course and pathogenesis of infection (Brownlie, 1991). BVDV infection has been a common disease for many years all over the world (Ridpath, 2010) and observed in Turkey for many years (Öncül et al., 1964; Alkan and Burgu, 1993; Çabalar and Karaoğlu, 1999; Duman et al., 2009; Okur et al., 2007; Tan et al., 2006; Yılmaz et al., 2012; Yeşilbağ et al., 2014; Yılmaz, 2016).

One of the most important results of BVDV infection is the presence of PI calves. PI calves are formed as a result of infected cows with noncytopathogenic virus in the first 3 months of fetal life (Stokstad and Loken, 2002). These calves born as virus carriers are a source of contamination throughout their lives. PI animals have an important role in the presence and persistence of BVDV infection in cattle populations (Moerman et al., 1993). Reported PI BVDV infection in cattle populations around the world is up to 2.00% (Brock, 2003; Peterhans et al., 2003; Smith et al., 2008; Stahl and Alenius, 2012; Newcomer et al., 2015; OIE, 2017). In Turkey, this ratio is not homogeneous, and it is reported to vary up to 6.50% (Şimşek, 1997; Burgu et al., 2003; Tan et al., 2006; Yeşilbağ et al., 2012; Yılmaz et al., 2012; Avcı and Yavru, 2013; Yavru et al., 2013; Şimşek et al., 2017).

It has been reported that persistent infected (PI) animals are responsible for the spread of the disease and have a role in direct and indirect transmission by spreading viruses throughout their lives (Houe, 1999). Therefore, early diagnosis and elimination of these PI animals in the population is important for disease control. To control and eradicate this disease, mostly "vaccination, systematic control, removal of PI animals, movement controls of infected herds, strict biosecurity, and surveillance" methods are recommended (Lindberg, 2003; Rypuła et al., 2013; OIE, 2017; Moening ve Becher, 2018). In recent years, it has become important to eliminate newborns with positive BVDV from the herd (Nelson et al., 2015). Also, foetal protection of newborns is very important to control the disease in herds.There are several publications about some vaccines providing fetal protection against BVDV infection (Bolin 1995, Brownlie et al., 2000, Patel et al., 2002, Dubovi 1992, McArthur, 2004).

Direct and indirect virological test methods are used in the diagnosis of the disease in infected or suspicious herds. For this purpose, Enzyme Linked Immunosorbent Assay (ELISA) and molecular tests are widely recommended methods (Edwars, 1990; Neill et al., 2014; OIE, 2017). In recent years, real-time RT-PCR is one of the mostly used molecular tests.

For many years, a single animal sample has often been used in tests, often said to be the best practice. On the other hand, using pooled samples is an alternative option for economic reasons. Pooling strategy was previously used in diseases that affect people with high testing costs (Kline et al., 1989). Pooled testing offers a cost-effective advantage, especially in disease cases with low (<10%) prevalence (Dorfman, 1943; Cowling et al., 1999). The pooling protocols have been presented by different researchers as a general application to screening using a sensitive diagnostic test to detect diseases or pathogens in populations for many years (Boulard and Villejoubert, 1991; Rodake et al., 1997; Munoz-Zanzi et al., 2000; Lanyon et al., 2014; Furstenau et al., 2020).

In this study, the aim is to determine whether the use of pooled samples and real-time RT-PCR are feasible for detecting BVDV in viremic or PI animals. The economic comparison was made according to the cost of commercially available individual AgELISA herd screenings. It is the first study to use

pooled samples and real-time RT-PCR together for BVDV diagnosis in Turkey.

MATERIAL and METHOD

Blood samples

Within the scope of the study, no extra sample collection was performed as blood samples that were brought to the Laboratory of Veterinary Virology Department in Aksaray University Faculty of Veterinary Medicine by dairy farms for diagnosis of BVDV disease were used. A total of 2701 EDTA blood samples from 62 different dairy farms with relatively good management practice in terms of biosecurity, located in seven regions of Turkey (Aegean, Black Sea, Central Anatolia, East Anatolia, Marmara, Mediterranean, South-East Anatolia) were tested. EDTA blood samples were combined in groups of maximum eight as a pool. The calculaton of pooling size is based on a publication (Munoz-Zanzi et al., 2000). This group calculated that 8 samples were economical for flocks with a prevalence of 2 percent (Munoz-Zanzi et al., 2000). 342 pools in total were created. The EDTA blood samples used in the study were stored at -20°C until they were analyzed without any pre-treatment.

Kits

RNA isolation from the pooled blood samples was performed using the Real PCR DNA/RNA Spin Column Kit (IDEXX, Montpellier, France) and a real-time RT-PCR test was performed using Real PCR BVDV RNA Test (IDEXX, Montpellier, France). Kits were used according to the manufacturer's instructions.

The test contains specific primers and probes designed for BVDV-1, BVDV-2, BVDV-3 and Border Disease Virus.

Controls

PCR positive control and BVDV positive blood samples in the stock of the Laboratory of Veterinary Virology Department in Aksaray University Faculty of Veterinary Medicine were used as positive controls. Distilled water and PCR negative control were used as negative controls. The internal controls (PCR positive control, PCR negative control, and internal controls) were included in the kit (IDEXX RealPCR BVDV RNA Test, IDEXX, Montpellier, France).

Statistical analysis

Statistical differences between pools, farms and regions for BVDV positivity were assessed through Pearson's Chi-square using SPSS v.22.0 software. The calculared "P" value less than 0.05 was regarded as statistically significant.

RESULTS

A total of 2701 EDTA blood samples of different ages, breeds, and sex were pooled in groups of maximum eight individual samples. As a result of the test, pestivirus nucleic acids were detected in 18 pools (5.26%). The 18 pools consisted of a total of 132 individual blood samples. Unfortunately, it was not possible to test those 132 blood samples individually. Therefore, the positive results detected were thought to indicate a possible BVDV infection (viremic or PI). If all are considered positive, this corresponds to 4.89% prevalence (Table 1). Eleven out of 62 (17,74%) farms were detected as positive for pestivirus antigens (Table 1).

Table 1. The distribution and test results of the samples used in the study

Geographical Regions of Turkey	Number of			Pestivirus Negative			Pestivirus Positive		
	Blood Samples	Pools	Farms	Blood Samples	Pools	Farms	Blood Samples	Pools	Farms
Mediterranean	237	30	9	221	28	7	16	2	2
East Anatolia	106	14	3	90	12	1	16	2	2
Aegean	381	48	7	365	46	5	16	2	2
South-East Anatolia	80	10	1	80	10	1	0	0	0
Central Anatolia	922	116	24	904	113	22	18	3	2
Black Sea	98	13	2	98	13	2	0	0	0
Marmara	877	111	16	811	102	13	66	9	3
Total Number and (%)*	**2701 (100)**	**342 (100)**	**62 (100)**	**2569 (95,11)**	**324 (94,74)***	**51 (82,26)**	**132 (4,89)**	**18 (5,26)***	**11 (17,74)**

*There was no significant differences between the regions where Pestivirus were detected in pools (P = 0.297).

DISCUSSION

BVDV infection has been detected in cattle in all countries where prevalence studies have been conducted (Lindberg, 2003). This situation is an indication that the virus is widespread in the world, and also expresses the spread potential of the agent with international live animal trade.

The control of BVDV infection is usually done by combining test and elimination, preventive vaccination, and strict biosecurity practices (Lindberg, 2003; Rypuła et al., 2013; OIE, 2017; Moening and Becher, 2018). With these methods, successful eradication programs are implemented in Europe (Switzerland, Germany, Austria, Luxemburg, Ireland) (Hanon et al., 2017). In these countries, breeders participate in the control-eradication programs on a mandatory or voluntary basis. According to the accessible literature data, there are no similar national programs in Turkey. However, conscious breeders apply similar practices in their own farms. The high cost of testing is shown as the most important reason why the majority keep their distance from this issue. In this respect, the use of pooled samples in tests should be considered as a very good alternative in order to reduce test costs and apply a sustainable combat strategy. Pooling strategy was first proposed to improve the efficiency of large-scale pathogen screening campaigns by Robert Dorfman (1943) during the second world war. It was previously used in diseases that affect people with high testing costs (Kline et al., 1989). Pooled testing offers a cost-effective advantage, especially in disease cases with low (<10%) prevalence (Dorfman, 1943; Cowling et al., 1999). The pooling protocols have been described by different researchers as a general application to screening using a sensitive diagnostic test to detect diseases or pathogens in populations for many years (Boulard and Villejoubert, 1991; Rodake et al., 1997; Munoz-Zanzi et al., 2000; Lanyon et al., 2014). It is widely used in disease-free or low prevalence herds in the USA (Kennedy et al., 2006). An evaluation in terms of current test prices can be made with the analysis fees announced by the laboratories affiliated to the Ministry of Agriculture and Forestry (URL 1). The AgELISA test fee has been announced as approximately 158 TL/sample and the real time RT-PCR test fee is approximately 454 TL/sample by the Ministry. Considering 8 sample pooling, the real time RT-PCR test fee can be calculated as approximately 56 TL/sample. The cost of per sample would be quite low even if retests were to be included. As can be seen, the proposed method with this study seems to be quite advantageous in herd-based BVDV control and eradication studies.

The results presented herein describe the prevalence of BVDV in EDTA blood sample pools tested by commercially available real-time RT-PCR. The test is ideal for use in low-prevalence situations using pooled samples as the detection limit of RT-PCR is very low.

Considering the results of previous studies on this subject in Turkey, the country can be considered as a country with low prevalence (<10%) in terms of disease.

In this study, viremic or PI (5.26%) animals were detected in 18 out of 62 farms (17.74%), in other words, 18 out of 342 pools of 2701 blood samples. However, we expected a higher prevalence of BVDV in Turkey based on previous serological studies which reported in average seropositivity of 50% and more (Yılmaz, 2016; Timurkan ve Aydın, 2019). The results from this study indicate a low prevalence in the tested farms. In particular, sample pooling is a very economical alternative to the general rule of "continuous monitoring of herds for BVDV and eliminating positive animals". In this study, demonstrating that there was no positivity in the farms sampled from the Black Sea and South-East Anatolia regions significantly reduced the test cost and data was obtained for these farms to start the vaccination program as soon as possible. Polak et al. (2016) especially indicate; "vaccination will be effective only when it is carried out correctly and preferably after previous determination of BVDV infection status". In order to reach a similar result, farms in regions other than Marmara will be able to get situations with a few tests and start vaccination programs safely.

The pooling method has been described in a thesis in relation to BVDV in Turkey. 160 blood samples were pooled in pairs and it was emphasized that two (2.5%) out of 80 pools were positive with RT-PCR (Sarıkaya et al., 2012). In our study, an important economic advantage is revealed by pooling in groups of eight with real time RT-PCR. Mars and Van Maanen (2005) estimated the optimum pool size as 36, based on the costs of RT-PCR versus the costs of individual AgELISA's in relation with the probability that a pool is PCR-positive. This estimation is based on Dutch cattle farming conditions with low BVDV prevalences. This means that also under the Turkish conditions with low BVDV prevalences, the number of individuals per pool may still be increased (more then 8 per pool) to make this test even more economically viable. For the detection of PIs in the Belgian eradication program, blood samples are gathered into pools of 30 and tested by RT-PCR (Laureyns et al., 2010).

In this study, the fact that 17.74% of the tested farms were BVDV positive confirms the lack of continuous monitoring and BVDV control among the breeders. The value of the results obtained in terms of controlling BVDV should not be underestimated as infected individuals should be quickly removed from the herd. It has been reported that 85% of the herd is in contact with the infection within 2 years following the introduction of a PI in a herd that was not immunized against BVDV infection and a very high rate of persistent viremic calf births were observed in the herd (Moerman et al., 1993).

Disease control including vaccination is an important preventive practice, so that herd immunity is kept at the highest level. Vaccines providing foetal protection can also be used for this purpose. However, vaccination should be done correctly in herds. In herds to be vaccinated, it is recommended to first determine the immune status of the herds in terms of the agent to be vaccinated (Polak et al., 2016). It will be useful to identify and eliminate carrier individuals in the herd before vaccination with the methods used in this study.

CONCLUSION

To conclude, the results obtained in this study confirm the presence of BVDV in cattle herds in Turkey. Periodic monitoring of cattle farms for presence of BVDV, elimination of PI individuals, and vaccination are recommended. Considering the relatively low prevalence (<10%) for BVDV infection, professional

support is strongly recommended to control BVDV in Turkish herds. Pooled-sample testing by real time RT-PCR lends itself to screening herds for low prevalence agents at an economically viable way. This may be of interest in animal disease surveillance, and herd certification programs. It is necessary to pay attention to some issues when working with sample pooling. Recent vaccination of herds with modified live BVDV vaccine must be taken into attention. Also, sensitivity and specificity of PCR are so important.

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Ethical approval: An ethical approval is not needed according Turkish regulations as only samples were taken for diagnostic purposes.

Conflict of interest: There is no conflict of interest.

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