Molecular Diagnosis and Seroepidemiology of Bovine Viral Diarrhoea Virus in Cattle with Abortion Problem

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Abstract: This study is a serological and virological investigation of the bovine viral diarrhoea virus (BVDV) infection in cattle with abortion problem from private small scale production units of less than 20 cattle per unit, in the Kars province of Turkey. For this purpose, blood sera from 188 cattle were tested for antibodies against BVDV using a commercial enzyme-linked immunosorbent assay (ELISA). Since the virus causes persistent infection, antigen-ELISA was also performed for this agent. Seropositivity rate was detected to be 60.63%. In addition, the BVDV antigen was not detected in seronegative sera samples. Reverse transcription polymerase chain reaction (RT-PCR) technique was used to determine the presence of BVDV nucleic acid. BVDV nucleic acid was found in 2 of 74 seronegative samples (2.7%) by RT-PCR. The results suggest that the infection was spreading in private small scale production units. Furthermore, recommendations for the control of BVDV infection are presented.

Keywords: Abortion, BVDV, Cattle, ELISA, RT-PCR.

INTRODUCTION

BVDV is a member of the genus Pestivirus, a group of small-enveloped, single stranded RNA viruses in the family *Flaviviridae*. At present, the genus Pestivirus comprises BVDV with its two genotypes BVDV1 and BVDV2, classical swine fever virus (CSFV), and border disease virus (BDV) [1-3]. All four pestivirus species are closely related genetically and antigenically. While BVDV-1 viruses have 16 subtypes, BVDV-2 viruses are classified into three subtypes [3-5]. For both species, cytopathogenic (cp) and non-cytopathogenic (ncp) virus strains are distinguished according to their growth characteristics in cultured cells [6]. The NCP biotype can cross the placenta to establish a persistent and lifelong infection. Furthermore, persistently infected (PI) animals are the single most important source of infection for other animals. Since there is no effective commercial BVDV vaccine, early detection and subsequent removal of PI animals is essential to a successful BVDV control programme. BVDV can cause repeat breeding, embryonic death, abortion, stillbirths and congenital defect in infected pregnant cattle [7,8]. Generally, in non-vaccinated herds, the seroprevalence differs among areas or countries, ranging between 20% and 90% [9,10]. It was also estimated that 1-2% of persistently infected animals were found in countries having no BVDV control program [10,11].

The RT-PCR has been a successful molecular tool for research purposes and diagnosis for BVDV infections. This has allowed the detection of pestivirus RNA in a variety of clinical samples such as blood, tissues, serum and swabs [12]. Several regions of the viral genome have been used for genetic typing of pestiviruses. At present, the 5' UTR, Npro and E2 regions are most often used [13,14].

Abortions in cattle herds are important problem in both the North-eastern Anatolia and Turkey. Especially, abortion rate caused by pestiviruses is dramatically increasing in the recent years. In this study, the seroprevalence and viroprevalence of BVDV infection, which has been shown to be active in western Turkey, was determined by ELISA in cattle with abortion problem in the Kars province in North-eastern Anatolia. In addition, BVDV nucleic acid was investigated in seronegative samples using RT-PCR. These virological and seroepidemiological data demonstrate the occurrence of BVDV in private small scale production units and emphasize the necessity of effective control measures. This study is the first molecular study to determine viroprevalence of BVDV infection in cattle in the Kars province of Turkey.

MATERIALS AND METHODS

Clinical Samples

Five selected cattle herds located in willages of central Kars province where frequent abortion cases were reported last a few years occurred. The time of abortion cases were estimated to be occurring mainly during first and second trimesters of the gestation by interviewing farmers in the herds subjected for the present study. A total of 188 blood samples were collected from unvaccinated cattle with abortion problem from private small scale production units (< 20

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cattle). The age of animals varied from 3 to 5 years. Blood samples were taken from the jugular vein. Blood tubes (without EDTA) were centrifuged at 3,000 × g for 10 min, and serum samples were transferred to sterile tubes and stored in −20 °C until used.

Enzyme Linked Immunosorbent Assay (ELISA)

Commercial ELISA (Pourquier ELISA BVD/MD/BD P80, France) used for the detection of antibodies against BVDV was carried out according to the manufacturer's instructions. A commercial ELISA kit (BVDV Ag Serum Plus HerdChek IDEXX Laboratories Westbrook, Maine 04092, USA) detecting Erns antigen in serum was used in the study according to the manufacturer's instructions. Both ELISA results were analyzed and calculated with an automated ELISA reader at 450 nm. The results are expressed as an inhibition percentage, calculated in equation.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

From seronegative samples, viral RNA was extracted using a GeneJet Viral DNA and RNA Purification Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's recommendation. The follow-up cDNA synthesis was carried out using a first strand cDNA synthesis Kit (Fermentas, Lituhania) as described by 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). Amplification of cDNAs by PCR was performed using the primer pairs 324/326 for 5′UTR The 324 (5´- ATG CCC TTA GTA GGA CTA GCA -3´) and 326 (5´- TCA ACT CCA TGT GCC ATG TAC-3´) primers flank a 288 bp DNA fragment for BVDV [14]. 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 (100 mM Tris-HCl pH 8.3 and 500 mM KCl), 2.4 µL of 25 mM $MgCl_2$, 10 pmole from of each of the primers, 0.5 µL of 10 mM dNTP mix, and 0.25 µL of Taq DNA polymerase (MBI, Fermentas, Lithuania), 19.85 µL of RNAse free distiled water. The PCR was initiated by a single denaturation step at 96 °C for 6 min, followed by 35 cycles of 45s at 94 °C, annealing at 55 °C for 1 min. The mixture was then incubated, as a single step, for 1 min at 50 °C and 10 min at 72 °C for final extension. Five microlitres of each PCR product were analyzed on 1% agarose (Prona, Spain) gel containing ethidium bromide (Sigma, USA). NADL (cytopathogenic reference virus) obtained from the Ankara University Faculty of Veterinary Medicine Virology Department was used as positive control.

RESULTS

A total of 188 serum samples were tested for BVDV specific antibodies using ELISA. Overall results revealed that 60.63% (114/188) of the cattle sampled were BVDV seropositive, while 39.36% of the samples (74/188) did not have antibodies aganist BVDV. To determine persistently infected cattle, serum samples with no antibody response to p80 protein were tested for viral antigen. For this purpose, 74 seronegative samples were tested for Erns antigen. None of seronegative samples were positive for BVDV antigen (zero%). To determine the BVDV antigen, 74 seronegative samples were tested by RT-PCR and 2 of 74 seronegative samples produced an expected 288 bp amplicons with 5′UTR-specific panpesti generic primers (Figure **1**).

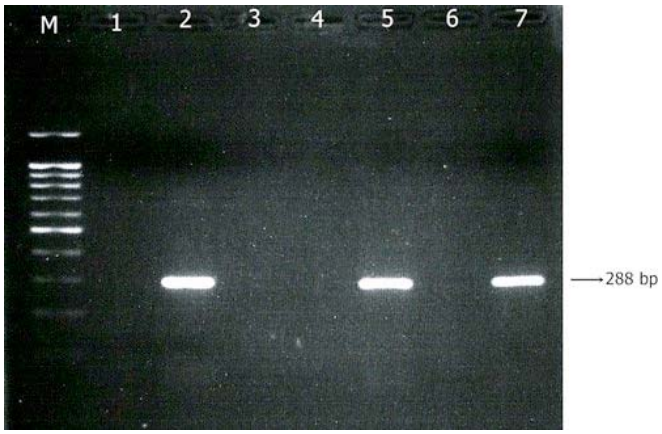

Figure 1: The result of RT-PCR in seronegative samples. Legends: Column M: 100 bp DNA Ladder; columns 1,3,4: negative samples; columns 2, 5: positive samples, column 6: negative control; column 7: positive control (288bp).

DISCUSSION

BVDV is a major cause of reproductive failure and immunosupression, and cause substantial economic losses in livestock industries through their impact on reproduction and health. Numerous studies [15-21] conducted with regard to the BVDV infection in Turkey have demonstrated that the infection is widespread in populations of cattle. These studies have reported that in various regions of Turkey BVDV seroprevalence varies between 14.3-100% in cattle. The seroprevalence of BVDV was investigated in cattle with abortion problems in the Kars province of Turkey. The

location for the study was chosen because Kars is located in the middle of an animal transit route between North-East Anatolia and Caucasus functioning as a bridge between Asia and Europe. The seropositivity of collected blood samples was found to be 60.63%. This percentage is higher than the percentages found in studies conducted in past years [16-19] and is lower than the percentages reported by Avci *et al*. [20] and Erol *et al*. [21]. This situation could be explained with the factors causing increase in the virus resistance. These factors include being kept in crowded and poorly-ventilated areas, inadequate poor-quality feed, stress, animal movements (entrance of contraband animals or transfers between operations or from other countries *via* importation), insufficient use of periodic laboratory screening and the fact that animal owners are not knowledgeable.

Many virological studies for BVDV have been performed in Turkey. The presence of BVDV has been reported between 0 % and 4.9% in cattle in a different part of Turkey [6-11]. An important factor for the spread of BVDV infection is the birth of persistently infected (PI) animals, which remains infected throughout their life. There are indications that 0.5 to 2% of the cattle in endemically infected countries are PI and 60-85% of adult cattle are antibody positive [10]. Simsek and Ozturk [22] reported 2 acute persistent infections by BVDV in 142 healthy cows by direct immunofloure-sance test (DIFT). Bulut *et al*. [18] reported 2 acute persistent infections by BVDV in 228 cattle with the history of abortion in a herd of Konya province of Turkey by monitoring the leucocyte samples with direct ELISA. Our results indicate a 2.7% rate of virus infected cattle in the Kars province of Turkey. In this study, two seronegative cattle were detected as positive for BVDV antigen. These two animals being antigen positive and antibody negative were sampled second time after two weeks. The same results were detected for two seronegative cattle. The animals detecting persistent infection status were suggested to slaughter. When seronegative cattle are infected with an ncp BVDV biotype, virus can be transferred easily into the foetus and infection in early period of gestation may produce PI calf. These animals show immunological tolerance to the carrier virus and maybe born apparently healthy [23]. In Turkey, there is no special effort to detect PI animals in herds and no efforts are exerted to eliminate cattle with PI BVDV. Consequently, there are no specific data on the frequency rate of PI cattle with pestivirus in the Kars province of Turkey.

Horner *et al*. study [24] comparing RT-PCR with ELISA and cell culture immunoperoxidase tests for the detection of ruminant pestivirus infections found RT-PCR to be more sensitive compared to the other tests. When 169 clinical samples were examined, the RT-PCR detected the most positives [42] compared to the ELISA [32] and the immunoperoxidase test (IPT) [20]. In this study, results indicated that from a total of 74 seronegative samples, zero (0%) and 2 (2.7%) were positive for presence of BVDV by antigen capture ELISA and RT-PCR assay, respectively. Our results showed that the molecular method was more sensitive than antigen capture ELISA.

BVDV control programs must first identify and remove PI animals from the breeding herd and prevent the infection of susceptible pregnant cows. As a control procedure, it may be advised that persistently infected animals can be kept together with other animals in the flock previous to gestation to reveal flock immunity and to prevent exposing sheep or goats to infection during pregnancy. Although immunization efforts have been successfully carried out in many countries to bring BVDV infections under control, still more work remains to be done in the fight against BVDV infections. An ideal vaccine should be suitable for administration to females before breeding for the prevention of transplacental infection. Most BVDV vaccines on the market in many countries, including in Turkey, contained BVDV 1-a and 1-b strains, some of them in combination with a BVDV 2 strain [25]. Ideally, a pestivirus control programme for Turkey should use a vaccine that contains not only BVDV 1 strains but also BVDV2. For efficacious BVDV immunization and protection in Turkey, predominant BVDV 1 subgenotypes (1-l and 1-f) are clear vaccine candidates [26].

As conclusion, BVDV can play an important role in abortion in cattle and it should be checked regularly. Furthermore it is always taken into consideration BVDV may also lead to reproductive problems on cattle breeding. The animals should be checked in terms of BVDV for being negative both antigen and antibody before accepting them to the herds. The control of abortions caused by BVDV infections vaccination should be practiced before the gestation time of cattle.

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