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Bovine viral diarrhoea: Ag ELISA and reverse transcription polymerase chain reaction as diagnostic tools in pooled serum samples from persistently infected cattle - short communication

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ABSTRACT

The applicability and reliability were investigated of antigen enzyme linked immunosorbent assay (Ag ELISA) and reverse transcription polymerase chain reaction (RT-PCR) for the diagnosis of bovine viral diarrhoea (BVD) in pooled serum samples of persistently infected cattle. In this study 968 serum samples were tested and persistent infection was confirmed by Ag ELISA in six animals by sampling twice at a four week interval. Positive samples were tested undiluted and diluted by Ag ELISA and RT-PCR. Sera were diluted (five-fold) and tested once again by both methods. All undiluted samples were tested positive by Ag ELISA, while the end point dilution was 1:5. When RT-PCR was used, samples were shown to be positive to 1:125, and in one sample in a dilution of 1:625. We can conclude that Ag ELISA can be useful in the diagnosis of BVD in a maximum five pooled serum samples from persistently infected animals. RT-PCR shows greater sensitivity and thus is more appropriate for screening purposes when pooling of samples is recommended.

Key words: bovine viral diarrhoea, diagnosis, pooled serum samples, Ag ELISA, reverse transcription polymerase chain reaction

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Introduction

Bovine viral diarrhoea (BVD) is an infectious disease of domestic and wild ruminants, with high economic impact in cattle. The virus belongs to the family *Flaviviridae*, genus *Pestivirus* (SIMMONDS et al., 2012). BVD viruses can be further classified into two genotypes: BVDV1 and BVDV2 (PELLERIN et al., 1994), today in two species (SIMMONDS et al., 2012). Both BVDV1 and BVDV2 strains may exist as one of two biotypes, cytopathic (cp) and noncytopathic (ncp) depending on the activity of the strain when propagated in cell cultures (GILLESPIE et al., 1961; RIDPATH, 2005).

The great majority of viruses (about 90%) isolated in the laboratory belong to the noncytopathic biotype (FULTON, 2005) and only this biotype is able to cause persistent infection. In the recent study a third biotype is proposed, the lymphocytopathic type (lcp) which can cause a serious clinical disease in cattle (RIDPATH et al., 2006). Biotype is associated with the ability to cause persistent infections in the foetus and with the appearance of mucosal disease in cattle. Persistent infection can arise if the foetus is infected with the ncp biotype in the first 120 days of pregnancy (ROEDER et al., 1986). This calf will be immunotolerant to the virus and can die in the first days of life, or it can appear healthy and achieve sexual maturity (EVERMAN and BARRINGTON, 2005). Such cows, if later used as reproductive animals, will also have calves that are persistently infected (EVERMAN and BARRINGTON, 2005). The possibility of persistent infection, with a lack of any evident symptoms in the infected animals, allows the infection to persist in the herd, as these animals are then constant sources of the virus, which multiplies unhindered and is excreted through secretions and excrement (HOUE, 1999).

Considering that persistently infected cattle are the main source of the virus, it is most important to identify and remove these animals from the herd. In acutely infected cattle, it is possible to identify the virus within only a couple of days depending on strain (HOUE, 1999). Also, considering that the virus is very rarely present in ear notch tissue samples, it is best to detect acute infections using reverse transcription polymerase chain reaction (RT-PCR) for testing of blood serum or buffy coat (GOYAL, 2005).

In persistently infected calves, antibodies from colostrums may have influences on results obtained by immunoperoxidase test and Ag ELISA (GOYAL, 2005). For this reason, it is best to use RT-PCR with samples of blood serum or to use ear notch tissue as diagnostic material for testing by RT-PCR, Ag ELISA and immunohistochemistry. The Virology Department of the Croatian Veterinary Institute routinely uses Ag ELISA to detect specific antibodies and viral antigens, and RT-PCR to detect viral genomes.

The objective of this paper was to establish how applicable it is to use pooled samples of blood serum in BVD diagnosis using Ag ELISA and RT-PCR, which can be implemented in BVD diagnosis at herd level.

Materials and methods

Samples. In order to detect persistent infection, a total of 968 samples of cattle blood serum were sampled. Sampling was conducted on ten herds suspected for BVD. Blood was taken from the tail vein into plastic test tubes and transported in a transport refrigerator to the laboratory. In the laboratory, blood was centrifuged at 2000 rpm for 10 minutes and the serum collected. One mL of serum was divided into four test tubes in order to avoid multiple freezing and thawing. The serum was then stored at -30 °C until testing. Sera from all positive cattle were sampled again after four weeks and tested by Ag ELISA or RT-PCR.

Ag ELISA. To detect viral antigen in the blood serum samples, the Ag ELISA was used according to the manufacturer's instructions. The Herdchek BVDV Ag/Serum Plus set (IDEXX, Liebefeld-Bern, Switzerland) was used with monoclonal antibody for glycoprotein E^{ms} absorbed to the walls of the microtitration wells. The data were analyzed on the basis of optical density (OD) value for each sample, and positive and negative controls according to the formula provided by the manufacturer.

RT-PCR. Viral RNA was isolated using the commercial kit QIAmp[®] Viral RNA Mini Kit (Qiagen, Germany) according to manufacturer's instructions. The 104F and 402R primers for the 5'-UTR region (BARLIĆ-MAGANJA and GROM, 2001) were used for amplification in a concentration of 10 µM. The entire RT-PCR procedure was conducted in a single step (one-step RT-PCR). The products of the PCR were examined using electrophoresis in 1.5% agarose gel stained with ethidium-bromide.

Dilution of blood serum samples. Blood serum samples from persistently infected animals, in which the presence of the virus was confirmed, were subjected to five-fold dilution in a sterile cell culture medium (DMEM, Dulbeccos Modified Eagle Medium-Sigma Aldrich Inc., USA). Serum dilutions from 1:5 to 1:3125 were obtained. All undiluted and diluted serum samples were then tested using Ag ELISA and RT-PCR.

Results

From the 968 blood serum samples, persistent infection was confirmed in six samples. By testing all six undiluted samples both methods showed positive results. The Ag ELISA method showed positive results for six 1:5 diluted samples (Table 1). For the six diluted samples tested with RT-PCR (Fig. 1) a positive result was obtained in all dilutions up to 1:125. In the 1:625 dilution, only one sample gave a positive result, while the remaining samples were negative (Table 1).

Table 1. Results of testing undiluted and diluted blood serum samples using Ag ELISA, with values of optical density (OD), and RT-PCR.

Serum No.	Method	Undiluted sample	Dilution				
			1:5	1:25	1:125	1:625	1:3125
3/163	*Ag ELISA (*OD)	pos. (3.76)	pos. (0.63)	neg. (0.15)	neg. (0.04)	neg. (0.05)	neg. (0.05)
	RT-PCR	pos.	pos.	pos.	pos.	neg.	neg.
3/237	*Ag ELISA (*OD)	pos. (3.49)	pos. (0.69)	neg. (0.13)	neg. (0.02)	neg. (0.02)	neg. (0.02)
	RT-PCR	pos.	pos.	pos.	pos.	neg.	neg.
5/59	*Ag ELISA (*OD)	pos. (3.61)	pos. (0.46)	neg. (0.11)	neg. (0.06)	neg. (0.05)	neg. (0.05)
	RT-PCR	pos.	pos.	pos.	pos.	neg.	neg.
7/64	*Ag ELISA (*OD)	pos. (3.57)	pos. (0.44)	neg. (0.14)	neg. (0.07)	neg. (0.06)	neg. (0.06)
	RT-PCR	pos.	pos.	pos.	pos.	neg.	neg.
7/65	*Ag ELISA (*OD)	pos. (3.31)	pos. (0.67)	neg. (0.19)	neg. (0.03)	neg. (0.03)	neg. (0.04)
	RT-PCR	pos.	pos.	pos.	pos.	neg.	neg.
9/30	*Ag ELISA (*OD)	pos. (3.43)	pos. (0.48)	neg. (0.15)	neg. (0.08)	neg. (0.05)	neg. (0.05)
	RT-PCR	pos.	pos.	pos.	pos.	pos.	neg.

*Observed OD value. OD value of the positive control in Ag ELISA was 1.13. The observed OD value for the negative control in Ag ELISA was 0.11. According to the manufacturer's instructions, all values >0.30 are considered positive and values ≤ 0.30 negative.

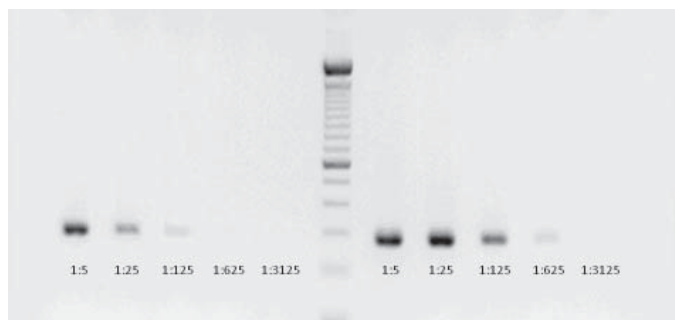


Fig. 1. The results of the RT-PCR for serum No. 7/65 and 9/30. The replication product of complementary DNA is visible in sample No. 7/65 at a dilution of 1:125, and at a dilution of 1:625 for sample No. 9/30.

Discussion

The World Organisation for Animal Health (OIE) has recommended several different methods for the etiological diagnosis of BVD/MD. Due to its simplicity, price and rapid performance, the commercially available Ag ELISA is very frequently used. However, virus isolation is still considered to be the gold standard (RIDPATH et al., 2002). RT-PCR can recognise fragments of the RNA virus genome even when the sample contains antibodies for the BVD virus, and is a reliable test in proving the genetic material of the virus. The main advantage of RT-PCR in the detection of BVDV in serum samples from calves, where other methods can be less efficient due to the presence of colostral antibodies. In this study we showed that RT-PCR is reliable for testing pooled serum samples.

During the testing of diluted samples, Ag ELISA succeeded in detecting all the positive samples in the dilution 1:5. However, the OD values of these samples were only just above the cut-off line of a negative test. This suggests the possibility of testing only five pooled samples, and consequently this application would not have a significant impact on the price and speed of the test.

Using RT-PCR on diluted samples, a positive result was obtained in the dilution of 1:125 for five samples, and 1:625 for one sample. These results are in line with similar studies, in which it was determined that RT-PCR is capable of detecting one positive animal among a hundred (KENNEDY et al., 2006). Though positive results were obtained up to dilutions of 1:125, it is our recommendation that RT-PCR not be used to test more than 25 pooled samples. The reason for this is that in this study, all samples were kept in optimal conditions and a minimum amount of time passed between sampling and sample storage. In field conditions, such timing is difficult to achieve, and samples for testing are often submitted after several days. BVD virus stay stable for virus isolation at

temperature of 4 °C for couple weeks. However, considering that this is an RNA virus, the genome is easily destroyed and such samples can become less suitable even for RT-PCR testing. Another point is that in the case of positive result the lower number of individually samples should be tested.

In conclusion, it can be said that Ag ELISA can be used in the diagnosis of BVD in a maximum of five pooled samples. According to the results presented here, RT-PCR is a more sensitive and specific method, and is very reliable, particularly in testing pooled samples (up to 125 samples) when it is necessary to determine the condition of the herd.

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SAŽETAK

Istražena je primjenjivost i pouzdanost Ag ELISA-e i lančane reakcije polimerazom uz prethodnu reverznu transkripciju (RT-PCR) u dijagnostici virusnoga proljeva u skupnom uzorku krvnog seruma perzistentno zaraženih goveda. Perzistentna zaraza dokazana je dvokratnim uzorkovanjem s razmakom od četiri tjedna. Ukupno je Ag ELISA-om bili pretraženo 968 uzoraka seruma goveda, a perzistentna je zaraza utvrđena u šest goveda. Uzorci seruma svih šest goveda bili su peterostruko razrijeđeni. Nerazrijeđeni i razrijeđeni uzorci bili su pretraženi usporedno Ag ELISA-om i RT-PCR-om. Svi nerazrijeđeni uzorci dali su pozitivan rezultat s obje metode. Razrijeđeni uzorci pretraživani Ag ELISA-om pokazali su pozitivan rezultat do razrijeđenja 1:5. Svi razrijeđeni uzorci pretraživani RT-PCR-om bili su pozitivni do razrijeđenja 1:125, a jedan uzorak do razrijeđenja 1:625. Zaključno se može reći da se Ag ELISA može rabiti u dijagnostici virusnoga proljeva goveda u skupnim uzorcima do najviše pet goveda. RT-PCR pokazao je veću osjetljivost i specifičnost u pretraživanju skupnih uzoraka krvnog seruma goveda.

Ključne riječi: virusni proljev goveda, dijagnostika, serum, skupni uzorci, Ag ELISA, lančana reakcija polimerazom
