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Original article

Detection of the bovine viral diarrhoea virus (BVDV) in young beef cattle in eastern and south-eastern regions of Poland

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Abstract

In view of the scarcity of information concerning viral diarrhoea virus (BVDV) infections in beef cattle in Poland, the aim of this study was to evaluate the presence of the BVDV in young beef cattle from selected herds in eastern and south-eastern regions of Poland. The material consisted of 78 sera obtained from beef cattle from 15 farms, aged 6-12 months. The anti-BVDV antibody level in the sera was estimated with an ELISA kit, and detection of the BVDV was carried out by standard PCR and one step Real-Time RT-PCR. The ELISA results showed a high degree (80%) of positivity in 5 of the 78 samples. In 7 samples the degree of positivity was in the very low range: $\leq 40\%$. Of the 78 cDNA samples, the presence of genetic material with a length of 288 bp was found by standard PCR in 3 sera. The genetic material of BVDV was also found in the sera of the same three calves by Real-Time HRM PCR. BVDV infection in young beef cattle in south-eastern Poland is not a significant problem. This was confirmed by the positive ELISA results for 6.4% of the animals and the positive PCR results for 3.9%. The percentage of positive beef herds was about 8.6%. However, due to the severe nature of the disease and rapid transmission of the virus, regular monitoring of BVDV should be carried out.

Key words: bovine viral diarrhoea virus, beef cattle, feedlot, RT-PCR

Introduction

Infections with bovine viral diarrhoea virus (BVDV) are widespread in cattle populations all over the world, resulting in economic losses due to decreased performance, loss of milk production, repro-

ductive disturbances, and increased risk of morbidity and mortality (Rypuła et al. 2011). The clinical outcome after infection is complex and depends on a number of factors. Host factors influencing the clinical outcome include pregnancy status, gestational age of the foetus at the time of infection, immune status

(passive or active, from natural exposure or vaccination), and the level of environmental stress at the time of infection (Grooms and Keilen 2002). The virus belongs to the family *Flaviviridae*, genus *Pestivirus*, and is classified into biotypes based on the presence or absence of observable cytopathology in infected cell cultures, as cytopathic (CP) or noncytopathic. The predominant biotypes found in isolates from samples collected from clinically sick cattle are noncytopathic strains. BVD viruses are differentiated on the basis of genomic differences, with 3 major subtypes: BVDV1a, 1b, and 2a (Corbett et al. 2011). As milk production in Poland has become increasingly widespread, obligatory diagnostic tests for infections caused by the BVDV are mainly conducted in dairy cattle herds. In the USA, however, where beef cattle production is the highest in the world, the potential risk of transmission of both BVDV 1 and 2 is reserved for persistently infected (PI) dairy and beef cattle entering the feedlot. A recent study demonstrated that of 21,743 cattle entering the feedlot, 0.4% were PI (Fulton et al. 2006). Among cattle herds (dairy and beef) seroprevalence varied between 19% and 89%, but the prevalence of PI animals in the cattle population ranged from 0.5% to 2% (Cowley et al. 2012).

Foetal consequences of infections in dairy and beef calves range from early embryonic death (with recycling), abortions, stillbirths, congenital malformations, and persistently infected calves. This depends on the time of infection (gestation period) and exposure of young cattle to PI calves. Calves with high levels of immunity to the BVDV were associated with increased protection from bovine respiratory diseases (BRD), expressed as lower BRD morbidity rates and treatment costs, and required fewer treatments for BRD (Baker 1995, Fulton et al. 2002, Loneragan et al. 2005). To achieve sufficient biosecurity it is necessary to manage BVDV transmission, especially with regard to PI calf identification in dairy and beef herds. The prevalence of bovine viral diarrhoea virus (BVDV) infection in dairy cattle has been studied by many research centres in Poland and elsewhere in the world. In Poland, the main research centre dealing with diagnosis of BVDV infection is the National Veterinary Research Institute in Pulawy (Kuta et al. 2013). The prevalence of the BVDV in dairy herds in Poland, as shown in a study conducted in 2010, was high, ranging from 53.9% to 100%, and depended on the size of the herd (Rypuła et al. 2010). However, no data are available concerning the occurrence of the BVDV in beef cattle.

Due to this lack of information, the present study attempted to evaluate the occurrence of the BVDV in young beef cattle in selected herds from eastern and south-eastern regions of Poland.

Materials and Methods

Animals used in the study

The material used for the study consisted of 78 blood samples obtained from healthy young beef cattle, aged 6-12 months, during a compulsory veterinary examination. The calves were of the breeds Simmental (n=41), Charolais (n=23), Belgian Blue (n=8), and Limousin (n=6), and were located on 15 farms in eastern and south-eastern Poland, after entering the feedlot. The calves were from non-vaccinated herds; the mother-cows were also not vaccinated against the BVDV.

Blood samples were collected from the external jugular vein using vacuum tubes for serum separation (Greiner Bio-One Int. AG, Ge). Serum was obtained from the blood after centrifuging at 3,000 x g for 15 min. at 4°C. The sera were stored at -20°C until analysis.

ELISA assay procedure

Anti-BVDV antibody detection in the sera was performed using an Elisa kit (BVDV indirect test, BioX Diagnostic, B) according to the manufacturer's instructions. Each blood serum sample was diluted in a 1 ml dilution buffer, and the positive serum was resuspended in 1/100 and distributed at 100 µl in microplate wells. The plate was incubated at 21°C for one hour. After incubation, the plate was rinsed three times with washing solution, and 100 µl of dilute conjugate solution was added to each well. The plate was incubated for 1 hour at 21°C. Next the plate was washed three times and 100 µl of chromogen solution was added to each well. After incubation for 10 minutes at 21°C, with protection from light, the reaction was stopped with 50 µl of stop solution. The plates were read using a plate reader (BIO-RAD, Ge) with a 450 nm filter. The results were calculated for each sample well using the corresponding positive control serum signal, multiplying this result by 100 to express it as a percentage according to the formula in the instructions. The intensity of the positive reactions was calculated using the "degree of positivity", which quantifies the reactivity of an unknown serum on a scale ranging from 0 (calculated as negative) to + + + + + as high positive (this formula was proposed by the manufacturer of the ELISA kit).

RNA extraction and PCR analysis

RNA was extracted from the cattle sera using an RNeasy Mini kit 250 (Qiagen, NL). PCR analysis was

carried out using a pair of primers specific to the highly conserved 5' non-coding/non-structural coding regions of pestivirus genomes 324 (5'-ATGCCCTTAG-TAGGACTAGCA-3') and 326 (5'-TCAACTCCAT-GTGCCATGTAC-3'), which enabled amplification of a DNA fragment consisting of 288 base pairs (Vilček et al. 2003).

Reverse Transcription PCR

A mixture of 5 µl of isolated total RNA, 9.5 µl of water and 1 µl of random hexamers (Invitrogen) was denatured at 65°C for 5 min. and then transferred to an ice bed for 5 min. Next, 5 µl of reverse transcriptase (Fermentas, Lithuania), 2.5 µl of deoxyribonucleotides (2mM dNTP, Fermentas, Lithuania), 1.0 µl of ribonuclease inhibitor (10 u/µl, Fermentas, Lithuania) and 1.0 µl of reverse transcriptase (200 u/µl, Fermentas, Lithuania) were added. Synthesis of cDNA was performed at 50°C for 30 min. in a Biometra (Gottingen, Ge) thermocycler. The reaction mixture was incubated at 94°C for 2 min.

Amplification of the resulting cDNA was carried out in 54 µl of the reaction mixture containing 37.4 µl of water, 5 µl of Tag polymerase buffer, 0.5 µl of dNTP (10 mM), 1 µl of each of the primers, 324 and 326 (at a final concentration of 50 pM), 3.6 µl of MgCl₂ (25 mM), 0.5 µl of Taq DNA Polymerase at a concentration of 5 u/µl (Fermentas, Lithuania), and 5 µl of the matrix (a previously synthesized DNA strand). PCR involved 40 cycles, each consisting of consecutive phases as follows: denaturing of the strand at 95°C for 60 s, binding of primers at 56°C for 60 s and strand prolongation at 72°C for 60 s (Vilček et al. 2003).

The products resulting from the RT-PCR were electrophoresed on 1% agarose gel in TBE buffer at 10 V/cm for 50 min. The sizes of the amplification products were determined by comparison with a standard 100 bp DNA Ladder (Gibco BRL, USA).

One-step Real-Time RT-PCR

One-step Real-Time RT-PCR was carried out for all the isolated RNA samples using a Corbett apparatus. A pair of 324 and 326 primers was used in the reaction – the same ones used in the conventional PCR method. The reaction in real time with SYBR Green 1 dye was carried out in thin-walled test-tubes with a capacity of 100 µl. A One-Step SYBR PrimeScript RT-PCR Kit (Takara, Japan) was used to enable a high-specificity reaction. The reaction mixture

was prepared according to the manufacturer's instructions.

The one-step Real-Time RT-PCR involved 2 stages: Stage 1 – Reverse transcription (synthesis of cDNA was performed at 50°C for 30 min. and then the reaction mixture was incubated at 95°C for 5 min.), and Stage 2 – the PCR reaction. This stage involved 40 cycles, with consecutive phases as follows: denaturing of the strand at 94°C for 15 s, binding of the primers at 53°C for 30 s and strand prolongation at 68°C for 30 s.

Measurement of the reaction mixture and determination of the Ct (Cycle threshold) indicator value (the number of amplification cycles after which the fluorescence intensity of the formed product is higher than the background fluorescence) was carried out in real time at the elongation stage of a helix complementary to the DNA matrix. To prove the specificity of the amplification, the melting temperature of the PCR products was defined (HRM – High Resolution Melting) by gradually increasing the reaction mixture temperature from 70°C to 95°C while continually measuring the fluorescence intensity. Additionally, after amplification was completed, all the RT-PCR products were analysed with 1% agarose gel.

Results

The ELISA test results showed the presence of a high "degree of positivity" in five of the 78 samples examined (Table 1). The highly positive calves were from three different herds. In seven samples the degree of positivity was at a very low level (+ or ++), in comparison to the positive control. The four calves which tested below 40% positive were from the same herd. For calves with antibody level = 40% positivity, two were from the same herd and one from another: these calves were treated as negative due to the lack of positive results in the RT-PCR test. For the remaining 66 samples the results were below 20%, yielding a negative mean for these samples (Table 1).

Table 1. The results of BVDV positivity (ELISA kit) in the serum samples obtained from young beef cattle, aged 5-12 months.

Degree of positivity	Number of serum samples
0; Value ≤ 20 % – negative	66
++; Value < 40% – negative	4
+++; Value = 40% – questionable result	3
++++; Value 40 ≤ 60% – positive	0
++++; Val 60 ≤ 80% – strongly positive	0
++++; Val 80 ≤ 100% – high positive	5

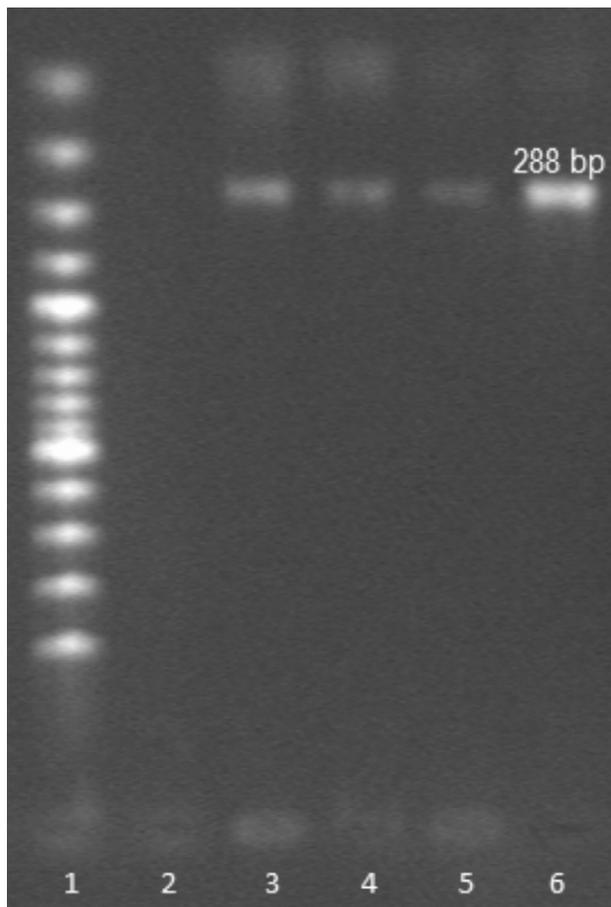


Fig. 1. PCR amplification of a partial sequence of the highly conserved 5' non-coding/non-structural coding regions of the pestivirus genomes (product size 288 bp) isolated from sera of calves. Lanes: (1) molecular weight marker = 100 bp (Fermentas, Lithuania); (2) negative control; (3) positive control; (4-6) samples examined.

Of the 78 cDNA samples the presence of genetic material from pestiviruses with a length of 288 bp was found by standard PCR in 3 of the sera (Fig. 1)

Genetic BVDV material was also found in the sera of the three calves examined by the Real-Time HRM PCR technique using SYBR Green I dye. These 3 PCR positive calves were also among the five calves that had the highest ELISA reading. The Ct values read from the amplification curve fluctuated around 30-35 cycles for all the samples examined. The Ct value for the positive control sample was about 25 cycles. The melting temperature (T_m) for all amplicons ranged between 81°C and 82°C (Fig. 2). The Real-Time PCR reaction products were visualized by the electrophoresis method in agarose gel. Their size (288 bp) was the same as for standard PCR.

Discussion

The results indicate the presence of the BVDV in beef cattle aged less than 12 months in the examined

herds. However, we cannot confirm that a similar level of presence of the virus would be observed in all beef cattle herds in Poland. In the present study, the BVDV was detected by both the PCR and ELISA techniques in 3 calves from 3 different farms. These results indicate that these three animals were indeed positive for BVDV, but in the case of the other two that tested positive in the ELISA test the PCR results were negative. However, the absence of clinical symptoms indicates that the calves could have been infected with BVDV strains of low virulence or that these calves could be PI. The fact that these animals could pose a direct danger of transmission of the BVDV among herds suggests that diagnosis of all calves should be obligatory.

Among all the beef cattle herds examined, three samples were positive for BVDV in the PCR test, and all three of these calves were from the same herd. The percentage of BVDV positive herds was about 8.6%, which suggests a low presence of the BVDV in the young beef cattle herds examined. These results could indicate the presence of PI calves in the herds, because the authors observed no clinical signs of disease.

Screening studies for prevalence of BVDV are primarily conducted using serological tests (Grom and Barlic-Maganja 1999). This is due to the fact that in asymptomatic animals at the time of examination the viral antigens may be absent in the analysed material, which may be serum, faeces or milk (Bachofen et al. 1995, Fan et al. 2012, Herlekar et al. 2013). This is confirmed by the observations of Grom and Barlic-Maganja (1999), who participated in a BVDV control and eradication programme in Slovakia. Of 7,968 samples from 354 herds, the authors found that 18% of the animals were antibody-positive, but only two of the 206 sera analysed by PCR reacted positively. The probability of detection of BVDV RNA is higher when the material is taken from animals showing clinical signs of infection. As reported by Shul'pin et al. (2003), in such cases viral genetic material can be detected in 40% of infected individuals.

The best way to obtain a credible result in epidemiological studies is to use both serological tests and molecular biology techniques. The results of the first enable determination of the degree of disease risk in the area studied, while the results of molecular studies make it possible to confirm an active infection, as well as providing material for phylogenetic studies.

It is difficult to compare the present results with those of other studies because of the lack of sufficient information on the presence of BVDV in beef cattle in Poland and elsewhere in Eastern Europe. However, there have been studies on dairy cattle in Poland and other European countries. In one study carried out on dairy cattle, high anti-BVDV antibody levels were

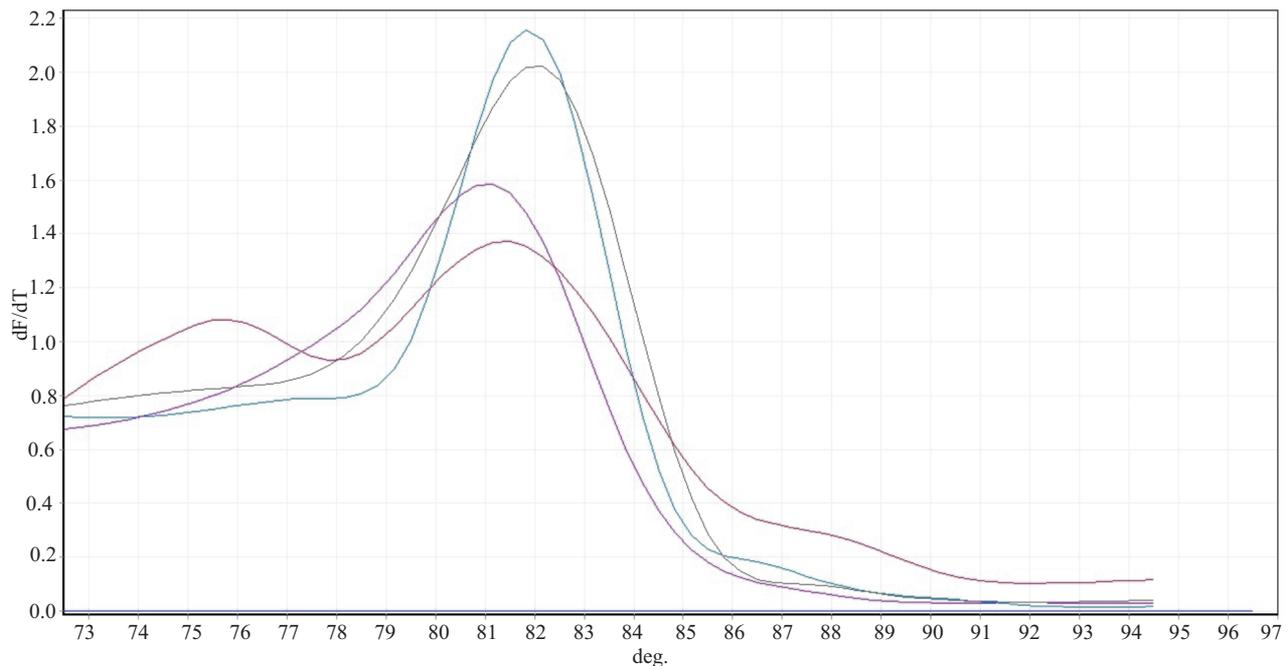


Fig. 2. Analysis of the BVDV amplicon melting curve obtained from the Real-Time PCR reaction. The melting temperature (T_m) of the products obtained ranged between 81°C and 82°C.

found in more than 52% of herds, which was a consequence of the lack of comprehensive immunoprophylaxis (Kuta et al. 2013). A similar study carried out by Rypuła et al. (2010) showed a high percentage of BVDV-positive animals, especially in large dairy herds. Studies carried out in other European countries (Paton et al. 1998, Cowley et al. 2012, Humphry et al. 2012) showed similar high anti-BVDV antibody levels (England, Wales and Scotland). In Ireland (Cowley et al. 2012) the true herd-level seroprevalence of the BVD virus approaches 100% in both non-vaccinating dairy and beef cattle herds, with no significant difference between dairy and beef herds (98.3% vs 98.8%). In Scotland, among 76 beef herds the prevalence of BVDV was more than 26.3% (Brulisauer et al. 2010).

The percentage of positive dairy herds was between 65% and 73%. Many European countries, including Denmark, Sweden, Norway, Finland, Austria, Germany, and Switzerland, have implemented BVDV eradication programs which have significantly reduced the presence of this virus in cattle herds (Obritzhauser et al. 2005, Houe et al. 2006, Lindberg et al. 2006).

In the Czech Republic, between three and six dairy farms tested negative for the presence of specific antibodies to BVDV infections in all cattle age groups (Jurhakovh et al. 2013). In the United States (Upper Peninsula, Michigan), in one beef cattle study (Corbett et al. 2011), positive results were observed only in one herd of 34 examined.

Conclusion

BVDV was found to be present in young beef cattle on the farms examined in south-eastern Poland. This is confirmed by the positive ELISA results for 6.4% of the tested animals and the positive PCR results for 3.9%. Due to the severe course of the disease and the rapid spread of the virus among cattle herds, regular monitoring for BVDV should be carried out.

Conflict of interest

The authors have no financial or personal relationships with other people or organizations that could influence their work. There are no conflicts of interest.

List of Abbreviations:

BVDV – Bovine Viral Diarrhoea Virus
 dNTP – deoxyribonucleotides
 cDNA – complementary deoxyribonucleic acid
 PCR – polymerase chain reaction
 RT-PCR – Real-Time polymerase chain reaction
 T_m – melting temperature
 HRM – High Resolution Melt
 PI – persistently infected
 Ct – cycle threshold
 CP – cytopathic

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