DETECTION OF BOVINE VIRAL DIARRHOEA VIRUS IN SALIVA SAMPLES

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Abstract

Forty-five cattle of different ages and gender were selected from three separate farms with a total number of 929 animals. Blood serum samples from each of the animals were tested twice at two-month intervals for bovine viral diarrhoea virus (BVDV) antigen (BVDV Ag) and BVDV antibodies (BVDV Ab) using ELISA. Five animals were found to be BVDV Ag positive and BVDV Ab negative. Therefore, their blood and saliva samples were subjected to further investigation. The samples of blood serum and saliva were additionally screened by a nested reverse transcription PCR (RT-nPCR), real-time PCR, and virus isolation to confirm BVDV persistent infection. Viral RNA was isolated from blood and saliva samples. The cDNA was synthesised and amplification of DNA was performed. The results of RT-nPCR were analysed by gel electrophoresis using ethidium bromide while those of real-time PCR were interpreted according to the amplification curve. Laboratory testing of blood and saliva samples revealed 5 persistently infected (PI) animals from one farm with 579 cattle (0.9% prevalence). The results were confirmed by RT-nPCR and real-time PCR screening samples of blood serum. The collection and testing of saliva is a simple and quick technique, and can be successfully applied in field conditions to identify PI animals, avoiding the risk of intervention while sampling blood or dependence on animal gender and lactation period while sampling milk or semen.

Key words: cattle, bovine viral diarrhoea virus, detection, saliva, PCR.

Bovine viral diarrhoea virus (BVDV) belongs to Pestivirus genus of the Flaviviridae family (4, 35). The virus infects mainly cattle and sheep, while other ruminants and swine are not so often infected (5). It possesses the ability to cross the placenta barrier (32). BVDV is one of mostly spread viruses in the world, as well as in Lithuania (33, 34). It can cause big financial losses because of reproduction disorders in the affected cattle (1). Half of them are due to persistently infected (PI) animals that are constantly shedding BVDV (23). Cultivation of BVDV in cell culture revealed two biotypes of this virus - cytopathic and non-cytopathic, depending on how it affects the cells. Both biotypes are pathogenic to cattle and may predetermine the course of the disease (8). Herds infected with BVDV suffer a wide range of losses due to infertility, immunosuppression, diarrhoea, thrombocytopenia, and drop in milk production (49). In pregnant cows, transplacental infection may cause abortion or stillborn birth, abnormal or PI infected calves, which remain infected for their entire life and are constantly carrying and shedding the virus (29). Their presence in herds may be considered as the most “favourable” possibility for spreading of the virus. In case of intensive virus replication, clinically healthy cattle during viraemia can also spread virus with their faeces, urine, saliva, nasal and eye secretions, and in bulls with semen (23, 24). PI animals usually do not have clearly expressed clinical symptoms; however, some calves are depressed, have difficulty in moving, lower weight, and shorter lifespan (11, 31, 37).

Different detection techniques can be applied for the identification of BVDV (43, 45). The virus infection is diagnosed by serological methods used to detect antibodies in blood serum (9, 15, 18, 25, 38). For identification of viraemic animals, the technique of virus isolation in cell culture and further identification of the isolates by immunoperoxidase method is used. BVDV Ag and BVDV Ab are detected by ELISA technique. The detection of viral nucleic acid is performed by PCR techniques (3, 20, 26, 38, 47, 50, 51). In order to control
BVD virus infection in herds, it is very important to identify PI animals in time (27, 28, 39). Most often all animals in the herd are tested by the use of blood samples. However, blood sampling may cause a drop in milk production and enhance the risk of a secondary infection. Therefore, it is advisable to use a safer sampling technique to screen samples and to identify PI animals.

Material and Methods

Forty-five animals (22 bulls and 23 heifers) were selected from three different farms (total number of cattle in farm I – 579, farm II – 317, farm III – 33), in which BVDV infection was earlier detected by serological methods (48). The age of the animals ranged from 4 to 24 months. The weight of some cattle was too low as compared with that of the healthy animals.

Selection of samples for testing. Blood and saliva samples were collected twice at two-month intervals and tested for BVD Ag and BVD Ab by ELISA. Five animals BVDV Ag positive and BVDV Ab negative were subjected to further investigation. The samples of blood and saliva were collected repeatedly and tested by RT-nPCR, real-time PCR, and by virus isolation in cell culture.

The saliva was sampled with Salivette swabs preserving RNA (Paxgene RNA tubes containing RNA preservative). Salivette swabs were kept under the animal’s tongue for 2 min. Later, they were put into sterile tubes that were closed, marked, and placed into a container for storing at room temperature. For each animal, a pair of disposal gloves, new Salivette swabs, and tubes containing RNA preservative were used. The saliva samples were then transported and stored in a container at room temperature.

Detection of BVDV Ag and BVDV Ab by ELISA. Forty-five blood serum samples were collected and screened for BVDV Ag and BVDV Ab by ELISA. Five animals BVDV Ag positive and BVDV Ab negative were subjected to further investigation. The samples of blood and saliva were collected repeatedly and tested by RT-nPCR, real-time PCR, and by virus isolation in cell culture.

Detection of BVDV in blood serum and saliva by RT-nPCR. Blood and saliva samples from BVDV Ag positive cattle were tested. RNA was isolated using “Total RNA Prep Plus” kit (A&A Biotechnology, Poland) as described by the manufacturer. The volume of 200 µl of blood serum and saliva was treated with 800 µl of phenosol to initiate the cell lysis. The next step was the incubation for 5 min at 50°C, then 200 µl of chloroform was added and the content was shaken intensively for 15 s by the MS1 minishaker. After keeping for 3 min at room temperature, the tubes were centrifuged at 10 000 rpm/min. The surface fraction was removed into a new tube and 250 µl of isopropanol was added, it was mixed and once again transferred into another tube containing a specific RNA immobilising membrane. Further, it was centrifuged at 10 000 rpm/min for 1 min. At this stage, viral RNA bound to the membrane was washed three times with a special solution containing 96% ethanol. The volume of 700 µl of the washing solution was poured onto the membrane containing RNA and centrifuged for 2 min at 10 000 rpm/min. After having it dried by centrifugation, 100 µl of DEPC-treated water containing no RN-ases or other inhibitors were added. The extracted RNA was used as a matrix for RT-nPCR and real-time PCR. The samples for negative (distilled water) and positive (BVDV, NADL strain) PCR control were used.

Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>The sequence of nucleotide primers 5’--3’</th>
<th>Position in nucleotide</th>
<th>Genome region</th>
</tr>
</thead>
<tbody>
<tr>
<td>V324 forward</td>
<td>ATGCCCT(T/A)TAGTGGGCACTGAGCA</td>
<td>90–110</td>
<td>5’UTR</td>
</tr>
<tr>
<td>V326 reverse</td>
<td>TCAACTCCATGTGCCCAGTGTAC</td>
<td>353–373</td>
<td>5’UTR/N\textsuperscript{PRO}</td>
</tr>
<tr>
<td>A11 forward</td>
<td>AGTAGGGTATGTCGTAGTGTTGTCG</td>
<td>161–186</td>
<td>5’UTR</td>
</tr>
<tr>
<td>A14 reverse</td>
<td>CAACTCCATGTGCCCAGTACAGCA</td>
<td>348–372</td>
<td>5’UTR/N\textsuperscript{PRO}</td>
</tr>
</tbody>
</table>
One-tube RT-nPCR method was used for the reaction. For the second nPCR stage, reagents have been prepared in the same one tube PCR. The solution contained 5 µl of 22% trehalose (Sigma) to fix other components of the reaction, 20 pmol/µl of nucleotide primers A11 and A14 (Table 1), 1 µl of dNTPs mixture (dATP, dCTP, dGTP, and dTTP) of 10 mM solution, and 0.25 µl of Taq DNA polymerase (1.25 U, Fermentas, Lithuania). The 8.25 µl volume of this mixture was pipetted directly into the cap of each sterile 0.2 ml Eppendorf tube and allowed to dry for 2 h at room temperature. PCR reagents prepared in such a way had a shelf life of 6 months.

At the next stage, the same 0.2 ml Eppendorf tubes with the caps containing dried mixture for nPCR were used. RT-PCR was performed at the bottom of these tubes. For the amplification, the reaction mixture of 50 µl containing 5 µl of RNA, 5 µl of 10xPCR buffer, 5 µl MgCl₂ 25 mM, 2 µl dNTPs 10 mM (Fermentas, Lithuania), 0.25 µl of each (5 pmol) upperstream primers V324 and V326, 1 µl of 10% triton-100 (Sigma), 0.5 µl (2.5 U) of taq DNA polymerase, 0.25 µl (10 U) of RNAAnin, 0.5 µl (100 U) of M-MLV transcriptase (Fermentas, Lithuania), and 30.25 µl of DEPC water was prepared. RT-PCR reaction mixture at the bottom of the tube was separated from nPCR components present in the cap of the tube by using mineral oil (Sigma). RT-nPCR was carried out in the amplifier Mastercycler® (Eppendorf). After mixing RNA and RT-PCR components, the reverse transcription was performed in a thermo-cycler at 42°C for 30 min. Reverse transcriptase was inactivated by heating at 95°C for 5 min, thus starting the 20 fold cycle reaction of the amplification: denaturation of cDNA at 94°C for 1 min, hybridisation using specific primers at 52°C for 1 min, cDNA elongation (extension) at 72°C for 1 min. At the end of the first stage of the amplification, the tubes were heated at 65°C for 2 min, turned upside down, and kept for 2 min in order to mix and lyse the components of nPCR at the cap of the tube with the RT-PCR products at the bottom of the tube. Afterwards, the tubes were centrifuged for a short time and nPCR of 30 amplification cycles was performed: at 94°C for 1 min, 52°C for 1 min, and at 72°C for 1 min. To effect the final amplification, the prolongation (extension) procedure at 72°C for 10 min was carried out. Thus, the product of 212 bp length was obtained.

The RT-nPCR amplification products were analysed in 2% agarose gel Top Vision™ GQ Agarose (Fermentas, Lithuania) stained with ethidium bromide (at the concentration of 1 µg/mL). The electrophoresis of 10 µl of PCR product was carried out in lxTBE (90 mM Tris, 90 mM boracic acid, 2 mM EDTA) or lxTAE buffer solution under 100 voltage for 45 min. The results of RT-nPCR were analysed using UV lamp to detect the specific fluorescence of DNA bands at the position of 212 bp molecular marker, GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, Lithuania) (47).

The detection of BVDV in blood serum and saliva by real-time PCR. The real-time PCR was carried out according to the protocol of ADIOGENE ADIAVET using BVD specific gene amplification (Q-PCR TEST). The reverse transcription was performed using RevertAidTM H minus First Strand cDNA Synthesis Kit (Fermentas, Lithuania) and random hexamer primers. RNA amplification was carried out according to ADIAVET instructions. BVDV amplification solution (23 µl) was added to each of PCR tubes. For each sample, 2 µl of cDNA was added to 23 µl amplification solution. Once all the tubes were prepared, real-time PCR amplification was performed in Applied Biosystems ABI Prism type 7900. The reaction cycles were programmed in the following: at 95°C for 15 min and 45 times (95°C 15 s and 60°C for 1 min). The results were interpreted according to the data of the amplification curve. For negative sample control, deionised water was used.

The isolation of BVD virus on cell culture. BVD virus was isolated from blood and saliva samples of PI animals. Bovine kidney cell line (MDBK) was obtained from the Federal Research Centre of Virus Diseases of Animals, Island Riems (Germany). The strain of BVD cytopathogenic virus NADL, which we used for control, was obtained from the Reference Laboratory, Institute of Virology (Germany). MDBK cells were cultivated in 96 well plates. The monolayer covering 80-90% of the surface was formed in 24 h. After sample centrifugation, the supernatant was collected and filtered through 0.45 µm pore size sterile membrane filter to infect cells. The cell growth medium was removed and the cells were washed three times with Hanks balanced salt solution. On the monolayer of MDBK cells in every one of four chambers, 200 µl of sample supernatant was added, while other two chambers contained cytopathogenic BVDV NADL strain. The inoculated cells were incubated for 2 h at 37°C. Following the incubation, 125 µl of prepared DMEM F-12 medium was added to every one of four chambers and the cultivation was prolonged for 5 d at 37°C in the presence of 5% CO₂. The cells were observed every second day for the cytopathic effect. After 5 d of incubation, the cells were fixed with 80% acetone. Following the second fixation they were labelled with Anti-BVDV fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (FITC anti-BVDV NS3-P80, Bio-X Diagnostics, Belgium) and incubated for 1 h at room temperature. Following the incubation, the cells were dried and analysed under fluorescent microscope. Blood samples were passaged once, while saliva samples – three times.

**Results**

From 45 animals selected for the study and tested by ELISA, 5 were found to be positive for BVDV Ag and negative for BVDV Ab. The remaining 40 blood samples were BVDV Ag and BVDV Ab negative. After two months, the investigations of 45 blood samples were repeated and the same results were obtained. The RT-nPCR and real-time PCR testing of blood serum and saliva samples revealed the presence of BVDV RNA in the same 5 animals, while the remaining 40 ones gave negative results. The data of RT-nPCR and real-time PCR are shown in Figs 1 and 2.
Fig. 1. 2% ethidium bromide stained agarose gel. The results of blood serum and saliva testing: 1 - 5 bovine blood serum, 6 - blood serum negative control, 7 – blood serum positive control, 8 - the marker of molecular mass, 9 - 13 bovine saliva, 14 – positive control of saliva, 15 - negative control of saliva, 16 - marker of molecular mass.

The intensity of fluorescence

Fig. 2. The amplification curve of real-time PCR results.

All positive and negative controls were carried out at the same time. By PCR technique, BVDV RNA in PI cattle was found in both blood serum and saliva. The study confirmed persistent infection with BVDV in one of three selected farms. On the farm I with 579 cattle, there were 5 PI animals (BVD prevalence – 0.9%) while on farm II and III with 317 and 33 cattle, respectively, no PI animals were found.

After performing RT-nPCR tests for all 5 PI cattle, saliva and blood serum samples were further investigated by real-time PCR to detect BVDV RNA. BVDV RNA was found in saliva of PI animals. The interpreted results of real-time PCR allow us to draw the conclusion that the amount of BVDV RNA was lower in saliva samples as compared with those of blood serum (Fig. 2). The threshold – 0.01 (ΔRn), the blood serum cycles (Ct) – 24.6, 25.3, 25.1, 25.4, 25.3 and saliva sample cycles (Ct) – 29.7, 35.8, 31.4, 28.2, 28.7.

To confirm the results of RT-nPCR and real-time PCR, the samples of blood serum and saliva from the same 5 PI animals were further subjected to the method of virus isolation. In the course of viral investigations, BVDV was isolated from the samples of blood serum and saliva. In MDBK cell culture, the BVD virus expressed its noncytopathogenic biotype. Blood and saliva-infected cells expressing BVDV in their cytoplasm showed green fluorescence. Saliva samples were passaged three times while blood samples only once to reach the same limit of fluorescence detection. The results showed that BVDV is constantly circulating in saliva, however its amount in blood could be considered higher.
Discussion

The main mean to eradicate BVD in bovine herds is the identification and elimination of PI animals (7, 29). For the detection of such animals, both serological and virological tests can be used. ELISA technique is mostly applied for fast identification of BVDV Ab and BVDV Ag in the samples of blood serum. However, it must be confirmed by other virological and molecular biology methods, such as virus neutralisation and virus isolation or viral nucleic acid detection (26, 44, 50, 51).

Today’s modern techniques allow for developing new practical schemes to control BVDV, which include testing of blood, milk, embryo liquid, and semen samples for the presence of the virus. However, some tests are gender dependent, i.e. semen samples can be collected only from bulls, and milk sampling depends upon the lactation period of the cow. Besides, blood collection increases the risk to infect the host organism (10, 17, 19, 20, 22, 36, 40).

The spread of the infection cannot be controlled when PI animals have a direct contact with the healthy ones. However, cattle at the initial stage of BVDV infection transmit it rather slowly. Saliva is constant secretion of the organism, thus it can be assumed that PI animals constantly secrete BVDV into the environment. Recent methodologies of laboratory tests deal a lot with saliva sampling to work out the monitoring schemes for the control of infectious diseases avoiding the risk of intervention and eliminating the incidence of infection (12, 16, 21, 41, 42). With the help of new laboratory techniques, it will be possible to carry out BVD control by saliva sampling, identifying PI cattle in herds, and eliminating them.

After the PCR method was developed, many scientists used it for virus detection in RNA genome amplification, or in other words, so-called reverse transcription polymerase chain reaction (6, 13, 14, 20, 30). In our study, the laboratory tests revealed BVD virus in blood and saliva samples from 5 PI animals. BVD virus RNA was detected by RT-nPCR, real-time PCR and also confirmed by virus isolation. All the time of investigation, these cattle were BVDV positive and secreted virus into the environment. The usual prevalence of PI animals in the herd is between 0.5% and 2% and our study revealed 0.9% prevalence in one of the farms. The benefit of saliva sampling lies in the simplicity of the method. Samples are easily collected by putting cotton swabs under the tongue of the animal and allowing them to be saturated with saliva. The animals suffer no stress and there is no intervention into the organism. Besides, saliva is collected disregarding animal gender and lactation period. However, there is some specificity in saliva sampling too. It is more difficult to take the samples from small calves because they secrete less saliva and the time to keep the swab under the animal tongue is longer. Saliva is a sensitive medium, therefore in order to detect BVDV PI animals more precisely, it is advisable to stabilise the viral RNA. At testing saliva samples by real-time PCR, the amount of virus obtained was lower and for virus isolation, three passages were needed to reach the same limit of fluorescence detection as that after only one passage at blood testing.

Saliva contains many degradative enzymes, some of which are capable of destroying an RNA template. This may reduce the already low copy number. The critical period for degradation is likely to be between the collection and freezing of the samples, as confirmed by some studies (41).

In summarising, we may assume that controlling the incidence of PI animals in herds is possible by working out a system of BVDV identification from saliva samples. It will render very similar results to those of testing blood serum. The benefits of saliva sampling lie in the simplicity of the method, its advantage in avoiding the intervention into the organism, and the possibility to collect samples independently on the period of lactation or animal gender. A carefully worked out practical scheme might serve as a good means for the identification and elimination of the BVDV infected animals from herds.

References

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