

The epidemiology of bovine viral diarrhoea virus infection on a dairy farm - clinical signs, seroprevalence, virus detection and genotyping

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ABSTRACT

In this study we investigated the presence of Bovine Viral Diarrhoea Virus (BVDV) antibodies and antigens in a dairy cow herd that had reported clinical signs related to BVDV infection. The aim of the study was to confirm BVDV infection on the dairy farm with 1500 Holstein-Friesian cattles, and genetically analyse the BVDV type/s circulating in the herd. Serum samples (n=233) were collected from clinically infected cattles and tested for the presence of BVDV antibodies and antigens. The virus neutralization test showed that 194 out of 233 sera of the tested cows were positive (83.26%), and by Ab-ELISA 203 sera samples of the tested cows were shown to be positive (87.12%). The presence of antigens was proven with the Ag-ELISA test in 2 out of 233 samples (0.86%). After RT-PCR and phylogenetic analysis of two BVD virus isolates, it was found that both isolates belonged to genotype 1, subtype BVDV-1d. This is the first time that the BVDV-1d subtype has been confirmed in Croatia, although the BVDV-1b and BVDV-1f subtypes have been detected previously on Croatian dairy farms.

Key words: BVDV; VNT; At-ELISA; Ag-ELISA; RT-PCR; sequencing; phylogenetic analysis; BVDV 1 subtype 1d

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Introduction

Bovine Viral Diarrhoea Virus (BVDV) is one of the viral agents with the most significant economic impact in the dairy and beef industry, mainly due to health and reproduction disorders (GRIFFIN, 1997; HOUE, 2003). BVDV is a positive-sense RNA virus of the genus *Pestivirus*, and *Flaviviridae* family (WENGLER et al., 1995). The genome is approximately 12.3 kb and consists of a single open reading frame, flanked by 5' and 3' untranslated regions (UTRs) (COLLETT, 1992). BVDV is classified into two genotypes, BVDV type 1 and BVDV type 2. On the basis of their in vitro cell culture characteristics and genetic differences, they are further classified as cytopathogenic (cp) and noncytopathogenic (ncp) genotypes. BVDV is highly variable, both antigenically and genetically, and is further genetically divided. BVDV type 1 is divided into at least 21 (1a-1u) subtypes, and BVDV type 2 into 3 (2a-2c) subtypes (BECHER et al., 1997; LIU et al., 2009; PETERHANS et al., 2010; DENG et al., 2012; ALPAY and YEŞILBAĞ, 2015). Worldwide distribution of BVDV-1 is significantly broader (88.2%) than the distribution of BVDV-2 isolates (11.8%) (YEŞILBAĞ et al., 2017). BVDV infections in cattle can be acute (transient) or chronic (persistent). Persistent infection (PI) develops when a fetus is infected in the early stage of gestation, during the first 120 days prior to the onset of immunological competence. The animals that maintain lifelong viremia and continuously shed large amounts of the virus play the main role in viral maintenance and transmission (HOUE, 1999; SMITH and GROTELUESCHEN, 2004). It is therefore important, for control of BVDV infection, to identify PI calves in the herd promptly and exclude them.

The sources of infection may be animals with no clinical signs of the disease. Humans can spread the disease through infected feed and water. Other ruminants (BECHER et al., 1999) and pigs (WANG et al., 1996), including wild animals, can also be infected with BVDV.

BVDV causes a complex of disease syndromes, that vary from subclinical to acute disease with possible fatal outcome (PELLERIN et al., 1994; BAKER, 1995; EVERMANN et al., 2005;

RIDPATH et al., 2006; COURTENAY, 2007). Symptoms include: transient fever, diarrhea, leukopenia, immunosuppression, depression, anorexia, respiratory disorders, lower milk production, absence of or reduced conception in cows, recurrence of infection, early termination of pregnancy, long service period, stillbirth with congenital malformations, abortion and mummification, immunotolerance and persistent infection, as well as the acute and chronic mucosal disease.

The mucosal disease caused by BVDV-1d with high morbidity and lesions is limited only to the upper digestive system and skin, and the absence of intestinal lesions has been studied. Thus, the epidemiological and pathological features of this form of mucosal disease may be similar to vesicular diseases, including foot and mouth disease (BIANCHI et al., 2017).

Previous research into BVDV in Croatia revealed the presence of genotype 1, subtype BVDV-1b and BVDV-1f (BEDEKOVIĆ et al., 2012), therefore the aim of this study was to detect the presence of BVDV on the dairy farm in question, and genetically analyse the BVDV type/s circulating in that herd.

Materials and methods

Sampling. In this study, 233 serum samples were collected between 2012 and 2016, from live bovine animals that manifested clinical signs related to BVDV infection (diarrhea, infertility, low milk production). The sample group consisted of 1500 dairy cows of different ages. Blood samples, approximately 10 mL per animal, were taken by puncture of the jugular vein (*v. jugularis*), and they were centrifuged in the laboratory at 2000 rpm for 10 minutes, and the sera 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.

Test procedures used. The seroprevalence of specific antibodies was determined using the Virus Neutralization test (VN-test) and Ab-ELISA test. The presence of antigens was proven by the Ag-ELISA test, and the presence of viral nucleic acid by a real-time RT-PCR method (HOFFMANN et

al., 2006). Positive samples were moreover tested by a PCR protocol (BARLIČ-MAGANJA and GROM, 2001). Phylogenetic analysis of BVDV isolates was based on a comparative analysis of the nucleotide sequences of the 5' UTR segment and N^{pro}.

Virus Neutralization test (VN-test). The test was performed as described in the accredited test protocol of the Virology Laboratory of the Faculty of Veterinary Medicine in Zagreb, according to the recommendations of OIE. All sera samples were inactivated at 56 °C / 30 min before testing. After that the tested sera were dribbled onto the plates in twofold dilutions, at an initial 1:2 to 1:256, and similarly positive and negative controls of standard sera. The diluent for the sera dilutions was a sterile cell culture medium (DMEM, Dulbeccos, Modified Eagle Medium-Sigma Aldrich Inc., USA). For each dilution of one serum sample, four wells of the microtiter plate were used. Bovine viral diarrhoea virus (BVDV) strain NADL was added with a titre of 100 TCID₅₀ / 50 µl. The incubation period was 60 minutes at 37 °C in the cell-incubator. After incubation, the cell suspension of Madin Darby Bovine Kidney (MDBK) cells, with a density of 1.2 x 10⁵ cells/1 ml, was added in a volume of 100 µm per well. The reciprocal of the highest serum dilution showed the complete inhibition of the cytopathic effect, and this was taken as the VNT titer.

Antibody-ELISA test (Ab-ELISA). Specific antibodies for BVDV were detected in the tested sera samples by the BVDV-Ab kit (Svanovir, Sweden). The test was performed following the manufacturer's instructions and the results were calculated after the optical density (OD) had been measured at 450 nm by spectrophotometer (Tecan, model Sunrise, GmbH Austria).

Antigen-ELISA test (Ag-ELISA). For BVDV antigen detection, the Herdchek BVDV Ag/Serum Plus set (IDEXX, Liebefeld-Bern, Switzerland) was used. This test was also performed following the manufacturer's instructions. The data were analyzed on the basis of the optical density (OD) value for each sample, and positive and negative controls according to the formula provided by the manufacturer. *Real time RT-PCR method.*

Viral RNA was isolated using a commercial kit (iPrep Total RNA, Invitrogen, SAD) according to the manufacturer's instructions, and amplified in a Mx3005P machine (Stratagene, USA) using a 1 Step RT qPCR Probe ROX L Kit (highQu, Germany) by the following cycles: 10 min/50°C; 2 min/95°C; 5 sec/95°C and 30 sec/60°C. The 104F and 402R primers for the 5'-UTR region (BARLIČ-MAGANJA and GROM, 2001) were used for amplification.

Positive samples were then tested by a further classical PCR protocol. The products of the 5'UTR region were amplified by Gene Amp PCR System 9700 (Applied Biosystems, SAD) in a cycle of 20 min/50°C; 2 min/95°C; 10 sec/55°C; 20 sec/72°C and 7min/72°C, and extracted by the Wizard SV Gel and PCR Clean-UP System (Promega, USA). Extraction and purification of the PCR-products from the agarose gels were performed by a commercial Wizard SV Gel and PCR Clean-UP System kit (Promega, SAD) according to the manufacturer's instructions. PCR products were sequenced by Macrogen Inc. (Amsterdam, Netherlands). The sequences of 294 and 441 base pairs were submitted to GenBank (accession no. MW057258-MW057259). Phylogenetic grouping and clustering were based on comparison with sequences retrieved from GenBank, using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov>). The sequences were aligned with ClustalW 1.6 and analyzed using MEGA7 (KUMAR et al., 2016), whereas the trees were generated using the neighbor-joining method, applying the Kimura 2-parameter evolutionary model. The bootstrap was calculated on the basis of 1000 repeats.

Results

The tested blood sera samples originated from 233 cows with noticeable clinical signs in their digestive system (diarrhea), reproductive system (infertility) and by drop in milk production. Examination of the medical record cards of the positive cows revealed that the most common clinical sign was diarrhea (91% of cases), followed by a drop in milk production (55%), infertility (52%), missbirths (19%), mastitis (18%), drying-off (12%), problems with rumination (8%), stillbirths

(4%), tarry excrement (2%) and pneumonia (2%), while the least represented were lactation oscillation and non-vital calves. The presence of observed clinical signs in cows is presented in Fig. 1.

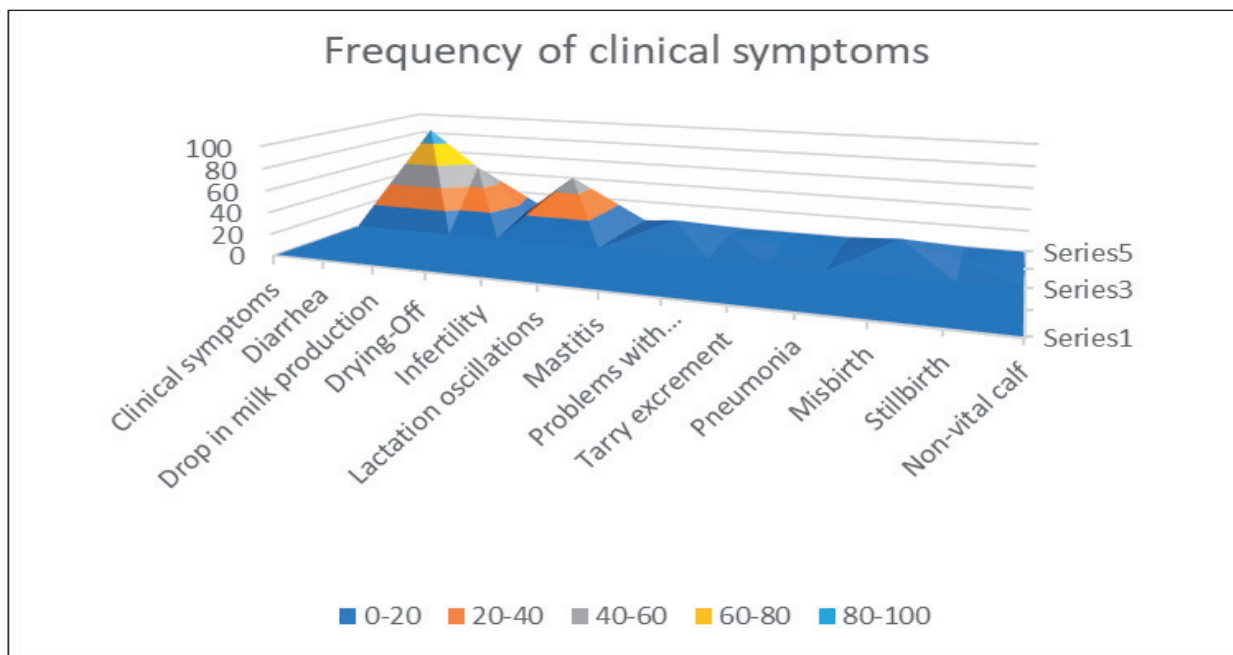


Fig. 1. Frequency of occurrence of certain clinical signs in searched cows

VN-test. The presence of antibodies for BVDV was confirmed by the VN-test in 194 out of 233 tested sera (83.26%). The VNT titer was calculated by the Reed-Muench method, and sera samples with VNT antibody titer $\geq 1:2$ were considered positive (Table 1).

Ab-ELISA. The presence of antibodies for BVDV was confirmed by Ab-ELISA in 203 cow sera samples (87.12%). According to the manufacturer's instructions, all values ≥ 1.20 were

considered positive and values < 1.20 negative (Table 1).

Ag-ELISA. The presence of BVDV antigens was proven with the Ag-ELISA test in 2 out of 233 samples (0.86%). The values of OD ≥ 0.30 were considered positive and values < 0.30 negative. Two animals that were shown to be positive for the presence of antigens by the Ag-ELISA test did not show any clinical symptoms of BVD (Table 1).

Table 1. Positive serum results in relation to test used

Test used	Positive on Ab	Positive on Ag	Negative on Ab	Negative on Ag
VN	194 (83,26%)	-	39	-
Ab ELISA	203 (87,12%.)	-	30	-
Ag ELISA	-	2 (0,86%)	-	231
RT-PCR	-	2 (0,86%)	-	-

RT-PCR method. Presence of BVDV RNA was proven with both RT-PCR procedures in 2 of 233 samples giving PCR products of the expected size (294 and 441 bp, respectively).

Phylogenetic analysis of nucleotide sequences of 5' UTR determined their affiliation to genotype 1, subtype 1d of BVDV.

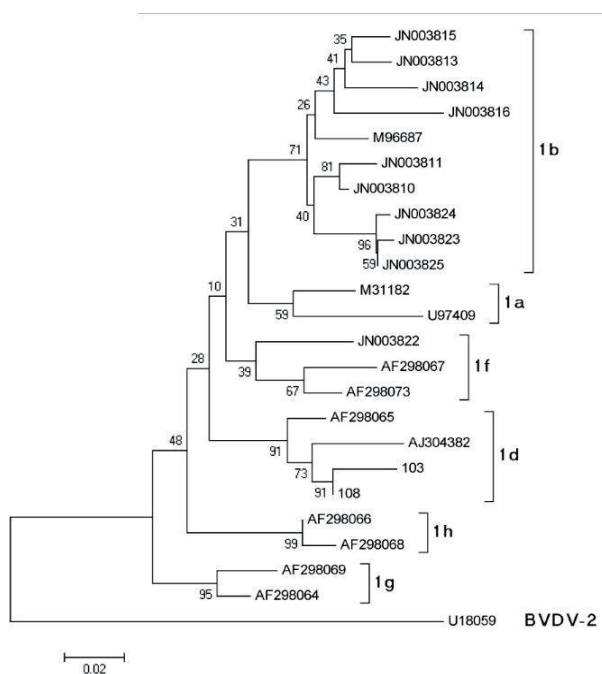


Fig. 2. Phylogenetic tree obtained by analysis of the 5' UTR fragment of the BVDV genome

The analysis (Fig. 2) was done by the neighbor joining method using the *Kimura-2 Parameter* model. The numbers in the node of the branches represent the values given by the *Bootstrap* statistical method in the 1000 repeats. The scale presents genetic distance ($d=0.02$). Phylogenetic groups are indicated on the right side of the angular brackets.

Our isolate was genetically very similar to the reference strain AJ304382-BVDV9466/91, genotype 1d, which was isolated in 2000 in Germany.

Our isolate was distinguished from reference strain AF298065 fragment 103 in 11 of 215 nucleotides, (5.11%), while the difference from the AF298065 fragment 108 was in 9 of 215 nucleotides (4.18%) (isolated in Europe in 2000).

Discussion

BVDV infection was confirmed in the studied herd. Antibodies for BVDV were found in 87% of the sampled animals. Two out of the 233 sera samples tested were positive for BVDV (about 0.86%). Both BVDV isolates belong to the BVDV-1, subtype 1d. Persistent infection was therefore determined in the investigated herd.

BVDV has been researched and its presence proven in Croatia since 1989. (MADIĆ et al., 1989.; BIUK - RUDAN, 1997.; BIUK - RUDAN et al., 1999.). There was a comprehensive study on the prevalence and genetic heterogeneity of local BVDV strains in Croatia, and it revealed the presence of the BVDV type 1, subtypes 1b and 1f in Croatia (BEDEKOVIĆ et al., 2012).

Similar research into BVDV genotypes has been conducted worldwide, and revealed the global nature of the virus. The same subtypes of BVDV can be found at different geographical locations, naturally in different ratios. According to the published data, the most prevalent BVDV group in Spain was 1b and to a lesser extent 1d, 1e and 1f (DIEGUEZ et al., 2017), which is very similar to the prevalence of BVDV genotypes in our country. Likewise, phylogenetic studies of BVDV in Polish dairy herds identified many different subtypes of this virus. A previous study indicated the predominance of BVDV-1b and BVDV-1d in Poland. In this study, seven subtypes of BVDV-1 species were identified: 1b, 1g, 1f, 1d, 1r and 1e (PAWEL and POLAK, 2019). Also, in Western Austria, the BVDV-1 genotypes were clustered into eight different subtypes, with the highest frequency of BVDV-1h, 1f, 1b and 1d (HORNBERG et al., 2009).

A random effect meta-analysis was performed to estimate the worldwide pooled bovine viral diarrhoea virus (BVDV) prevalences of persistently infected (PI), viraemic (VI) and antibody-positive (AB) animals and herds. The meta-analysis covered 325 studies in 73 countries that determined the presence or absence of BVDV infections in cattle from 1961 to 2016. The worldwide pooled PI prevalences at animal level ranged from low (<0.8% Europe, North America, Australia), medium (>0.8% to 1.6% East Asia) to high (>1.6%

West Asia). The PI and AB prevalences in Europe had decreased over time, while BVDV prevalence increased in North America. The highest mean pooled PI prevalences at animal level were identified in countries that had failed to implement any BVDV control and/or eradication programmes (SCHARNBOECK et al., 2018). In a dairy cattle herd in Brasil, the phylogenetic analyses revealed that PI animals were infected by three different BVDV subgenotypes (BVDV-1a, BVDV-1b and BVDV-1d) (RODRIGO et al., 2014), which also corresponds with the results of this study.

Eleven dairy cattle herds with nearly 20,000 dairy cows were analyzed to investigate persistent infection (PI) with BVDV along with its coexistence with BVDV antibody titer and BVD virus in the blood of tested cows. The results revealed that 98.56% cows were positive for BVDV antibodies, while the BVDV antigen was detected in only 1.42% cows, which were negative for BVDV antibodies and so were considered as persistently infected cows (GAROUSSI et al., 2019). These results are also very similar to the results of this study. Since there is still no specific cure beyond symptomatic and prophylactic treatment of affected animals or those at risk, a logical approach to minimize the prevalence of BVDV is to develop vaccines, which over the years have reduced losses due to acute BVDV infections, and protection measures to prevent fetal infections (TORSTEIN, 2004).

The results show that BVDV infection in the dairy cow herd examined is excessively present because of the high seroprevalence, and persistently infected cattle. The two BVDV isolates belong to BVDV genotype 1, subgenotype 1d, which is the first evidence of BVDV-1d in Croatia. These data show that the prevalence of BVDV is very similar to that in other European countries, especially the presence of BVDV-1d genotype, which is mainly found in Europe.

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SAŽETAK

U mliječnim krava s kliničkim simptomima virusnog proljeva goveda (VVPG) analizirana je prisutnost uzročnika s obzirom na protutijela i antigen. Na farmi s 1500 krava holštajn-frizijske pasmine, cilj istraživanja bio je potvrditi infekciju s VVPG i to na onim grlima kod kojih su klinički znakovi upućivali na bolest. Zadatak je bio izdvojiti virus i genetski ga analizirati s obzirom na tipove i podtipove koji cirkuliraju na navedenoj farmi. U tu svrhu su sakupljena 233 uzorka seruma od oboljelih životinja, kako bi se testirali na prisutnost protutijela i antigena VVPG. Pomoću virus neutralizacijskog testa (VN test) prisutnost protutijela za VVPG je dokazana u 194 serumska uzorka (83,26%). Ovaj rezultat je potvrđen pomoću antibody-elisa testa (Ab-ELISA test) u 203 serumska uzorka (87,12%). Prisutnost antigena je istražena pomoću antigen-elisa testa (Ag-ELISA test) i dokazana u 2 serumska uzorka (0,86%), što je potvrđeno i RT-PCR testom. Nakon sekvenciranja uzoraka, filogenetskom analizom potvrđeno je da su to izolati VVPG-a i da pripadaju genotipu 1, podtipu 1d. Ovo je prvi dokaz VVPG-1d u Republici Hrvatskoj u kojoj su dosad na farmama mliječnih krava prepoznati podtipovi VVPG-1b i VVPG-1f.

Ključne riječi: VVPG; VN-test; At-ELISA; Ag-ELISA; RT-PCR; sekvenciranje; filogenetska analiza; VVPG 1 podtip 1d
