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ISOLATION AND CONFIRMATION OF BOVINE VIRAL DIARRHOEA VIRUS IN SERBIA AND COMPARATIVE TYPING WITH RECENT SLOVENIAN ISOLATES

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The results of an investigation on bovine viral diarrhoea virus (BVDV) in fetal calf serum (FCS), whole blood and pathological material obtained from sick or dead cattle in Serbia are presented. Whole blood and FCS from sick animals were screened for BVDV antigen (E^{rns}) by ELISA. ELISA positive samples and pathological material from dead animals were inoculated into primary cell cultures of fetal calf testis (FTTe). After threefold passage in FTTe cells, BVDV was detected by direct immunofluorescence and indirect immunoperoxidase tests and by reverse transcriptase-polymerase chain reaction (RT-PCR). Among 64 individual samples of FCS, two were positive for noncytopathogenic BVDV. One cytopathogenic BVDV was isolated from a whole blood sample from a heifer with clinical signs of mucosal disease. The 5'-untranslated region (5'-UTR) of three Serbian BVDV isolates was amplified by RT-PCR, sequenced and, together with 15 recent Slovenian BVDV isolates, characterised by phylogenetic analysis. All isolates were classified as BVDV genotype 1 viruses. The majority of the BVDV isolates were of the 1f (Serbia - 2 isolates, Slovenia – 7 isolates) and 1d subtypes (Slovenia - 7 isolates), whilst one Serbian and one Slovenian isolate were genotyped as BVDV 1b.

Key words: bovine viral diarrhoea virus; RT-PCR; genotyping; phylogenetic analysis

INTRODUCTION

Bovine viral diarrhoea (BVD) is a viral disease of cattle that is common worldwide. The disease is caused by the BVD virus (BVDV) that belongs to the genus, *Pestivirus*, of the *Flaviviridae* family (Heinz *et al*., 2000). BVDV is genetically and antigenically related to two other *pestiviruses*; classical swine fever virus and border disease virus (BDV). BVDV is a small enveloped virus with a genome consisting of a single, positive stranded RNA molecule approximately 12.3 kb in length. A single open reading frame (ORF) is flanked at either end by 5'- and 3'- untranslated regions (5'-UTR, 3'-UTR). The ORF encodes a polyprotein of about 4.000 amino

acids, which is posttranslationally cleaved by viral and cellular proteases to 11-12 structural and nonstructural proteins (Collett *et al*., 1988).

Natural domestic animal hosts for BVDV are ruminants and swine. Since there is no absolute virus specificity for one species, there is also a possibility of interspecies infection (Terpstra *et al*., 1988).

On the territory of former Yugoslavia BVDV infection was described for the first time by Đuricković *et al.* (1966) based on clinical symptoms and pathomorphological findings and it was serologically confirmed by Cvetnić and coworkers in 1968 (Cvetnić, 1983). Since the first description of BVD in Serbia, only a few authors have dealt with this problem. Belić *et al*. (1973) detected BVD antibody in six herds and out of 224 examined serum samples 166 (74%) were seropositive. The percentage of seropositive animals on different farms ranged from 38.8% to 91%. Kurčubić (1993) presented data on serological examination of cattle on two farms. On a farm with fattening cattle (6 to 7 months of age) the author found 55.81% seropositive animals, while on a farm with dairy cows the percentage of seropositive animals ranged between 30.55% and 52.24% depending on the age of the animals.

During the last 4 years, the laboratory for virology at the Scientific Veterinary Institute "Novi Sad" has intensively investigated the prevalence of BVD infection (Petrović, 2002). In the period from 1999 to 2000 the first large investigation was undertaken to detect antibodies against BVDV in sera from cattle in the area of Southern Backa and Srem district by the virus-neutralization (VN) test. Serum samples from breeding cattle older than 6 months were collected in the whole epizootiological area. The presence of VN antibodies to the C24V strain of BVDV was found in 46.50% of the 2546 sera samples examined. Positive results for VN antibodies to the NADL strain were found in 50.96% samples out of examined 2657 sera, while VN antibodies to the AD-8 strain were detected in 50.77% out of 2657 sera samples (Petrović, 2002). Beyond the similarity in percentage of seropositive animals, some statistically important differences were discovered regarding the VN antibody titres obtained against different BVDV strains. In all the examined cattle herds, the lowest VN titres were discovered against the C24V virus strain. In the majority of the examined herds, the highest VN titres were against the NADL strain, and in some the AD-8 strain. In the majority of the examined cattle herds, the difference in VN titre levels was statistically significant. Taking into account the above mentioned results, as well as data from the literature, it may be concluded that in this epizootiological area local BVDV strains are antigenically different (Petrović, 2002).

Different regions of the pestivirus genomes have been used to study their genetic diversity, including variable parts of the 5'-UTR as well as the genes encoding E2 and N^{pro} (Ridpath *et al*., 1994; Wolfmeyer *et al*., 1997; Baule *et al*., 1997; Vilček *et al*., 1999; Jones *et al*., 2001; Nagai *et al*., 2001). In most cases, the 5'-UTR was analyzed, but analysis of other genomic regions led to essentially the same grouping (Vilček *et al*., 2001). Short sequences derived from highly conserved 5'-UTR can be used for segregation of BVDV isolates into two genotypes, BVDV 1 and BVDV 2, and further subdivision into defined subgroups. Cluster analysis of combined nucleotide sequences from the 5-UTR and N^{pro} regions recently di-

vided BVDV 1 into at least 11 genetic groups (Vilček *et al*., 2001). It is important to note that BVDV 2 appears to be a newly emerging variant that has become increasingly common in North America since 1990 (Ridpath *et al*., 1994). During the last few years it has also been described in many European countries (Wolfmeyer *et al*., 1997; Letellier *et al*., 1999; Pratelli *et al*., 2001; Vilček *et al*., 2002; Drew *et al*., 2002; Vilček *et al*., 2003).

In this study, we examined samples from fetal calf sera (FCS), whole blood and pathological material obtained from sick or dead animals in Serbia by an antigen ELISA, isolation of the virus using cell cultures and by the reverse transcriptase-polymerase chain reaction (RT-PCR) method, with the aim to isolate and characterise Serbian BVDVs.

MATERIAL AND METHODS

Serum samples from 64 individual cattle foetuses collected for batch production of foetal calf serum (FCS) were tested individually for BVDV antigen before being pooled. The dams of these foetuses all came from the South Backa region in Serbia. A second group of 35 samples consisted of whole blood and sera from aborted fetuses and sick animals, plus tissue samples from dead animals, where clinical signs indicated possible infection with BVDV. The latter group of samples came from cattle in the South Backa and Srem regions and also in the wider epizootic region of Vojvodina.

An ELISA for detection of BVD viral protein E^{rns}, produced by IDEXX (HerdChek antigen/serum kit) was used, according to the instructions of the manufacturer, for analysis of FCS, heparin blood and serum samples. Tissue samples and antigen ELISA positive blood and sera were inoculated into primary fetal calf testis (FTTe) cell cultures, which previously had been tested by direct immunofluorescence (FITC conjugated anti-BVDV polyclonal antibody; American Bioresearch, USA), and found to be free from BVDV. A standard protocol described in the OIE "Manual of Standards for Diagnostic Tests and Vaccines" (1996) was followed for isolation of BVDV. After three passages, the FTTe cultures were fixed and stained for noncytopathogenic BVDV by indirect immunofluorescence (IFAT) or immunoperoxidase staining (IPX) using pestivirus-specific monoclonal antibodies (MABs) WB103/WB105 (Veterinary Laboratories Agency, Weybridge, UK) as primary antibodies.

BVDV nucleic acid was detected in the samples by (RT-PCR), following the procedures described by Barlič-Maganje and Grom (2001) and Toplak (2002), which targeted the 5'-UTR of the viral genome. PCR products were separated on 1.5% agarose gels stained with ethidium bromide. DNA bands obtained from BVDV positive samples were excised from the gels, purified and used as templates in direct sequencing reactions. After assembly of consensus sequences, 245 nucleotide long regions were aligned with corresponding sequences of 15 recent isolates from Slovenia, plus relevant BVDV reference strains representing all currently known genetic subgroups of BVDV type 1, and analysed phylogenetically as described by Toplak (2002).

RESULTS

Two out of 64 foetal serum samples collected during the 3 year period tested positively for BVDV by antigen ELISA. Both positive samples came from herds in the Southern Backa district, which were monitored for production of FCS. FTTe cell cultures inoculated with these two sera showed no cytopathic effect during three passages, but BVDV antigen was detected in the cytoplasm of infected cells by IFAT (Fig. 1), or by IPX (Fig. 2). First-passage noncytopathogenic BVDV isolates were thereafter obtained by repeated inoculation of FTTe cell cultures with aliquots of the original two foetal serum samples, and named BVDV 0016 and BVDV 0017. All negative control cell cultures processed in parallel with those inoculated with field samples remained negative for BVDV antigen by IFAT or IPX.

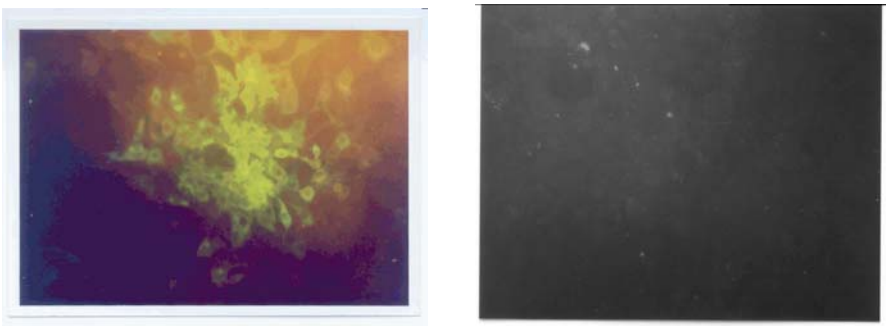

Figure 1. Detection of BVDV by direct immunofluorescence. On the left side FTTe cell cultures infected by BVDV Beograd, on the right side parallel uninfected FTTe cell cultures

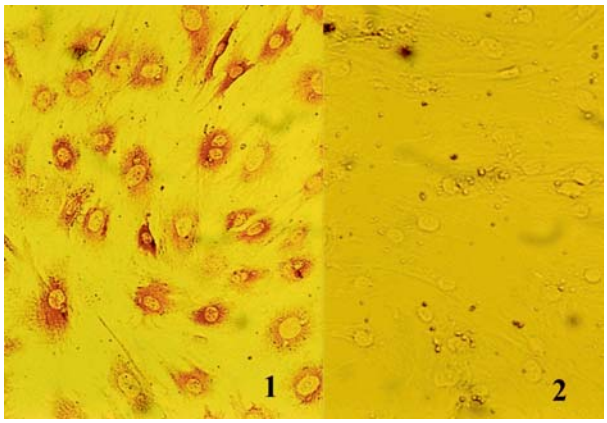

Figure 2. Indirect immunoperoxidase test. On the left side (1) are FTTe cells infected by BVDV 0016, and on the right side (2) uninfected parallel cell cultures

From the second group of 35 samples from clinically affected animals, another two samples were positive by antigen ELISA. One of these was blood from a heifer in the Čenta (Banat) region, that had shown clinical signs of mucosal disease. After three passages through FTTe cell cultures inoculated with blood from this heifer, a cytopathic effect appeared. A cytopathogenic BVDV was detected by indirect immunostaining methods, as described above for the BVDV 0016 and BVDV 0017 isolates. The cytopathogenic (cp) isolate was named BVDV Beograd.

The last antigen ELISA positive sample, was found during screening of blood samples from a herd in the Western Backa district. However, this sample was negative by both virus isolation test and by RT-PCR.

Original material from the three cases of positive virus isolation was further tested by RT-PCR, which resulted in PCR products of the same size as the BVDV reference strain (Fig. 3).

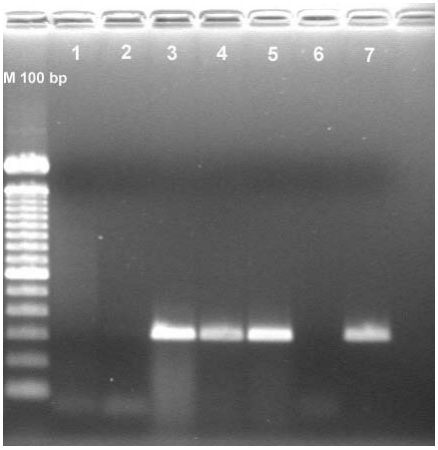

Figure 3. Agarose gel detection of PCR products. Lanes 1 and 6 are PCR negative controls (water template), lane 2 is the antigen ELISA positive sample that tested negatively by cell culture inoculation/IPX, lanes 3 – 5 are samples from which the BVDV isolates 0016, 0017 and Beograd were obtained, whilst lane 7 is BVDV reference strain NADL. The molecular weight marker to the left is a 100-base pair ladder

Direct sequencing of PCR products, alignment and phylogenetic analysis showed that the 0016 and Beograd isolates were of genetic subtype BVDV-1f, whilst the 0017 isolate was of genetic subtype 1b (Fig. 4). The Slovenian isolates were of subtypes 1d, 1f and 1b. No BVDV type 2 or BDV were found. Closer examination of the aligned sequences showed that the nucleotide differences between the three Serbian BVDV isolates and the 15 recent Slovenian BVDV isolates occurred mainly in two variable regions of the 5'-UTR (regions II and III, according to Deng and Brock, 1993). In the latter of these variable regions, the viruses typed as subtype 1f isolates had deletions of either four or five nucleotides, when compared with the reference strains.

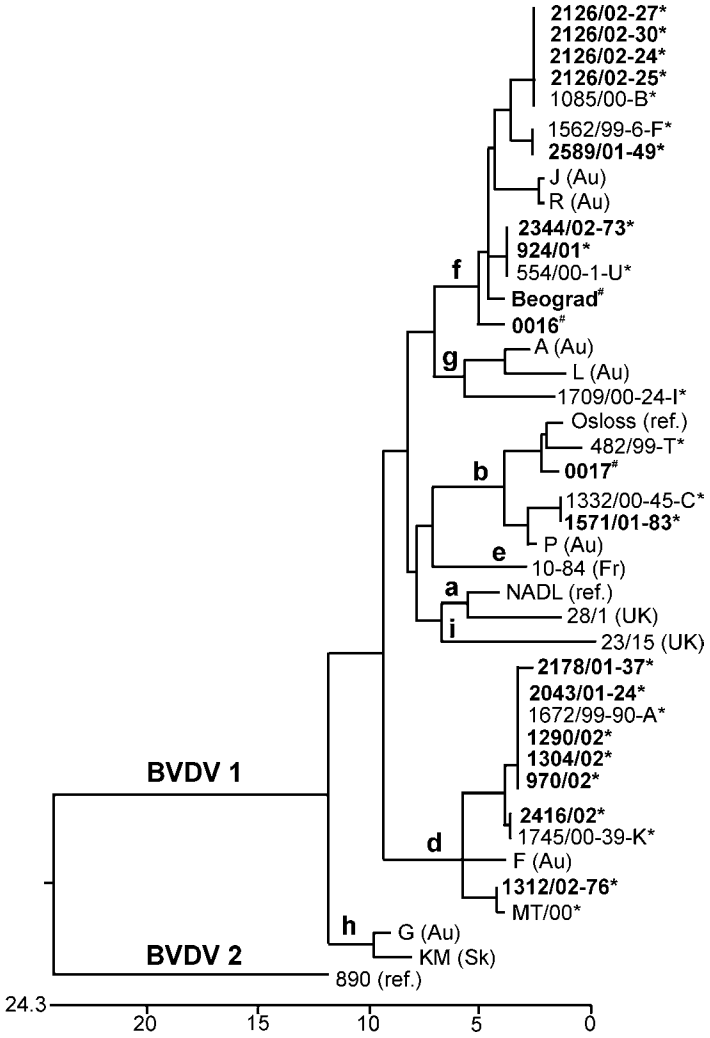

Figure 4. Phylogenetic tree of 245 nucleotide long 5'-UTR sequences of BVDV strains, 15 from Slovenia (*) and 3 from Serbia (#). BVDV isolates described in this study are indicated in bold. Published sequences of BVDV 1 were taken from GenBank AY323871, AY323873, AY323877, AY323878, AY323879, AY323880, AY323881, AY323891, AY323895, AF298054, AF298059, AF298061, AF298064, AF298065, AF298066, AF298067, AF298068, AF298069, AF298070, AF298071, U18059, M96687, M31182

DISCUSSION

This paper describes the first isolation of infectious BVDVs from cattle in Serbia. Previous serological investigations suggested that BVD has been common

throughout the territory of Serbia from the early seventies (Belić *et al*., 1973), and has remained so until this millennium (Petrović, 2002; Milošević, 2003).

The identity of the BVDV virus isolates BVDV 0016 and BVDV Beograd was confirmed by a number of laboratory tests in three laboratories (Scientific Veterinary Institute "Novi Sad", Serbia; Department of Virology, Veterinary Laboratories Agency, Weybridge, United Kingdom and the Virology Unit of Institute for Microbiology and Parasitology at the Veterinary Faculty in Ljubljana, Slovenia). By confirmation from the OIE reference laboratory for BVDV (VLA, Weybridge, UK-letter of December 9, 2002) the presence of this infection of cattle on the territory of Serbia and Montenegro is officially registered. Based on the association between infection with BVDV and clinical disease recorded in other countries, we have reason to believe that cattle in Serbia and Montenegro are similarly affected by significant BVDV-induced losses in the form of reproductive failure, respiratory and gastrointestinal disease, in addition to more severe clinical presentation of other infections due to immunosuppression by BVDV.

The genetic differences we found between the three isolates from Serbia and 15 recent BVDV isolates from Slovenia were based on nucleotide sequencing of the 5'UTR of the genome. These isolates belonged to three already defined subgroups within the BVDV 1 genotype (1b, 1d, 1f), and differed from each other by up to 14 % of the analysed nucleotide sequence. The isolate BVDV 0017 from Serbia was most similar to the BVDV reference strain Osloss, which belongs to subgroup 1b of BVDV. Isolate 1571/01-83 (Slovenia) from subgroup 1b was 100 % homologous to the previously typed isolate 1332/00-45-C sampled from the same herd (Toplak *et al.*, 2002). The similarity between 482/99-T from Slovenia (Toplak *et al*., 2002) and BVDV 0017 from Serbia was greater (97.6 %) than the between BVDV 0017 and isolate P originating from Austria (94.7 %). The remaining Serbian BVDV isolates described in this paper grouped together with the Austrian isolates J and R (Vilček *et al*., 2001) in the BVDV 1f subtype. However, the deletion of a group of five nucleotides seen in six Slovene strains (2126/02-24, 25, 27, 30, 2344/02-73, 924/01) and in strain BVDV 0016 from Serbia were not seen in the J and R strains from Austria. The 1f group of isolates from this study were similar to the known strains from Austria typed as 1f, but they branched separately (Fig. 4). The Slovenian BVDV isolates that were typed as subgroup 1d viruses were very similar or identical to previously characterized viruses, which supports a history of frequent trade with PI animals with consequent spread of BVDV in Slovenia.

The Serbian BVDV isolate "Beograd" is a cytopathogenic biotype of BVDV. This is the first report of a cp BVDV belonging to the 1f subgroup. The detection of a cp BVDV 1f supports the hypothesis that the origin of cp BVDVs, is the result of a random genetic recombination leading to expression of the NS3 protein separate from the NS2-3 expressed by the noncytopathogenic biotype. Such genetic recombinations are likely to occur independent of the evolutionary history of the virus.

When compared to the genetic prevalence of BVDV in the rest of Europe and beyond, a matching pattern of epidemiology of BVDV is evident, even with the small number of virus isolates genotyped in this paper. Subtype 1b BVDVs have been reported from all over the world, whereas the 1f subtype has been described

so far only in Central Europe (Austria, Germany, Hungary, Italy, Slovakia, Slovenia, Serbia), plus Mozambique in cattle imported from Austria (Baule *et al*., 1997). Both BVDV subtypes are common in central European countries, together with primarily 1d, 1g and 1h subtypes. Concerning BVDV-2, this virus appears to be rare in central and eastern Europe, and thus a larger number of isolates needs to be typed before conclusions about whether it is present or not can be drawn. During the last 10 years cattle have not been imported into Serbia, so it may be assumed that the BVDV subtypes described in this paper have been present for a long time.

Our data do not allow us to conclude why one of the blood samples tested false positive for BVDV antigen by ELISA. In some animals persistently infected with BVDV, low titres of BVDV may occasionally be seen in blood. Additionally, specific antibodies of either maternal origin or resulting from superinfection with antigenically different BVDV strains may interfere with virus isolation in cell cultures. The animal in question may also have been acutely infected with BVDV, and in the process of seroconverting. However, the sensitivity of the RT-PCR assay employed is sufficient to detect BVDV neutralised by specific antibodies and the low titres usually seen in acutely infected animals. Thus our conclusion is a false positive reaction, the reason for which should be looked into with continued use of this ELISA. These observations emphasise the need for diagnostic tests based on different detection principles to back up large scale diagnostic studies.

The fact that the present study on three BVDV isolates from Serbia revealed the existence of two distinct genetic groups of viruses within BVDV 1, when compared with the situation in other countries, indicates the presence of diverse BVDV strains, and more subgroups of BVDV-1 are expected to be found in this region. The results emphasize the need for further studies, first of all in the field of phylogenetic differences between more isolates of BVDV, to allow firm conclusions to be drawn regarding the diversity of Serbian BVDVs. Also, practicing veterinarians need to be aware that BVDV has been detected in the country and consequently to note the various clinical signs that infection with this virus can give rise to in cattle.

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Petrović T *et al*. Isolation and confirmation of bovine viral diarrhoea virus
in Serbia and comparative typing with recent Slovenian isolates

IZOLACIJA I POTVRDA VIRUSA GOVEĐE DIJAREJE (BVD) NA PODRUČJU SRBIJE I UPOREDNA TIPIZACIJA SA SKORAŠNJIM SLOVENAČKIM IZOLATIMA

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U radu su prikazani rezultati ispitivanja prisustva virusa goveđe virusne dijareje (BVDV) u uzorcima fetalnog telećeg seruma (FCS), pune krvi obolelih i patološkog materijala uginulih životinja sa područja Srbije. Za utvrđivanja prisustva BVD virusnog antigena (E^{rns}) u FCS-u i punoj krvi bolesnih životinja korišćen je ELISA test. Pozitivni uzorci u ELISA testu i uzorci patološkog materijala uginulih životinja su zatim inokulisani na primarnu kulturu ćelija fetalnog telećeg testisa (FTTe). Nakon trostruke pasaže na FTTe, prisustvo BVDV je potvrđivano metodom direktne imunofluorescencije, metodom indirektnog imunoperoksidaznog testa i metodom reverzne transkripcije-polimeraza lančane reakcije (RT-PCR). Ispitivanjem 64 uzorka FCS-a utvrđena su dva pozitivna necitopatogena BVDV. Jedan citopatogeni BVDV je izolovan iz pune krvi govečeta sa kliničkim znacima bolesti sluznica. 5' nekodirajući deo genoma (5' UTR) tri virusna izolata iz Srbije je umnožen pomoću RT-PCR metode,sekvencioniran i filogenetski analiziran sa skorašnjih 15 slovenačkih izolata. Većina BVDV izolata je bila 1f (Srbija – 2 izolata, Slovenija - 7 izolata) i 1d podtipa (Slovenija 7 izolata), dok je jedan izolat iz Srbije I jedan izolat iz Slovenije genotipiziran kao BVDV 1b.