GENETIC ANALYSIS OF A BOVINE VIRAL DIARRHEA VIRUS 2 ISOLATE FROM SLOVAKIA

M. NOVÁČKOVÁ[1], A. JACKOVÁ[1], M. KOLESÁROVÁ[2], Š. VILČEK[1*]

[1]University of Veterinary Medicine, Komenského 73, 041 81 Košice, Slovak Republic; [2]P.J. Šafárik University, Faculty of Natural Sciences, Košice, Slovak Republic

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Summary. – The identification and genetic characterization of bovine viral diarrhea virus (BVDV) isolate 17237 detected in western Slovakia is described. The analysis of 5'-untranslated region (5'-UTR), autoprotease (N^{pro}) gene, and structural genes (C, E^{rns}, E1, E2) was carried out. The percentage of nucleotide and deduced amino acid identity in analyzed genes implied that the isolate was closely related to the bovine viral diarrhea virus 2 (BVDV-2). Furthermore, the phylogenetic analysis revealed that this isolate fall into BVDV-2b subtype that is sporadic in Europe. The cleavage sites between viral proteins were similar to the ones of a reference strain of BVDV-2.

Key words: bovine viral diarrhea virus 2; RT-PCR; sequence analysis; phylogenetic analysis

Introduction

Bovine viral diarrhea viruses 1 and 2 (BVDV-1, 2) belong to the genus *Pestivirus,* the family *Flaviviridae* (Fauquet *et al*., 2005). Pestiviruses are important infectious agents of diseases affecting enteric, respiratory, and reproductive systems of cattle, swine, and sheep. Pestivirus infections cause considerable economical losses in livestock industry.

The pestivirus genome is usually 12.3 kb in size and consists of positive single-stranded RNA containing a single ORF encoding about 3,900 amino acids. This polyprotein is co- and post-translationally cleaved into 11 or 12 proteins. The 5'-UTR close to the 5'-terminus is followed by the N^{pro} gene coding for autoprotease and with four genes for structural proteins C, E^{rns}, E1, E2. Remaining part of genome comprises genes for non-structural proteins (NS) and 3'-untranslated region (3'-UTR). The structure of the entire pestivirus genome is as follows: 5'-UTR-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3'-UTR (Meyers and Thiel, 1996; Collet *et al*., 1988).

Genetic typing of BVDV isolates is usually based on the nucleotide sequence comparison of conserved 5'-UTR, N^{pro}, and E2 regions (Couvreur *et al*., 2002; Tajima *et al*., 2001; Vilček *et al*., 2001; Becher *et al*., 1999, 1997). The phylogenetic analysis revealed that BVDV-1 viruses could be classified into at least 12 subtypes termed as BVDV-1a to BVDV-1l (Jacková *et al*., 2008; Vilček *et al*., 2001). BVDV-2 isolates were classified into two phylogenetic subtypes termed as BVDV-2a and BVDV-2b (Flores *et al*., 2002).

BVDV-2 was originally identified in Canada and USA. Highly virulent BVDV-2 strains were responsible for severe disease outbreaks with the occurrence of hemorrhagic syndrome associated by thrombocytopenia, leucopenia, fever, and diarrhea (Pellerin *et al*., 1994; Ridpath *et al*., 1994). Other BVDV-2 strains could cause enteric, respiratory, and reproductive signs in cattle similar as for BVDV-1 strains. BVDV-2 isolates were also detected in sheep (Pratelli *et al*., 2001).

While BVDV-1 and BVDV-2 isolates are approximately equally distributed throughout the cattle populations in US,

*Corresponding author. E-mail: vilcek@uvm.sk; fax: +4215-52981011.

Abbreviations: BVDV-1,2 = bovine viral diarrhea virus 1,2; C = core protein; N^{pro} = autoprotease; NS = non-structural protein; 5'-UTR = 5'-untranslated region

the BVDV isolates detected in Europe belong predominantly to the species BVDV-1 (Vilček *et al*., 2001). The BVDV-2 isolates were only sporadically identified in Germany, Belgium, France, Italy, Slovakia, Austria, and Portugal (Barros *et al*., 2006; Vilček *et al*., 2003, 2002, 2001; Pratelli *et al*., 2001; Letellier *et al*., 1999; Wolfmeyer *et al*., 1997).

In Slovakia, the first BVDV-2 isolate was identified in eastern part of the country (Vilček *et al*., 2002). Two years later, analyzing BVDV isolates coming from western Slovakia, we detected another identical or very similar BVDV-2 isolate labeled as 17237. Since the occurrence of BVDV-2 isolates in Europe is sporadic and genetic data for those viruses are limited, we decided to analyze the new BVDV-2 isolate 17237 more thoroughly.

In this study we report genetic characterization of the 5'-UTR, N^{pro}, and structural genes C, E^{rns}, E1, and E2 of the BVDV isolate 17237. The obtained genetic data were compared with those of BVDV-1 and BVDV-2 reference strains.

Materials and Methods

Origin and preliminary detection of BVDV isolate. The BVDV isolate 17237 originated from a set of samples containing various organs (kidney, lung, lymph node) collected from the diseased cattle in the farm of western Slovakia. The animals suffered from diarrhea, respiratory problems, and a loss of appetite. Based on anamnesis and clinical signs, the animals were suspected of BVDV infection. The presence of BVDV antigen in the samples was detected by ELISA at the State Veterinary Institute in Bratislava, Slovakia. The BVDV antigen was detected only in one sample. However, the ELISA used could not differentiate between BVDV-1 and BVDV-2 species, thus the isolate 17237 was analyzed at the genetic level.

Isolation of total RNA and synthesis of cDNA. The total RNA was extracted from 200 µl of the organ sample using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was started with denaturation of RNA and binding of gene specific reverse primer 3422R or 5175R (Arnal *et al*., 2004).

Sequencing strategy. The 5'-UTR – E2 region was amplified using single, nested, or semi-nested PCR employing pestivirus-specific and BVDV-2 specific primers selected from the data obtained during sequencing of the isolate 17237 with pestivirus-specific primers. Thus, at the first stage genomic fragments were amplified with pestivirus-specific primers. A part of 5'-UTR sequence was obtained by PCR using 324/326 primers (Vilček *et al*., 1994). The N^{pro}-C gene fragment was obtained using 324/1400R and BD1/BD2 primers and a part of E1-E2 region using the 2256/3422R primers (Arnal *et al*., 2004; Vilček *et al*., 2001; Becher *et al*., 1999). In addition, inner part of those fragments was amplified and sequenced using five primers designated from the partial nucleotide sequences of the isolate 17237. The 3'-end of E2 region was amplified using the BSK7 and BSK9R primers. While primer BSK7 (5'-ACAGTACAGGTCATCGCCAC-3') was selected from the partial sequence of the isolate 17237, the BSK9R (5'-GCC CATGACTCCTTGCTCAG-3') was selected from the alignment of E2 region of BVDV-2 sequences deposited in GenBank (Acc. Nos. U18059, AF144612).

RT-PCR. The PCR mixture (50 µl) for the first round of PCR contained 5 µl of reaction buffer (Finnzymes), 5 µl of dNTPs (2 mmol/l), 2.5 µl of $MgCl_2$ (50 mmol/l), 300 nmol/l of each primer and 1 U of Phusion High-Fidelity DNA Polymerase (Finnzymes) and 4 µl of cDNA. The first round of nested/semi-nested RT-PCR was carried out in 30 cycles under following conditions: initial denaturation at 98°C for 1 min, then denaturation at 98°C for 10 secs, annealing at 52°C for 30 secs, extension at 72°C for 1 min, and final extension was prolonged to 10 mins. In the second round of nested/semi-nested PCR, 2 µl of PCR product were amplified using internal primer pairs. Nested/semi-nested PCR was running under the same conditions as first PCR, but the number of cycles was increased to 35.

Sequencing of PCR products. The sequencing of specific DNA fragments was performed in both directions using PCR primers on ABI PRISM sequencing device employing fluorescently labeled ddNTPs at The Comenius University in Bratislava. The nucleotide sequences were proofread in both directions using SeqManII program of DNASTAR software package (DNASTAR Inc., Lasergene). The 5'-UTR-E2 nucleotide sequence of the BVDV isolate 17237 was deposited in GenBank under Acc. No. EU747875.

Analysis of the nucleotide sequences. The alignment of nucleotide sequences was done with the Clustal W program (Thompson *et al*., 1994). The percentage of nucleotide identity was calculated using MegAlign from the DNASTAR package.

Phylogenetic analysis. The phylogenetic tree was constructed using the NEIGHBOR program from the PHYLIP inference package that was based on the neighbor-joining algorithm, using Kimura-2 parameters (Felsenstein, 1993; Saitou and Nei, 1987; Kimura, 1980). Statistical analysis of the tree was performed by bootstrapping carried out on 1000 replicates using the SEQBOOT and CONSENCE programs from the PHYLIP package (Felsenstein, 1985). To type the BVDVs, the sequences from the representative BVDV-1 and BVDV-2 strains and isolates were included in the phylogenetic analysis (Acc. Nos: NADL-M31182, Osloss-M96687; SD1-M96751; 890-U18059; 1373-AF145967; Gi-4-AF144468; Soldan-U94914; NADL-M31182, Osloss-M96687; A, F, G, W, 26-Fr – Vilček *et al*., 2001; 9-Br – Vilček *et al*., 2004).

Results

Analysis of 5'-UTR region

To confirm the presence of the specific pestivirus sequences in the isolate 17237, we amplified 288 bp DNA fragment from 5'-UTR by RT-PCR employing the pestivirus-specific 324/326 primers. The detection of expected DNA fragment on agarose gel indicated the presence of the pestivirus in the organ sample. Nucleotide sequencing of the PCR product revealed that the nucleotide sequence was completely identical with the sequence obtained from the BVDV-2 isolate Kosice_SK (Acc. No. EU360934) that was

the first BVDV-2 isolate identified in Slovakia (Vilček *et al*., 2002).

The phylogenetic tree constructed from 5'-UTR nucleotide sequences of selected BVDV-1 and 2 strains indicated that the isolate 17237 was classified as BVDV-2 falling into BVDV-2b branch together with some North and South American isolates (Fig. 1). The isolates from Germany, France, and the reference BVDV-2 strain 890 originating from USA fall into BVDV-2a subtype. Additionally, matching results were obtained also from the nucleotide sequence identity analysis of 5'-UTR region (Table 1). The isolate 17237 was closer related to the 890 strain (88% of nucleotide sequence identity) than to the BVDV-1 reference NADL and Osloss strains (78% of nucleotide sequence identity).

Analysis of N^{pro} gene

Next, we performed the analysis of the N^{pro} gene coding the viral autoprotease. Again, the phylogenetic analysis confirmed typing of the isolate 17237 as BVDV-2 (data not shown). The nucleotide and amino acid identity values were also closer to the BVDV-2 strain 890 (81 and 83%, respectively) than to the BVDV-1 strains NADL and Osloss, 68–71% and 70%, respectively (Table 1).

The alignment of the entire 168 amino acid N^{pro} sequence for the isolate 17237 and other BVDV-1, 2 isolates is presented in Fig. 2. All six cystein residues of the isolate 17237 occurring at positions 69, 112, 134, 138, 161, and 168 were conserved (with an exception of cystein located at position 161 for BVDV-1 isolate A). Three amino acid residues E22, H49, and C69 representing the putative catalytic triad, were conserved in all pestivirus isolates analyzed. The cleavage site between N^{pro} and C gene was conserved for BVDV-2 isolates (Fig. 3). This cleavage site had similar but not identical characteristics as for BVDV-1 isolates. No insertion/deletions were found in the N^{pro} region.

Analysis of structural genes

Phylogenetic analysis based on the alignment of nucleotide and amino acid sequences confirmed that the isolate 17237 was classified as BVDV-2 in all structural genes C, E^{rns}, E1, and E2 exactly as was determined for 5'-UTR and N^{pro} regions (data not shown).

This conclusion was supported by the nucleotide and amino acid identity analysis in all structural genes. The data for the isolate 17237 were more related to the BVDV-2 strain 890 varying in the entire C-E2 region in the range 82–90%, than to BVDV-1 reference strains NADL and Osloss that varied from 61 to 71% (Table 1). The most conserved region especially at the amino acid level was observed for C gene coding core protein (90% for strain 890 and 76% for strains NADL and Osloss). The E2 gene coding immunodominant envelope protein represented the most variable region (83% for strain 890 and 61 and 62% for strains NADL and Osloss, respectively, Table 1).

Comparison of the cleavage sites between proteins for BVDV-1 and BVDV-2 revealed several amino acid changes (Fig. 3). Similar disposition was observed also for the N^{pro}/C cleavage site between the isolate 17237 and BVDV-1 strains.

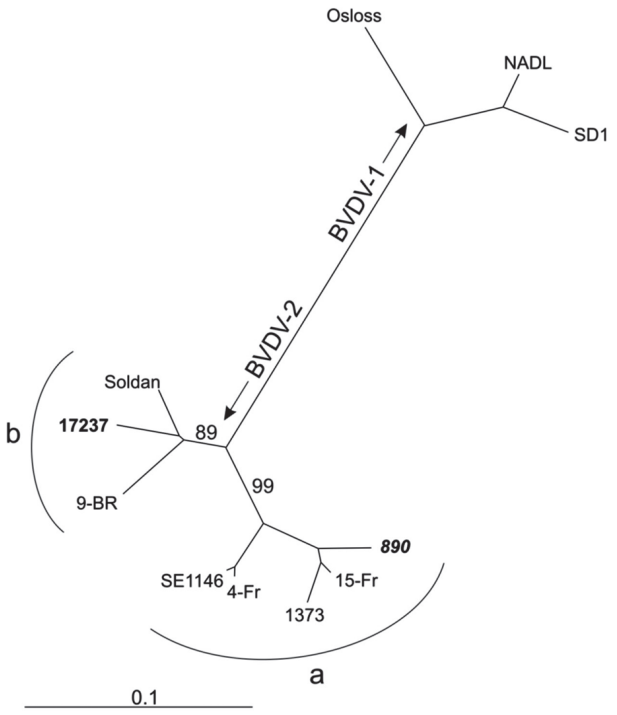

Fig. 1

Phylogenetic tree of BVDV-1 and 2 strains including isolate 17237 based on the nucleotide sequence of 5'-UTR region

Table 1. Nucleotide and amino acid sequence identity in % between the isolate 17237 and reference strains BVDV-1 and BVDV-2

Reference strain	Analyzed region*					
	5'-UTR	N^{pro}	C	E^{rns}	E1	E2
890 BVDV-2	88.2	81.2 *83.3*	85.6 *90.2*	85.3 *90.3*	82.7 *89.2*	82.0 *82.8*
NADL BVDV-1	78.4	68.3 *69.6*	65.4 *75.5*	71.1 *79.7*	70.6 *81.0*	65.0 *62.1*
Osloss BVDV-1	78.3	70.8 *70.2*	64.1 *76.5*	71.2 *78.0*	70.3 *80.0*	64.1 *61.3*

*Nucleotide (Roman script) and amino acids (italic script) sequence identities.

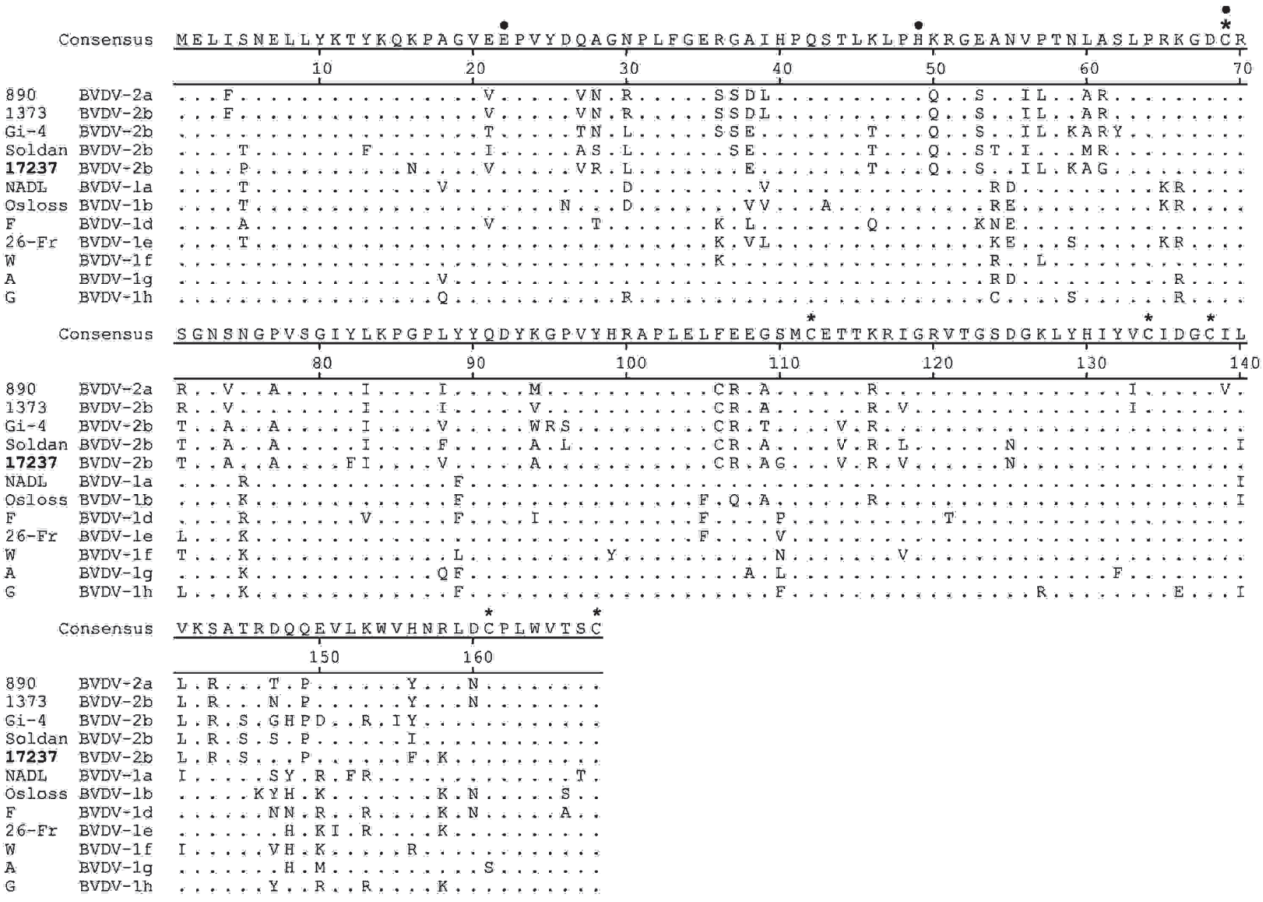

Fig. 2

Alignment of amino acid sequence of N^{pro} region of the 17237 isolate and various BVDV-1 and BVDV-2 strains

(*) – conserved cystein residue; (•) – amino acids of the catalytic triad.

	N^{pro}/C	C/E^{rns}	E^{rns}/E1	E1/E2
	▼	▼	▼	▼
890 – BVDV-2	LWVTSC SDEGSK	LQLVTG ENITQW	WFGAHA ASPYCG	ITGAQG FPECKE
17237 – BVDV-2	LWVTSC SDEGGK	PQPVTG ENITQW	WFGAHA ASPYCD	ITGAQG FPECTE
NADL – BVDV-1	LWVTTC SDTKEE	FQVTMG ENITQW	WFGAYA ASPYCD	ITGVQG HLDCKP
Osloss – BVDV-1	LWVSSC SDTKAE	FQVAVG ENITQW	WFGAYA ASPYCE	ITGAQG LPVCKP

Fig. 3

Cleavage sites between individual proteins of the isolate 17237 and BVDV-1 and BVDV-2 reference strains

The cleavage sites were adopted from experimentally determined sites in related classical swine fever virus strain (Rumenapf *et al.*, 1993; Stark *et al.*, 1993).

Discussion

The isolate 17237 analyzed in this work represented the second cattle pestivirus isolate originating from Slovakia that was identified as BVDV-2. The BVDV-2 isolate identified earlier and labeled as Kosice_SK was detected on cattle farm in eastern Slovakia in winter season 2000, where serious clinical problems of animals were reported. The animals carrying BVDV-2 died from catarrhal pneumonia, bronchopneumonia, haemorrhagic liver and

spleen, and from other symptoms. The presence of BVDV-2 was detected by ELISA and by RT-PCR in several organs (Vilček *et al*., 2002).

The isolate 17237 investigated here represented the BVDV-2 isolate originating from cattle farm in western Slovakia far from the place where the earlier BVDV-2 isolate Kosice_SK was identified. In this farm, the animals with mild general clinical symptoms of diarrhea and respiratory tract problems were screened for viruses. BVDV-2 was detected in a single animal by ELISA and by RT-PCR. The identical nucleotide sequence of 5'-UTR for both isolates suggested that this BVDV-2 isolate circulated in cattle farms in Slovakia for several years causing severe or mild clinical symptoms. Similar situation was observed in North America where single BVDV-2 strain was responsible for many outbreaks that plagued cattle farms in the period 1993–1995 (Ridpath *et al*., 2006).

In the last decade, identification of the BVDV-2 isolates in EU countries (Barros *et al*., 2006; Vilček *et al*., 2003, 2001; Pratelli *et al*., 2001; Lettelier *et al*., 1999; Wolfmeyer *et al*., 1997) indicated that clinical signs observed in the infected cattle were not too harsh. That was the reason why some European epidemiologists have not paid enough attention to the occurrence of BVDV-2 in cattle or sheep farms. However, it was known that highly virulent BVDV-2 isolates in North America led to the serious economical loss in cattle farming. In fact, the BVDV-2 was originally discovered in Canada and USA due to the recognized severe clinical signs (Pellerin *et al*., 1994; Ridpath *et al*., 1994). Taking into account the serious problems that can arise with the occurrence of BVDV-2 infection in animals, there is an urgent need to monitor the epidemiological situation in Europe thoroughly.

Since several BVDV-2 isolates previously found in Europe were analyzed in 5'-UTR, N^{pro} or E2 gene only, our results represented the first report about the European BVDV-2 isolate analyzed in longer 5'-UTR-E2 region involving all structural genes. Overall, we analyzed over 3,400 bp long nucleotide sequence. The precise genetic typing revealed that the isolate 17237 belonged to the BVDV-2b subtype, while BVDV-2 isolates originating from other European countries were classified as BVDV-2a subtype similarly as the BVDV-2 reference strain 890. The isolate 17237 is the first European BVDV-2 isolate classified as BVDV-2b subtype. It is worth pointing out that most BVDV-2b isolates were found on the American continent. The origin of BVDV-2 isolate 17237 in Slovakia is unknown, what is true for most of the European BVDV-2 isolates. Hypothetically, an artificial insemination carried out in the farm might be a source of viral infection.

The genetic analysis of the isolate 17237 carried out in the 5'-UTR-E2 region suggested that genetic organization of this virus was similar to the remaining BVDV-2 isolates as well as to other pestivirus isolates analyzed so far. The genetic examination of the isolate 17237 genome revealed that the C gene showed the highest level of amino acid conservation in comparison to the reference strain 890. On the other hand, most variability within BVDV-2 isolates was found within the E2 gene. This finding was plausible, since E2 gene encoding envelope protein is the gene with a high mutation rate also in other pestivirus isolates as well (Tajima *et al*., 2001; Becher *et al*., 1999).

Lower frequency of mutations in some parts of the viral genome led to a hypothesis that some nucleotide or amino acid positions were necessary for proper virus functions. In analyzed isolate 17237, the positions of cysteine residues and catalytic triad in N^{pro} region composed of conserved E22, H49 and C69 residues were well conserved. The cleavage sites between structural proteins showed common and specific pestivirus characteristics.

The genetic data presented in this work contribute to the general knowledge about the pestivirus genome especially about BVDV-2 isolates circulating in Central Europe. The molecular data can be useful for the development of molecular diagnostic assays for pestivirus infection with special attention to the detection of BVDV-2 in clinical samples, as well as for the development of molecular epidemiology of BVDV-2 infection in cattle and sheep population.

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