

Genotyping of bovine viral diarrhoea virus isolates from the Czech Republic

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ABSTRACT: This study was focused on the genetic typing of bovine viral diarrhoea virus (BVDV) isolates obtained from 41 serum samples of persistently infected cattle in the Czech Republic in the period of 2004 to 2007. For the differentiation of BVDV isolates, the 5'-UTR and N^{pro} regions were selected. A 288-bp fragment from 5'-UTR and 428-bp fragment from N^{pro} of the selected isolates were amplified by RT-PCR and subsequently sequenced and analysed by computer-assisted phylogenetic analysis. The isolates belong to BVDV-1 genotype and the following subtypes were identified: b ($n = 16$), d ($n = 16$), e ($n = 2$) and f ($n = 7$). In this collection of viral samples, no isolate belonged to BVDV-2 genotype.

Keywords: BVDV; phylogenetic analysis; genotyping; cattle

The bovine viral diarrhoea virus (BVDV) is a significant pathogen of cattle that occurs worldwide. Different manifestations of BVDV infection have been reported, e.g. diarrhoea, respiratory problems, abortion and immunosuppression. In pregnant cows, the resorption of the foetus can also occur (Radostits and Littlejohns, 1988). Viral infection during the first trimester of pregnancy in cows can result in the development of persistently infected (PI) animals. PI animals carrying virus during their lifetime are immunotolerant to BVDV and become a source of infection for healthy cattle in their proximity (Nettleton and Entrican, 1995). The number of PI individuals in herds ranges between 0.1 and 1.8% (Houe, 1999).

According to the accepted taxonomy, BVDV type 1 (BVDV-1) and BVDV type 2 (BVDV-2) species are recognised. BVDV-2 causes similar clinical symptoms as BVDV-1. Further, highly viraemic strains may develop a serious haemorrhagic syndrome with high mortality of cattle (Pellerin et al., 1994; Ridpath et al., 1994).

BVDV-1 and BVDV-2 are single-stranded positive sense RNA viruses forming together with classical swine fever (CSFV) and border disease virus (BDV) the genus *Pestivirus* in the Flaviviridae family (Fauquet et al., 2005). The length of RNA genome is approximately 12.3 kbp. The viral genome comprises a single open reading frame (ORF) encoding about 4 000 amino acids (Collet et al., 1988; Demoerlooze et al., 1993). At the 5' and 3' ends, the genome is surrounded by untranslated regions (UTR). The 5'-UTR region consists of approximately 380 nucleotides. This part of the genome is highly conservative and it has been used as a target sequence for diagnostic RT-PCR (Vilcek et al., 1994; Letellier and Kerkhofs, 2003). Since 5'-UTR also contains variable regions and it is easy to obtain a fragment by RT-PCR for sequencing, it was widely used for the preliminary genetic typing of pestiviruses. In addition, the N^{pro} and E2 regions were used for the typing of BVDV isolates at the genetic level (Becher et al., 1997, 1999; Tajima et al., 2001; Vilcek et al., 2001; Couvreur et al., 2002).

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The present phylogenetic analysis revealed that BVDV-1 is divided into 12 or even more subtypes (Vilcek et al., 2001; Jackova et al., 2008; Nagai et al., 2008). Despite BVDV the subtypes (subgenotypes) were not accepted by the international taxonomy committee, they have been widely reported in the molecular epidemiology papers (Tajima et al., 2001; Falcone et al., 2003).

The BVDV-1 subtypes are spread worldwide in cattle population. In the case of BVDV-2 species, the highest occurrence is reported in the USA and Canada (Pellerin et al., 1994; Ridpath et al., 1994), partially in Japan (Nagai et al., 2001), South America (Flores et al., 2002) and occasionally in some European countries (Wolfmeyer et al., 1997; Vilcek et al., 2002, 2003; Pizarro-Lucero et al., 2006). No thorough genetic typing of the BVDV subtypes occurring in the Czech Republic has been done yet. This work is focused on the analysis of a larger collection of BVDV isolates obtained from infected cattle in the Czech Republic. The results of the present study are important for the control/eradication strategies regarding cattle herds affected by BVD virus.

MATERIAL AND METHODS

Virus samples

In the scope of the systematic BVDV control/eradication program in the Czech Republic, serum samples of cattle kept in 55 herds without vaccination were collected. In the period 2004 to 2007, the active infection was identified in 41 herds and the presence of PI animals was proved. From each herd, one PI animal was randomly selected for the genetic analysis. A total of 41 samples of BVDV isolates were obtained in this way. The PI animals were identified in a classical way. During the control program, serum samples were analysed for the antibodies against BVD virus by ELISA kit (BVD-MD IgG ELISA, TEST-Line, Czech Republic). The serologically negative samples were examined by ELISA for the specific BVDV antigen (BVDV Ag/Serum, IDEXX Laboratories). If the result of the BVDV Ag-ELISA technique was positive, the blood of the animal was taken once more 14 days later. If the positive result was confirmed, the serum sample was stored at -70°C for the genetic analysis. The field virus isolates were not passaged on cell culture.

Isolation of RNA and PCR

Total RNA was isolated from 140- μl serum samples by means of QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The isolated RNA was eluted into 40 μl of H_2O . Two fragments were amplified: 5'-UTR of BVDV₂ genome flanked by primers 324/326 (Vilcek et al., 1994) and in the case of 16 selected isolates N^{pr}o flanked by primers BD1/BD3 (Vilcek et al., 2001). Reverse transcription and amplification of the 288-bp (5'-UTR) and 428-bp (N^{pr}o) products were performed by *C. therm.* Polymerase One-Step RT-PCR System (Roche). The reaction mixture consisted of 0.4mM dNTP, 5% DMSO, 5mM DTT, 0.3 μM of each primer, 0.4 IU of Rnazin, 1 \times reaction buffer and 2 μl of enzyme mixture. A 5- μl volume of the eluted RNA was used as a template diluted by double distilled H_2O to reach the final reaction mixture volume of 50 μl . The thermal profile of RT-PCR was as follows: $60^{\circ}\text{C}/30$ min, $94^{\circ}\text{C}/2$ min, 35 cycles at $94^{\circ}\text{C}/30$ s, $55^{\circ}\text{C}/1$ min, and $70^{\circ}\text{C}/1$ min. The final step was $70^{\circ}\text{C}/10$ min. The PCR products were detected in 1% agarose gel.

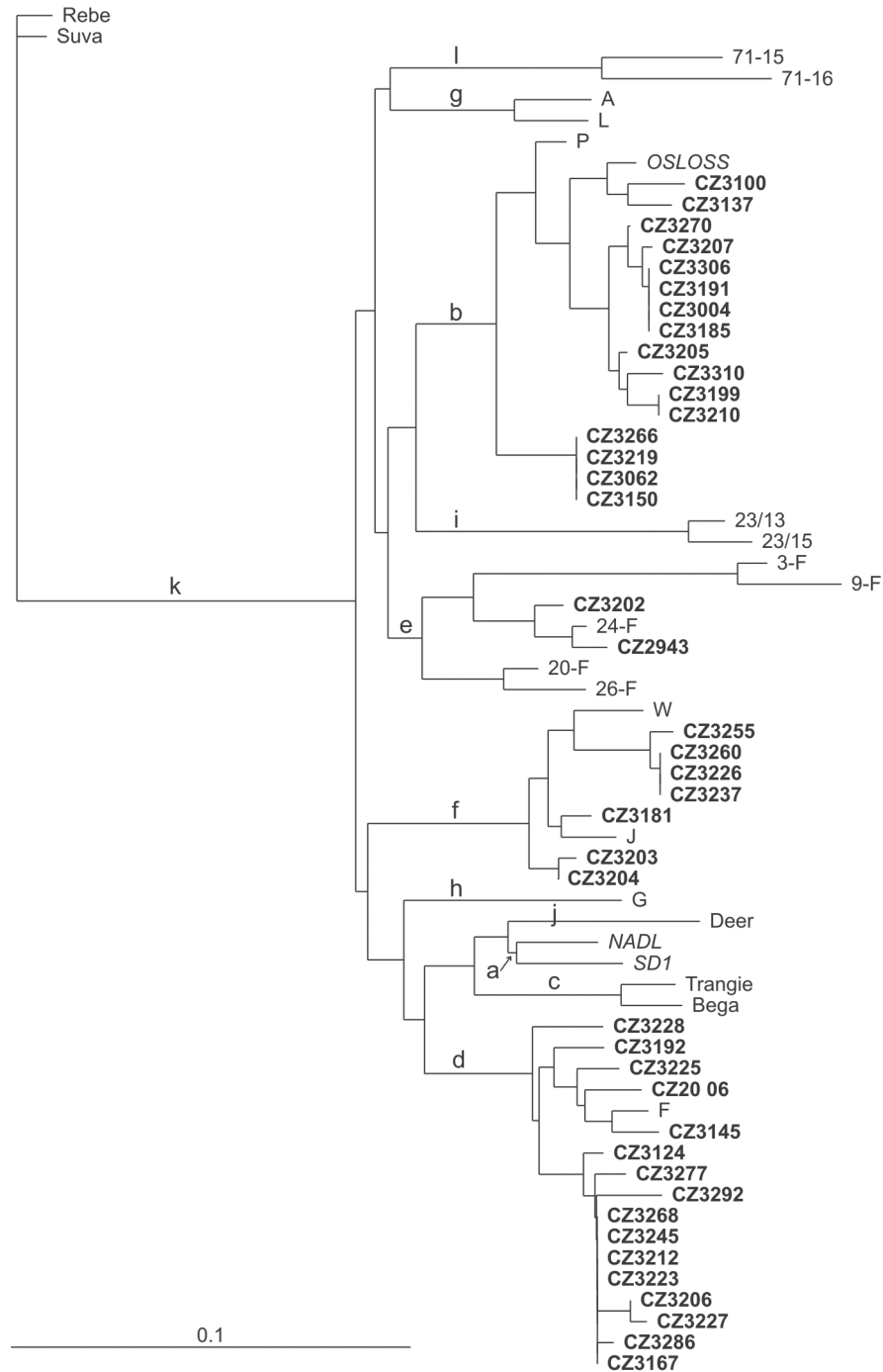
Sequencing and phylogenetic analysis

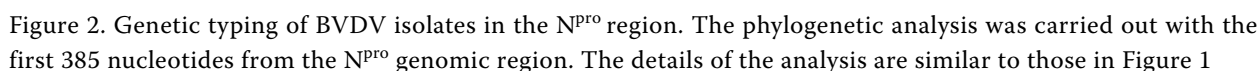
The amplification products obtained by RT-PCR were purified by QIAquick PCR Purification Kit (Qiagen). The purified cDNA was eluted into 30 μl of H_2O . BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used for direct sequencing of PCR products. The reaction mixture contained 8 μl of the eluted purified cDNA, 8 μl of the master mix kit, 3.5 μl of 1 μM primer and 0.5 μl of H_2O . The thermal profile of the sequencing reaction was as follows: 30 cycles at $96^{\circ}\text{C}/20$ s, $50^{\circ}\text{C}/20$ s and $60^{\circ}\text{C}/4$ min.

The nucleotide sequences were proof-read using SeqManII program from DNASTAR (Dnastar, Lasergene). Sequence alignment was carried out by Clustal W program. Phylogenetic tree was prepared using the neighbour joining algorithm (Kimura 2-parameter method) and visualised by Treeview program. More details of the analysis are given elsewhere (Vilcek et al., 2001).

RESULTS AND DISCUSSION

Our study was focused on the genetic typing of BVDV isolates found in the cattle population in





In the genetic analysis, each herd was represented by a virus sample obtained from one randomly selected PI animal. Theoretically, several different BVDV-1 strains that would possibly belong to the same or different type or subtype could be circulating in one herd due to their supposable independent introduction to the farm. In order to verify this hypothesis, five isolates obtained from PI animals of two large cattle farms were analysed. The sequencing of PCR products from 5'-UTR of all viral samples confirmed that on a particular farm there occurs only one characteristic strain without any mismatch in the nucleotide sequence. A herd-specific isolates have also been reported in other studies (Paton et al., 1995; Hamers et al., 1998).

As concerns the BVDV-1 subtypes circulating in the Czech Republic, the epidemiological situation is in principle similar to that of the neighbouring countries. A higher genetic diversity of BVDV-1 isolates was also observed in Germany, Austria and Slovakia (the data from Poland are not available). While in the Czech Republic four subtypes (BVDV-1b, BVDV-1d, BVDV-1e and BVDV-1f) with predominant BVDV-1b and BVDV-1d subtypes are circulating, in Austria eight subtypes occur: BVDV-1a, BVDV-1b, BVDV-1d, BVDV-1e, BVDV-1f, BVDV-1g, BVDV-1h, and BVDV-1k with a prevalence of BVDV-1h and BVDV-1f subtypes (Hornberg et al., 2009). A dissimilar situation was

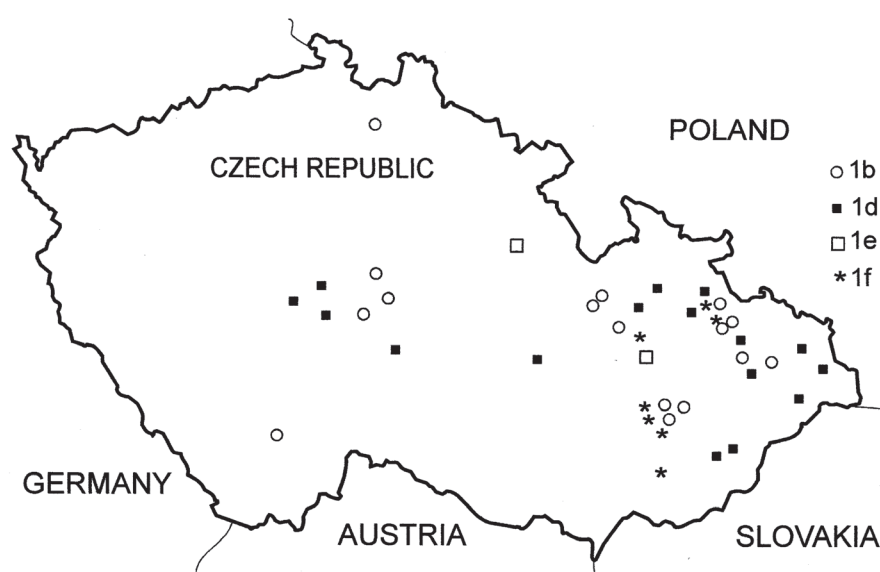


Figure 3. Occurrence of BVDV-1 subtypes in the Czech Republic

observed in Germany, where five subtypes BVDV-1a, BVDV-1b, BVDV-1d, BVDV-1f and BVDV-1g were found with predominant subtypes BVDV-1b and BVDV-1d (Tajima et al., 2001). In Slovakia, BVDV-1d, BVDV-1f and BVDV-1g subtypes were identified (Vilcek et al., 2001).

On the other hand, BVDV-2 isolates were identified in neighbouring Germany (Wolfmeyer et al., 1997), Austria (Vilcek et al., 2003) and Slovakia (Vilcek et al., 2002). We have not found any BVDV-2 in our collection of viruses so far. However, we cannot exclude the occurrence of BVDV-2 in the Czech Republic in future since BVDV-2 subtypes circulating in Europe do not often develop any significant clinical signs that would be similar to those observed in the USA and Canada.

In conclusion, the four subtypes that were found in cattle herds in the Czech Republic belong to BVDV-1. The results can contribute to a better understanding of the epidemiology of BVDV infection in the Central-European region, improve the strategy of the BVDV control program in cattle herds and stimulate the introduction of a national BVDV/MD eradication program.

REFERENCES

- Becher P., Orlich M., Shanon A.D., Horner G., König M., Thiel H.J. (1997): Phylogenetic analysis of pestiviruses from domestic and wild ruminants. *Journal of General Virology*, 78, 1357–1366.
- Becher P., Orlich M., Kosmidou A., König M., Baroth M., Thiel H.J. (1999): Genetic diversity of pestiviruses: identification of novel groups and implication for classification. *Virology*, 262, 64–71.
- Collet M.S., Larson R., Belzer S., Retzel E. (1988): Proteins encoded by bovine viral diarrhoea virus: the genome organization of a pestivirus. *Virology*, 165, 200–208.
- Couvreux B., Letellier C., Collard A., Quenon P., Dehan P., Hamers C., Pastoret P.P., Kerkhofs P. (2002): Genetic and antigenic variability in bovine viral diarrhoea virus (BVDV) isolates from Belgium. *Virus Research*, 85, 17–28.
- Demoerlooze L., Lecomte C., Brownshimmer S., Schmetz D., Guiot C., Vandenberghe D., Allaer D., Rossius M., Chappuis G., Dina D., Renard A., Martial J.A. (1993): Nucleotide-sequence of the bovine viral diarrhoea virus Osloss strain – Comparison with related viruses and identification of specific DNA probes in the 5' untranslated region. *Journal of General Virology*, 74, 1433–1438.
- Falcone E., Cordioli P., Tarantino M., Muscillo M., La Rosa G., Tollis M. (2003): Genetic heterogeneity of bovine viral diarrhoea virus in Italy. *Veterinary Research Communications*, 27, 485–494.
- Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (2005): *Virus Taxonomy*. 8th Report of the International Committee on Taxonomy of Viruses. Elsevier-Academic Press, Amsterdam.
- Flores E.F., Ridpath J.F., Weiblen R., Vogel F.S., Gil L.H. (2002): Phylogenetic analysis of Brazilian bovine viral diarrhoea virus type 2 (BVDV-2) isolates: evidence for a subgenotype within BVDV-2. *Virus Research*, 87, 51–60.
- Hamers C., Lecomte C., Kulcsar G., Lambot M., Pastoret P.P. (1998): Persistently infected cattle stabilise bovine

- viral diarrhea virus leading to herd specific strains. *Veterinary Microbiology*, 31, 177–182.
- Hornberg A., Fernandez S.R., Vogl C., Vilcek S., Matt M., Fink M., Kofer J., Schropf K. (2008): Genetic diversity of pestivirus isolates in cattle from Western Austria. *Veterinary Microbiology*, 135, 205–213.
- Houe H. (1999): Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Veterinary Microbiology*, 64, 89–107.
- Jackova A., Novackova M., Pelletier C., Audeval C., Gue-
neau E., Haffar A., Petit E., Rehby L., Vilcek S. (2008):
The extended genetic diversity of BVDV-1: Typing of
BVDV-1 isolates from France. *Veterinary Research
Communications*, 32, 7–11.
- Letellier C., Kerkhofs P. (2003): Real-time PCR for si-
multaneous detection and genotyping of bovine viral
diarrhea virus. *Journal of Virological Methods*, 114,
21–27.
- Nagai M., Ito T., Sugita S., Genno A., Takeuchi K., Ozawa
T., Sakoda Y., Nishimori T., Takamura K., Akashi H.
(2001): Genomic and serological diversity of bovine
viral diarrhea virus in Japan. *Archives of Virology*, 146,
685–696.
- Nagai M., Hayashi M., Itou M., Fukutomi T., Akashi H.,
Kida H., Sakoda Y. (2008): Identification of new genetic
subtypes of bovine viral diarrhea virus genotype 1 iso-
lates in Japan. *Virus Genes*, 36, 135–139.
- Nettleton P.F., Entrican G. (1995): Ruminant pestivi-
ruses. *British Veterinary Journal*, 151, 615–642.
- Paton D.J., Carlsson U., Lowings J.P., Sands J.J., Vilcek
S., Alenius S. (1995): Identification of herd-specific bo-
vine viral diarrhoea virus isolates from infected cattle
and sheep. *Veterinary Microbiology*, 43, 283–294.
- Pellerin C., van den Hurk J., Lecomte J., Tussen P. (1994):
Identification of a new group of bovine viral diarrhea
virus strains associated with severe outbreaks and high
mortalities. *Virology*, 203, 260–268.
- Pizarro-Lucero J., Celedon M.O., Aguilera M., de Calisto
A. (2006): Molecular characterization of pestiviruses
isolated from bovines in Chile. *Veterinary Microbiol-
ogy*, 115, 208–217.
- Radostits O.M., Littlejohns I.R. (1988): New concepts in
the pathogenesis, diagnosis and control of diseases
caused by the bovine viral diarrhea virus. *Canadian
Veterinary Journal-Revue Veterinaire Canadienne*, 29,
513–528.
- Ridpath J.F., Bolin S.R., Dubovi E.J. (1994): Segregation
of bovine viral diarrhea virus into genotypes. *Virology*,
205, 66–74.
- Tajima M., Frey H.R., Yamato O., Maede Y., Moennig V.,
Scholz H., Greiser-Wilke I. (2001): Prevalence of gen-
otypes 1 and 2 of bovine viral diarrhea virus in Lower
Saxony, Germany. *Virus Research*, 76, 31–42.
- Vilcek S., Herring A.J., Herring J.A., Nettleton P.F., Low-
ings J.P., Paton D.J. (1994): Pestiviruses isolated from
pigs, cattle and sheep can be allocated into at least
three genogroups using polymerase chain reaction and
restriction endonuclease analysis. *Archives of Virol-
ogy*, 136, 309–323.
- Vilcek S., Paton D. J., Durkovic B., Strojny L., Ibata G.,
Moussa A., Loitsch A., Rossmanith W., Vega S., Sci-
cluna M. T., Palfi V. (2001): Bovine viral diarrhoea
virus genotype 1 can be separated into at least eleven
genetic groups. *Archives of Virology*, 146, 99–115.
- Vilcek S., Durkovic B., Bobakova M., Sharp G., Paton
D.J. (2002): Identification of bovine viral diarrhoea
virus 2 in cattle in Slovakia. *The Veterinary Record*,
151, 150–152.
- Vilcek S., Greiser-Wilke I., Durkovic B., Obritzhauser W.,
Deutz A., Kofer J. (2003): Genetic diversity of recent bo-
vine viral diarrhoea viruses from the southeast of Austria
(Styria). *Veterinary Microbiology*, 91, 285–291.
- Wolfmeyer A., Wolf G., Beer M., Strube W., Hehnen H.
R., Schmeer N., Kaaden O.R. (1997): Genomic (5'UTR)
and serological differences among German BVDV field
isolates. *Archives of Virology*, 142, 2049–2057.

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