GENETIC ANALYSIS OF INDIAN BOVINE VIRAL DIARRHEA VIRUS 1 ISOLATES IN N^{PRO} AND ENTIRE GENE REGION CODING STRUCTURAL PROTEINS

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Summary. – Three Indian Bovine viral diarrhea virus 1 (BVDV-1) isolates were analyzed at genetic level in N^{pro} (viral autoprotease) and entire gene region coding structural proteins, namely capsid (C) protein, E^{rns}, and envelope proteins E1 and E2. All these isolates were found to be of b subtype based on the entire 504 nt region of N^{pro} and 1119 nt region of E2. However, in comparison with other isolates of this subtype, they were allocated inside the BVDV-1 subtype b cluster to a separate clade with a longer distance. Of six cysteine residues in N^{pro} only three were totally conserved in all three isolates. The isolates showed 94.9–99.3% and 92.2–99.0% identities for the entire C-E2 gene region at nucleotide and amino acid levels, respectively. The lowest identity values (88.5–91.7%) were observed for E2 amino acid sequences. The identity of the isolates with Osloss, a reference BVDV-1 subtype b strain, was in the range of 82.1–89.9% for nucleotide and 78.6–89.2% for amino acid sequences in the C-E2 region. The N^{pro}/C and E^{rns}/E1 cleavage sites were highly conserved. The C/E^{rns} and E1/E2 cleavage sites were more conserved from the N-end of E^{rns} and the C-end of E1, respectively. These findings suggest that some unique mutations have occurred in the described Indian BVDV-1 isolates, though they all belong to the BVDV-1 subtype b.

Key words: Bovine viral diarrhea virus; pestivirus; autoprotease; structural proteins; genetic analysis; phylogeny

Introduction

BVDV-1 (the species *Bovine viral diarrhea virus 1*) and BVDV-2 (the species *Bovine viral diarrhea virus 2*) belong to the genus *Pestivirus* in the family *Flaviviridae* (Fauquet *et al*., 2005). BVDV-1 is widespread among cattle and causes significant economic losses to the livestock industry. The BVDV-1 infected cattle develop respiratory, enteric or reproductive clinical signs and rarely fatal mucosal disease (Brownlie *et al.*, 1987). Highly virulent BVDV-2 isolates may cause severe hemorrhagic syndrome with high mortality (Carman *et al*. 1988).

The genome of both BVDV-1 and BVDV-2 has similar organization. A 12.3 kb single-stranded RNA of positive polarity is flanked with 5' and 3' untranslated regions (5'-UTR, 3'-UTR) (Colett *et al*., 1988). The first 5'-UTR gene is N^{pro}, which encodes nonstructural viral autoprotease. The following four genes code for viral core (C) protein and three envelope structural proteins, E^{rns}, E1 and E2. The E2 glycoprotein is the most important immunodominant envelope component. The other part of the genome encodes non-structural proteins (Meyers and Thiel, 1996).

Variability of nucleotide and amino acid sequences of pestivirus genome depends on genome regions (Ridpath and

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Abbreviations: BVDV-1 and BVDV-2 = Bovine viral diarrhea virus 1 and Bovine viral diarrhea virus 2; C = capsid protein; E^{rns} = envelope protein (ribonuclease secreted); E1, E2 = envelope proteins; N^{pro} = N-terminal autoprotease; p7 = non-structural protein linked to E2 glycoprotein; UTR = untranslated region

Bolin, 1997). Whereas the most conserved nucleotide sequences were found in 5'-UTR, the highest genetic variability of structural genes was observed in the E2 region. Traditionally, pestiviruses have been usually classified on the basis of nucleotide sequences of the 5'-UTR, N^{pro} and E2 regions (Baule *et al*., 1997; Becher *et al*., 1999; Mahony *et al*., 2005; Tajima *et al*., 2001; Vilček *et al*., 2001). Recently, a more precise genetic typing based on the phylogenetic analysis has divided BVDV-1 isolates into eleven subtypes and BVDV-2 into two-three subtypes (Becher *et al*., 1997; Vilček *et al*., 2001). An analysis of nucleotide sequence variability is useful for genetic typing of pestivirus isolates, molecular epidemiology as well as understanding of the virus evolution.

To date, all BVDV-1 isolates originating from India have been typed as subtype b (Mishra *et al*., 2004). The aim of this work was to analyze genetically three selected Indian BVDV-1 isolates and to compare them with reference BVDV-1 strains NADL (subtype a) and Osloss (subtype b). The analysis was focused on the N^{pro} and structural genes C, E^{rns}, E1 and E2.

Materials and Methods

Viral isolates. Three cattle BVDV-1 isolates, namely Ind S-1226 from Gujarat (2001), Ind S-1449 from Orissa (2002) and Ind S-1456 from Uttar Pradesh (2002) were examined. They were grown in bovine turbinate cells free of any adventitious pestiviruses by RT-PCR in Glasgow Minimal Essential Medium (GMEM, Sigma) with 2% of horse serum (Gibco BRL).

RT-PCR. RNA was isolated by using the RNeasy Minikit (Qiagen) according to the manufacturer's recommendations. The synthesis of cDNA was carried out in total volume of 20 µl by using random hexamer primers (Promega) and the Superscript II reverse transcriptase (Life Technologies). Four overlapping DNA fragments spanning nt 179–3577 of the genome of NADL strain (Fig. 1) were obtained by PCR using the following primers: Ol 100 (5'-GAG TACAGGACAGTCGTCAG-3', nt 179–198) and 1400 R (5'-ACCA GTTGCACCAACCATG-3', nt 1448–1430) (Becher *et al*., 1997; Becher *et al*., 1998) produced a 1.2 kb fragment from 5'-UTR covering N^{pro}, C and a part of E^{rns}; P1 (5'-AACAAACATGGTTG GTGCA ACTGGT-3', nt 1424–1448) and P2 (5'-CTTACACAGA CATATTT GCCTAGGTTCCA-3', nt 2250–2222) (Sullivan and Akkina, 1995) generated a C-terminal part of E^{rns} and a N-terminal part of E1 (826 bp); TS3 (5'-GGGGGTCACTTGTCGGAGG - 3', nt 2027–2045) (Sullivan and Akkina, 1995) and gp (5'-GTCTC CAGGCACACAAGTCC-3', nt 3040–3021) (Hertig *et al*., 1991) produced a 1013 bp fragment covering the E^{rns}-E1-E2 region; P5 (5'-TGGA ACGCTGCAACAACTACTG-3', nt 2354–2375) and P3 (5'-GGC CTTCTGTTCTGATAAGACC-3', nt 3577–3556) (Toth *et al*., 1999) generated a 1223 bp fragment covering E2 and a part of E1 and p7. The DNA fragments were purified by means of Wizard PCR prep columns (Promega) and were cloned by using the pGEMT Easy TA Cloning Kit (Promega). The nucleotide sequencing was done in a SQ3 sequencer (Hoefer) by using the fmol Cycle Sequencing Kit (Promega) The corresponding overlapping sequences were assembled together by using the SeqMan Program of the DNASTAR Computer Program Package (USA). The obtained sequences were deposited at the GenBank database under the following Acc. Nos.: AY911672 (Ind S-1226), AY911670 (Ind S-1449) and AY911671 (Ind S-1456).

Computer-assisted analysis. The nucleotide and deduced amino acid sequences were aligned and the identity values were calculated by using the MegAlign Program (DNASTAR). The cleavage sites between viral proteins were used as found for CSFV (Rumenapf *et al*., 1993; Stark *et al*., 1993). Phylogenetic and bootstrap analyses were performed by using the NEIGBOR and SEQBOOT Programs from PHYLIP (Felsenstein, 1993; Vilček *et al*., 2001).

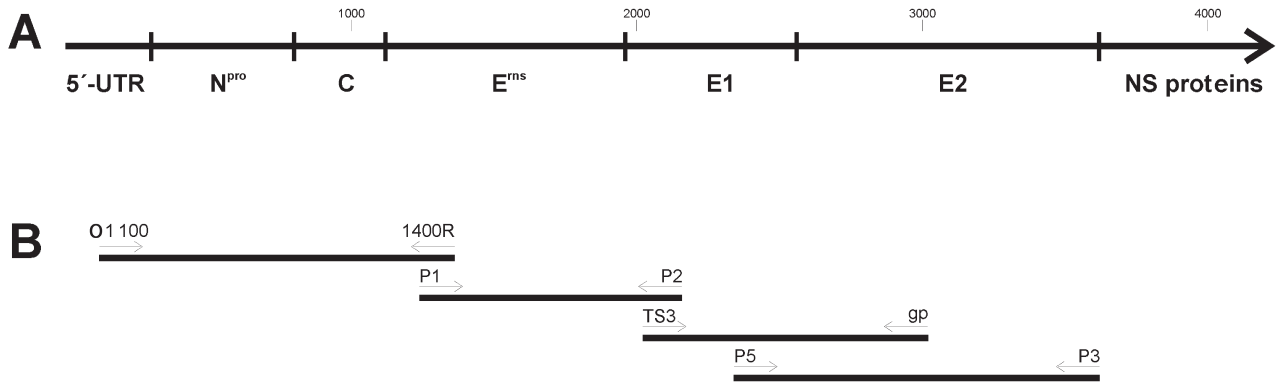

Fig. 1

Strategy for the amplification of 5'-UTR-E2 region of BVDV-1 genome

The map of the 5'-UTR-E2 region (A) and the fragments generated by RT-PCR (B).

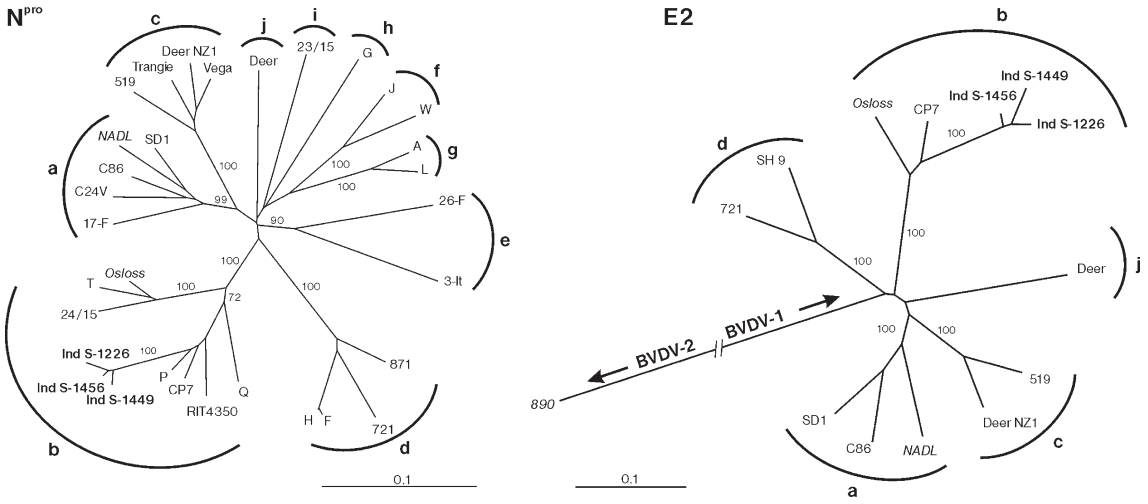

Fig. 2

Phylogenetic trees of Indian BVDV-1 isolates based on N^pro and E2 regions

The trees were based on entire N^pro (left, N^pro) and E2 (right, E2) regions. The three Indian isolates analyzed in this work are in bold.

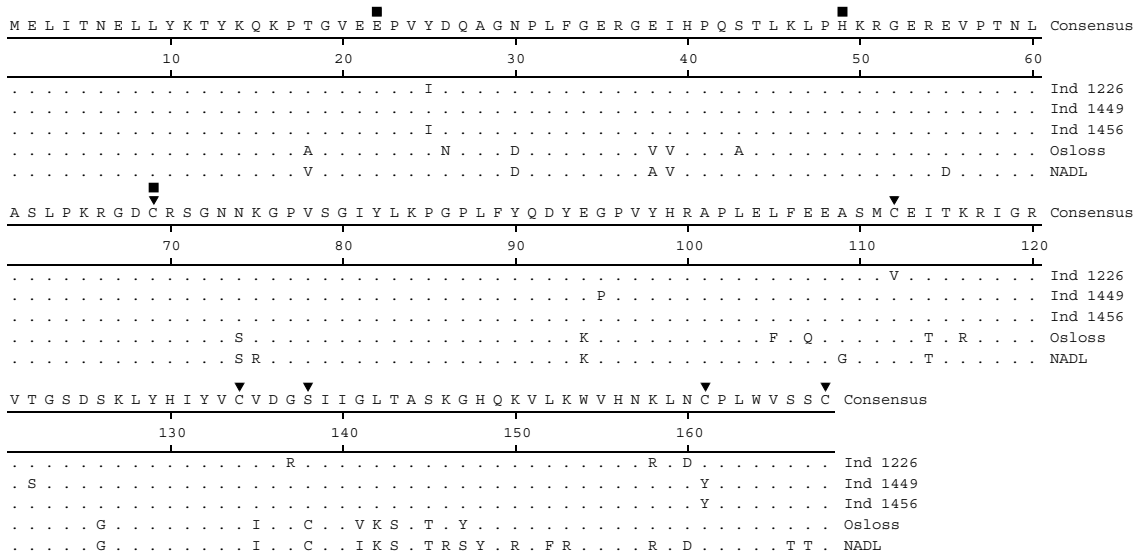

Fig. 3

The alignment of the amino acid N^pro sequences of the three Indian isolates and the reference Osloss and NADL strains of BVDV-1

Dots represent the same amino acid as those in the consensus sequence. Cysteine residues are marked by arrowheads. Squares indicate the amino acids of a catalytic triad.

Results

The three BVDV-1 isolates analyzed in this work were initially assigned to subtype b using partial N^pro nucleotide sequences (385 nt). The same result was obtained when entire N^pro sequence (504 nt) was examined (Fig. 2). However, in comparison with other isolates of this subtype, they were allocated inside the BVDV-1 subtype b cluster to a separate clade with a longer distance. This type of clustering was highly supported by the bootstrap analysis.

The alignment of amino acid sequences of the entire N^pro region (168 amino acids) of the isolates with those of the Osloss and NADL strains is shown in Fig. 3. Of six cysteine residues found in N^pro only three at the positions 69, 134 and

	N^{pro}/C	C/E^{rns}	E^{rns}/E1	E1/E2
	▼	▼	▼	▼
S-1226	PLWVSSC SDTGTEG	LTQVTVE ENITQWN	TWFGAYA ASPYCEV	LITGAQG YPDCKPD
S-1449	PLWVSSC SDTGTEG	WTQVTVG ENITQWN	AWFGAYA ASPYCVV	LITGAQG YPDCKPD
S-1456	PLWVSSC SDTGTEG	LTQVTVG ENITQWN	TWFGAYA ASPYCVV	LITGAQG YLDCKPE
Osloss	PLWVSSC SDTKAEG	LFQVAVG ENITQWN	TWFGAYA ASPYCEV	LITGAQG LPVCKPG
NADL	PLWVSSC SDTKEEG	LFQVTMG ENITQWN	TWFGAYA ASPYCDV	LITGVQG HLDCKPE

Fig. 4

Alignment of the amino acid sequences in cleavage sites of the three Indian isolates and the reference Osloss and NADL strains of BVDV-1

The cleavage sites are marked by arrowheads.

168 were strongly conserved. Also the glutamic acid (E22) and histidine (H49) residues were found conserved in the three isolates. The isolates showed a high identity both at nucleotide (96.6–98.8%) and amino acid (95.2–98.2%) level.

For determination of putative BVDV-1 cleavage sites, comparative analysis of the sequences around the cleavage sites of N^{pro}, C and the glycoproteins as reported for other pestivirus, namely CSFV was used. The predicted cleavage sites between viral proteins N^{pro}/C, C/E^{rns}, E^{rns}/E1 and E1/E2 are presented in Fig. 4. The proteins C, E^{rns}, E1 and E2 consisted of 102, 227, 195 and 373 amino acids, respectively. While the N^{pro}/C and E^{rns}/E1 sites were entirely conserved, that of C/E^{rns} was conserved only from the N-end of E^{rns} protein and that of E1/E2 was conserved only from the C-end of E1 protein.

The isolates were also highly similar in the region of C-E2 genes. The identity values reached 94.9–99.3% at nucleotide and 92.2–99.0% at amino acid levels. The lowest values (88.5–91.7%) were observed for E2 amino acid sequences.

A comparison of the entire gene region analyzed of the three isolates with that of the Osloss strain, a representative of BVDV-1 subtype b, showed identity values of 81.9–89.9% for nucleotide and 78.6–89.2% for amino acid sequences (Table 1). The lowest values were observed for the E2 region. When Osloss and NADL (a representative of BVDV-1 subtype a) strains were compared, the identity values for the entire region varied in the lower range, namely 69.5–79.6% for nucleotide and 77.7–87.3% for amino acid sequences; the lowest values were obtained for the E2 region (69.5% and 77.7%, respectively).

The lengths of E2 gene and E2 protein of the three isolates were similar to those of the Osloss strain (1119 nt and 373 aa, respectively). The E2 protein of the NADL strain compared to the Osloss strain was shorter by one amino acid, with a deletion at the position 140 in the E2 protein of the Osloss strain. Eleven to twelve cysteine residues were found in the E2 protein of the three isolates. Despite a genetic variability is the highest in the E2 region, the genetic typing of the three isolates gave the same result for entire E2 region, and partial or entire N^{pro} region (Fig. 2).

Table 1. Nucleotide and amino acid identities of the three Indian isolates and the NADL strain of BVDV-1 with the Osloss strain of BVDV-1 based in various gene regions

Isolate	Gene regions				
(strain)	N^{pro}	C	E^{rns}	E1	E2
IndS-1226	81.9	86.3	89.0	87.7	83.3
	85.1	*88.2*	*88.7*	*88.7*	*78.6*
IndS-1449	83.7	85.9	88.0	87.2	82.1
	86.3	*85.3*	*86.2*	*86.2*	*76.1*
IndS-1456	83.9	86.9	89.9	86.3	83.3
	86.9	*89.2*	*84.6*	*84.6*	*80.4*
NADL	78.0	78.8	79.6	73.3	69.5
	87.5	*87.3*	*85.1*	*85.1*	*77.7*

The Osloss strain sequences requested 100%.

Discussion

Different regions of the BVDV-1 genome have been used for typing and comparative sequence analysis in several studies. In general, the genes encoding N^{pro} and E2 are mostly used targets in phylogenetic analysis of pestiviruses (Becher *et al*., 1997, 1999; Vilček *et al*., 1997; Tajima *et al*., 2001). Initially, BVDV-1 has been divided into two subtypes, a (NADL-like) and b (Osloss-like) on the basis of the 5'-UTR analysis (Pellerin *et al*., 1994). Further genetic studies using BVDV-1 isolates from different countries have divided BVDV-1 into 3–11 phylogenetic entities (Becher *et al*., 1997, 1999; Mahony *et al*., 2005; Nagai *et al*., 2004; Tajima *et al*., 2001; Vilček *et al*., 1997, 2001). We have reported previously a prevalence of BVDV-1 of subtype b in India (Mishra *et al*., 2004). In order to determine the relatedness of Indian BVDV-1 isolates with the two reference strains, three recent cattle BVDV-1 isolates originating from different geographical locations (eastern, western and northern) in India were characterized at molecular level.

The sequencing of the first third of the genome of the three isolates clearly demonstrated that these isolates are not unique but belong to BVDV-1 subtype b which has been also found in other parts of the world (Tajima *et al*., 2001; Vilček *et al*., 2001). However, on the basis of both N^{pro} and E2 regions, they were placed together with some other Indian isolates into a separate phylogenetic clade located within the basic branch b. This indicates unique mutations in these isolates. However, it should be pointed out that many Indian BVDV-1 isolates stand in the branch b but out of the separate clade mentioned above (Mishra *et al*., 2004).

A high conservation of amino acid sequences flanking the N^{pro}/C junction of the three isolates further supports the earlier finding of the conserved N^{pro} self-cleavage site (Rumenapf *et al*., 1993; Stark *et al*., 1993). The conservation of glutamic acid (E22), histidine (H49) and cysteine (C69) residues in the N^{pro} protein also supports the hypothesis that these amino acids represent a catalytic triad responsible for N^{pro}-C proteolytic cleavage (Rumenapf *et al*., 1998). Despite various hypervariable amino acid stretches have been found earlier in deduced E2 amino acid sequences in BVDV-1 (Becher *et al*., 1999), we found in the three isolates only 11–12 instead of 15 cysteine residues (data not shown). This finding indicates the incidence of unique mutations in these isolates

On the other hand, we observed a relatively high level of similarity of the three isolates despite they had been collected from geographically distant regions of India. A theoretical possibility of a laboratory contamination or a contamination originating from fetal calf serum during cultivation of viruses in cell cultures is highly improbable, because all precautions recommended to exclude contamination during manipulation with viral isolates have been taken (Belák and Ballagi-Pordany, 1993). The viruses were cultivated in cells in 1–2 passages, but instead of fetal calf serum horse serum was used. Therefore, the low genetic diversity of the three BVDV-1 isolates reflects their real variability but not laboratory artefacts.

In conclusion, the genetic analysis of the entire region of N^{pro}-E2 genes of three recent Indian BVDV-1 isolates showed that these isolates belong to subtype b with some unique nucleotide mutations as it has also been documented by their assignment to a separate phylogenetic clade.

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References

Baule C, van Vuuren M, Lowings JP, Belak S (1997): Genetic heterogeneity of bovine viral diarrhoea viruses isolated in Southern Africa. *Virus Res.* **52,** 205–220.

Becher P, Orlich M, Shannon AD, Horner G, Konig M, Thiel HJ (1997): Phylogenetic analysis of pestiviruses from domestic and wild ruminants. *J. Gen. Virol.* **78,** 1357–1366.

Becher P, Orlich M, Thiel HJ (1998): Complete genomic sequence of border disease virus, a pestivirus from sheep. J. Virol. **72,** 5165–5173.

Becher P, Orlich M, Kosmidou A, Konig M, Thiel HJ (1999): Genetic diversity of pestiviruses: identification of novel groups and implications for classification. *Virology* **262,** 64–71.

Belák S, Ballagi-Pordany A (1993): Experiences on the application of the polymerase chain reaction in a diagnostic laboratory. *Mol. Cell. Probes* **7,** 241–248.

Brownlie J, Clarke MC, Howard CJ, Pocock DH (1987): The pathogenesis and epidemiology of bovine viral diarrhoea virus in cattle. *Ann. Rech. Vet.* **18,** 157-166.

Carman S, van Dreumel T, Ridpath J, Hazlett M, Alves D, Dubovi E, Tremblay R, Bolin S, Godkin A, Anderson N (1998): Severe acute bovine viral diarrhoea in Ontario. J. *Vet. Diagn. Invest.* **10,** 27–35.

Colett MS, Larson R, Gold C, Strick D, Anderson DK, Purchio AF (1988): Molecular cloning and nucleotide sequence of the pestivirus bovine viral diarrhoea virus. *Virology* **165,** 191–199.

Fauquet CM, Mayo MA, Maniloff J, Dessellberger U, Ball LA (Eds) (2005): *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses.* Elsevier-Academic Press, Amsterdam.

Felsenstein J (1993): PHYLIP (Phylogeny Inference Package), version 3.5c, Department of Genetics, University of Washington, Seattle.

Hertig C, Pauli U, Zanoni R, Peterhans E (1991): Detection of bovine viral diarrhoea virus (bvdv) using polymerase chain reaction. *Vet. Microbiol.* **26,** 65–76.

Mahony TJ, Mccarthy FM, Gravel JL, Corney B, Young PL, Vilcek S (2005): Genetic analysis of bovine viral diarrhoea viruses from Australia. *Vet. Microbiol.* **106,** 1–6.

Meyers G, Thiel, HJ (1996): Molecular characterisation of pestiviruses. *Adv. Virus Res*. **47,** 53–118.

Mishra N, Pattnaik B, Vilcek S, Patil SS, Jain P, Swamy N, Bhatia S, Pradhan HK (2004): Genetic typing of bovine viral diarrhoea virus isolates from India. *Vet. Microbiol.* **104,** 207–212.

Nagai M, Hayashi M, Sugita S, Sakoda Y, Mori M, Murakami T, Ozawa T, Yamada N, Akashi H (2004): Phylogenetic analysis of bovine viral diarrhoea viruses using five different genetic regions. *Virus Res.* **99,** 103–113.

Pellerin C, Hurk JVD, Lecomte J, Tijssen P (1994): Identification of a new group of bovine viral diarrhoea virus strains associated with severe outbreak and high mortalities. *Virology* **203,** 260–268.

Ridpath JF, Bolin SR (1997): Comparison of the complete genomic sequence of the border disease virus BD31, to other pestiviruses. *Virus Res.* **50,** 237–243.

Rumenapf T, Unger G, Strauss JH, Thiel HJ (1993): Processing of the envelope glycoproteins of pestiviruses. *J. Virol.* **67,** 3288–3294.

Rumenapf T, Stark R, Heimann M, Thiel HJ (1998): N-terminal protease of pestiviruses: identification of putative catalytic residues by site directed mutagenesis. *J. Virol.* **72,** 2544–2547.

Stark R, Meyers G, Rumenapf T, Thiel HJ (1993): Processing of pestivirus poly protein: cleavage site between auto-protease and nucleocapsid protein of classical swine fever virus. *J. Virol.* **67,** 7088–7095.

Sullivan D G, Akkina RK (1995): A nested polymerase chain reaction assay to differentiate pestiviruses. *Virus Res.* **38,** 231–239.

Tajima M, Frey HR, Yamato O, Maede Y, Moennig V, Scholz H, Greiser Wilke I (2001): Prevalence of genotypes 1 and 2 of bovine viral diarrhoea virus in Lower Saxony, Germany. *Virus Res.* **76,** 31–42.

Toth RL, Nettleton PF, McCrae MA (1999): Expression of the E2 envelope glycoprotein of bovine viral diarrhoea virus (bvdv) elicits virus type specific neutralising antibodies. *Vet. Microbiol.* **65,** 87–101.

Vilček Š, Nettleton PF, Paton DJ, Belák S (1997): Molecular characterisation of ovine pestiviruses. *J. Gen. Virol.* **78,** 725–735.

Vilček Š, Paton DJ, Durkovič B, Strojný L, Ibata G, Moussa A, Loitsch A, Rossmanith W, Vega S, Scicluna MT, Pálfi V (2001): Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. *Arch. Virol.* **146,** 99–115.