SZENT ISTVÁN UNIVERSITY
POSTGRADUATE SCHOOL OF VETERINARY SCIENCE

Molecular characterisation of bovine viral diarrhoea virus with special regard to cytopathogenicity

Doctoral thesis

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Abbreviations

BDV	border disease virus
BHV-1	bovine herpesvirus-1
bp	base pair
BRSV	bovine respiratory syncytial virus
BT	bovine turbinate
BVD	bovine viral diarrhoea
BVDV	bovine viral diarrhoea virus
C	capsid protein
cDNA	complementary DNA
cINS	cellular insert
cp	cytopathogenic
CPE	cytopathic effect
CSFV	classical swine fever virus
ds	double-stranded
DIP	defective interfering particle
DNA	deoxyribonucleic acid
E1	envelope glycoprotein 1
E2	envelope glycoprotein 2
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
E^{rns}	envelope glycoprotein with RNase function
HCV	hepatitis C virus
IFA	immunofluorescence assay
INF	interferon
IPX	immunoperoxidase assay
IRES	internal ribosomal entry site
JIV	J-domain interacting with viral protein
kb	kilobase
kDa	kilodalton
MAb	monoclonal antibody
MD	mucosal disease
M-MLV RT	Moloney-murine leukaemia virus reverse transcriptase
ncp	non-cytopathogenic
N^{pro}	N-terminal autoprotease
NS	nonstructural protein
NTPase	nucleoside triphosphatase
ORF	open reading frame
p7	protein of 7 kDa
PCR	polymerase chain reaction
PI	persistently infected
PI-3	parainfluenza-3
p.i.	post infection
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS PAGE	sodium-dodecyl-sulphate polyacrilamide gel electrophoresis
UCH	ubiquitin carboxy-hydrolase
UTR	untranslated region
VN	virus neutralisation

Abstract

Bovine viral diarrhoea virus is a major pathogen of cattle that causes significant economic losses worldwide in the cattle industry. Based on the ability to cause cytopathic effect in cell cultures, BVDV strains can be classified as cytopathogenic or non-cytopathogenic biotypes. The cp biotypes are generated in the persistently infected cattle by genomic alterations of the ncp biotype. Cytopathogenicity of BVDV has been shown to correlate with the presence of insertions of cellular sequences, duplication of viral genomic regions with or without insertions, deletions and point mutations in the genomes of cp strains. These genetic alterations are termed cytopathogenicity markers. In most cp BVDV strains the role of these cp markers is well defined, but in some cases further genetic and functional analyses are needed to elucidate the role of these genomic alterations.

Since the Hungarian cp BVDV isolates have not been characterised so far, in the first study the possible cytopathogenicity markers in the genomes of six "archive" cp BVDV strains isolated in the 1970s have been examined. At that time a live BVDV vaccine (termed here for ethical reasons BVDV-X) was introduced and widely used in Central Europe until the beginning of the 1990s. The viruses were selected as representatives of various forms of BVDV infections: enteritis and mucosal disease presumably associated with the use of the live attenuated vaccine, as well as respiratory syndrome. The complete NS2-3 coding region of the six isolates and the vaccine virus were amplified by RT-PCR and were sequenced. The results showed that new cp markers were found in all cp BVDV strains at nucleotide position 4355 in the NS2 gene. These cp markers resemble to a very rare cp marker that is present in only reference strain BVDV CP7. The NS2-3 region of four isolates originated from the vaccination accidents and of the BVDV-X vaccine virus was identical proving that the vaccine caused early onset of mucosal disease. The cp marker proved to be a 45-nucleotide viral insertion that encodes 15 amino acids of the NS4B/NS5A junction region in a normal BVDV genome. In respiratory isolate H3887, a 21-nucleotide insertion of non-viral origin was found, which also located at nucleotide position 4355. The insertion had high similarity with a gene coding for murine interferon-induced guanylate-binding protein 1, and represented the first non-viral insertion identified at this position of the NS2 coding region. Respiratory isolate H3142 contained a 42-nucleotide viral duplication at close proximity of nucleotide position 4355. The insertion was identical to a part of the NS5B gene. This isolate also had a deletion of three nucleotides approximately 90 nucleotides downstream of the insertion. The genome rearrangements found in these isolates occurred preferentially at position 4355, suggesting that this part of the genome could represent a potential hot spot for recombination events in ncp BVDV, and may be termed position C.

In the second study, recently isolated cp BVDV isolates were characterised. The use of BVDV-X vaccine contributed to the "positive effect" of causing MD in PI animals, reducing the natural source of BVDV. This may explain the phenomenon that cp BVDV strains were not isolated for nearly 30 years. Two cp BVDV strains have been recently isolated from MD cases in Hungary. The strains were examined for cytopathogenicity markers to check whether the newly found genomic alterations show any common feature with those of the recent cp BVDV isolates. In the genome of strain H4956, a jiv-like insertion was found similar to those described in reference strain NADL and in other BVDV 1, BVDV 2 and BDV strains. The jiv-like nucleotide sequence coding 133 amino acids was inserted at nucleotide position 4984, nine nucleotides upstream of that of strain NADL. The insertion showed 96% amino acid sequence identity with the cellular Jiv protein. In the genome of cp BVDV strain H115/PCR, an ubiquitin-containing duplication was found. The duplicated sequence started at nucleotide position 7978 in the NS4B gene. The duplication contained a complete ubiquitin monomer of 76 amino acids and the complete NS3 gene. The duplication located further downstream of the known ubiquitin-containing genomic regions of cp BVDV strains. The insertions and duplication of the recently isolated two cp BVDV strains further confirmed that recombinations occurring at positions A and B are the most common mechanisms leading to the development of BVDV cytopathogenicity.

In the third study, complete genomic analysis of the BVDV-X vasccine was carried out. The BVDV-X vaccine was marketed many years ago (and not any more) as a derivate of the Oregon C24V strain. However, sequencing the whole NS2-3 region of the BVDV-X vaccine and of the cp BVDV strains originated from MD cases suggested recombinations between the vaccine and wild type variants of BVDV during the vaccine production. The analysed nucleotide sequences seemed to be distinct from BVDV Oregon C24V, therefore the genome of a pre-registration (termed here BVDV-Xpre) and of a marketed (BVDV-X) batches of the vaccine was analysed. Results of the complete genome analysis of BVDV-Xpre confirmed that the original virus strain used at the start of the vaccine production was Oregon C24V. Surprisingly, analysis of the complete nucleotide sequence of the BVDV-X marketed vaccine revealed that this strain belongs to the BVDV 1b subgroup, with a 93.7% nucleotide sequence identity with BVDV reference strain Osloss. The identity to BVDV Oregon C24V was significantly lower (77.4%), and a thorough sequence scanning showed that the genome of BVDV-X had not derived from Oregon C24V. These data indicate the very likely scenario that a strain different from Oregon C24V was picked up during the *in vitro* or *in vivo* passages for vaccine development. Despite the virus-switch, the BVDV-X vaccine continuously maintained its innocuity and efficacy, as proven by the regular quality testing data, and the presence of the alien virus remained unnoticed over many years. The results of this work emphasize that the contamination of commercially available live vaccines with exogenous BVDV strains is a real risk factor, and an unequivocal analysis, including molecular methods is needed to verify their authenticity.

In the further two studies, the role of the cytopathogenicity marker found in the genome of BVDV-X was examined. Since the complete molecular analysis showed that other possible factors contributing to the cytopathogenicity of BVDV-X are not present in the genome, in the first step the role of the 45-nucleotide insertion in the expression of NS3 was investigated in the case of the vaccine virus. The whole NS2-3 gene of this virus and a PCR-directed mutagenesis-generated insertion-negative variant were cloned in pCI mammalian expression vector, and were expressed in BT cells. Western blot analysis revealed that the insertion contributed to a partial cleavage of NS2-3 generating NS3, the marker protein of cytopathogenicity. In addition, it was also demonstrated that the NS4B/NS5A junction of the insertion that is cleaved in the BVDV polyprotein is not processed in this case.

In order to further examine the possible role of the 45-nucleotide insertion in the cytopathogenicity of BVDV-X, in the final step, a full-length infectious cDNA clone of the BVDV-X vaccine was generated. The recovered virus, BVDV-XR showed slight retardation in growth in comparison with the wild-type BVDV-X, but was appropriate for further reverse genetic studies. Since the natural ncp counterpart of the vaccine virus was not available, an ncp mutant was generated by PCR-directed mutagenesis. The recovered virus, BVDV-XR-INS- also showed the same growth characteristics as its cp counterpart, and caused no CPE. This observation gave a final proof that the insertion is indispensable in the cytopathogenicity of BVDV-X.

In summary, these studies provide novel information on the biology of BVDV from aspects of virus recombination, which has an important impact, both on basic and applied research of veterinary virology.

Key words: BVDV, pestivirus, vaccine, cytopathogenicity, NS2-3, infectious cDNA clone

1 Introduction

1.1 General background

The viral aetiology of BVD was first described by Olafson et al. (1946). A few years later the fatal disease of unknown origin, called mucosal disease was described (Ramsey and Chivers, 1953). Gillespie et al. (1961) proved that the two clinical diseases are caused by the same virus, termed BVDV. The close antigenic relationship between this agent and classical swine fever virus was not elucidated until the 1960s (Mengeling et al., 1963). In the 1970s an ovine virus, the causative agent of border disease was identified as an additional member of this group (Plant et al., 1973). The term *pestivirus* was given by Horzinek (1981) to designate this group of viruses.

BVDV occurs in most cattle-producing countries and causes significant economic losses to the cattle industry. This led in several countries to carry out epidemiolgical as well as cost/benefit studies and initiate eradication or control programmes. The seroprevalence in the EU ranged from less than 1% (Finland) through 19% (Norway), 46% (Swedent), 64% (Denmark) to 95% (England) before starting these programmes (Greiser-Wilke et al., 2003).

In Hungary, BVDV was suspected to cause severe respiratory, enteritis and abortion cases in the late 1950s (Áldásy and Szabó, 1959), but the virus was isolated only a few years later (Manninger et al., 1963). Although exact data are not available, the seroprevalence of BVDV in Hungary was 40-50% in the 1970s, 60-70% in the 1980s, and is almost 100% at present (Kudron, 1999). The estimated annual losses caused by the virus at national level reach 630 million HUF/year (Ózsvári et al., 2001). Starting of eradication programmes against the disease will be inevitable in the near future.

1.2 Taxonomy

*Pestivirus*es were previously classified as members of the *Togaviridae* (Westeway et al., 1985). However, molecular characterisation showed that genome organisation and strategy of gene expression are much closer to those of *Flaviviridae*. Based on these results, *pestivirus*es were re-classified as an additional genus in the *Flaviviridae* family that also consists of *Flavivirus* and *Hepacivirus* genera (Wengler et al., 1995). *Pestivirus*es were initially classified according to host specificity, but proof of transmission of *pestivirus*es between cattle and sheep (Carlson, 1991; Vilcek et al., 1996), ruminants and swine (Vilcek and Belák, 1996; Becher et al., 1999; Kulcsár et al., 2001) and serological analyses (Paton, 1995; van Rijn et al., 1997) confirmed the significance of antigenic and genetic relationship-based taxonomy of *pestivirus*es. Currently, the genus *Pestivirus* comprises the four approved species BVDV 1, BVDV 2, CSFV, BDV and one tentative fifth species represented by a single strain (H138) isolated from a giraffe in Kenya more than 30 years ago (Becher et al, 1997). Recently, the existence of BDV-1, BDV-2 and BDV-3 as major genotypes within the species BDV has been proposed (Becher et al., 2003).

1.3 Structure of the virion and genomic organisation

The BVDV virion is 40-60 nm in diameter. The core is composed of a single RNA molecule covered with the nucleocapsid protein that is surrounded with a lipid envelope. Two glycoproteins (E1 and E2) are anchored into the membrane and a third glycoprotein (E^{rns}) is loosely associated to the envelope (Rümenapf et al., 1993).

BVDV has an uncapped and unpolyadenylated positive-stranded RNA genome of about 12.5 kb (Collett et al., 1988a; Deng and Brock, 1992), but genomes of DIPs of 7.5 kb (Behrens et al., 1998) and with large duplications of 16 kb (Qi et al., 1992) have also been described. As seen in Fig. 1, the genome of BVDV comprises a single ORF that is flanked by 5' and 3'UTRs (Collett et al., 1988b).

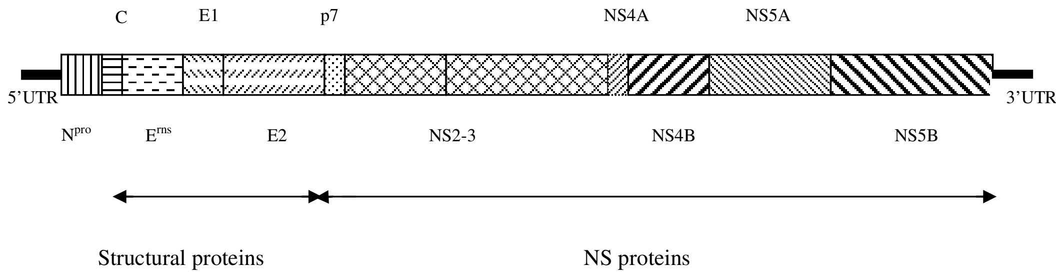

Fig 1. Organisation of the BVDV genome.

The 5'UTR of *pestivirus*es and HCV, functioning as IRES, promotes translation of the viral polyprotein by cap-independent internal ribosomal entry (Poole et al., 1995; Pestova and Hellen, 1999). The 5'UTR of BVDV ranges from 383 to 385 nucleotides (Collett et al., 1988a; Deng and Brock, 1992). The 5'UTR is highly conserved nucleotide sequence among *pestivirus*es, however, this sequence contains also three variable regions (Deng and Brock, 1993). The conserved regions allow the design of pan-*pestivirus* detection systems (Vilcek et al., 1994), while variable regions can serve as the base of identification of species in the genus *Pestivirus* (Letellier and Kerkhofs, 2003). The secondary structure of 5'UTR forms a stem-loop structure consisting of four domains: domain Ia, Ib, II, and III. The 5' border of the BVDV IRES is located at the end of stem-loop Ib, and the 3' border extends into the 5' region of the ORF (Chon et al., 1998). The region upstream of the IRES element is important for the second step of BVDV replication, the positive-strand viral RNA synthesis (Becher et al, 2000; Yu et al., 2000). Mutations in hairpin Ia or lacking Ia and part of Ib result a growth-restricted phenotype that forms smaller plaques and has lower growth rates than the parent virus.

Sequence and structural elements residing in the 3'UTR of the BVDV genome are considered as *cis*-acting elements that are necessary for the first step of viral replication, i.e., the synthesis of the complementary negative-strand RNA (Yu et al., 1999). The *pestivirus*es have a relatively long 3'UTR, 188 nucleotides for BVDV Osloss and approximately 228 for nucleotides for other BVDV strains (Collett et al, 1988a; Deng and Brock, 1992). A variable and a conserved region were identified in the 3'UTR of *pestivirus*es. The 5' part of the 3'UTR shows remarkable heterogeneity in size, and it is involved in the coordination of the viral translation and replication (Isken et al., 2004). In contrast, the 3' part of the 3'UTR comprises a number of characteristic RNA motifs: the 3' terminus of the *pestivirus* genome consists of four single-stranded C residues, upstream of this sequence, a 70 nucleotid long strech is found which comprises of a stem-loop structure SL I and further upstream an intervening sequence of single stranded nucleotides, nine of which (AGCACUUUA) are identical among all *pestivirus*es. The remarkable stability and conservation of structure as well as sequence characteristics of the SL motifs suggest that these elements represent well-defined interaction sites for viral and/or cellular proteins during RNA replication (Yu et al, 1999).

The ORF encodes a polyprotein of about 4000 amino acids that is co- and post-translationally processed by viral and cellular proteases into 11 or 12 mature viral proteins: NH_2-N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A and NS5B-COOH (Rice, 1996; Thiel et al., 1996). The structural proteins (C, E^{rns}, E1, E2,) are encoded at the N-terminal part of the BVDV genome, while the NS proteins, except for the N^{pro}, are encoded at the C terminal two third of the genome.

1.4 Genome replication

BVDV replicates efficiently only in cells derived from *Artylodactyla*. Replication is optimal in bovine, ovine, or goat cells, whereas replication is less efficient in swine cells (Bolin et al., 1994). Rabbits are susceptible to infection with rabbit-adapted BVDV, and are the only known laboratory animal host for the virus. Replication of BVDV takes place entirely in the cytoplasm of the cell. Each infected cell releases 100 to 1000 infectious virions (Donis, unpublished).

BVDV enters cells by receptor-mediated endocytosis, followed by an acid-dependent step that delivers the viral genome to the cytosol. The initial interaction between the virus and susceptible cell is mediated by the E2 glycoprotein that attaches to a cellular surface receptor protein CD46 (Xue and Minocha, 1993; Maurer et al., 2004), and to actin-binding molecules (Schelp et al., 2000). The E^{rns} also plays role in the initial binding of BVDV to the host cell by interacting with cell surface heparane sulphate glycosaminoglycans (Iqbal and McCauley, 2002). Cell lines of bovine, swine, human, murine, simian and canine origin interact with BVDV E^{rns}. However, BVDV infection of mammalian cells showed species specificity. This suggests that infection of cells by BVDV may be a multistep process in which virus attachment to cells and internalisation are two distinct steps requiring different cell surface receptors. This assumption was further supported when Agnello et al. (1999) described the role of low-density lipoprotein (LDL) receprors in mediating the entry *flavivirus*es, and proved that a cell line that is BVDV resistant (Flores et al., 1996) lacks LDL receptor activity.

After the initial virion attachment and endocytosis, the replication cycle is continues with the release of the ribonucleoprotein complex into the cytosol of the cell. The complex comes in contact with ribosomes, but signals directing its intracellular trafficking are not known. Translation of the viral genome requires at least partial stripping of the capsid protein off the viral RNA. The genomic RNA serves as an mRNA, translation and polyprotein processing take place mainly in association with the ER. Starting from the N-terminus of the polyprotein, the first cleavage, between N^{pro} and C is catalised intramolecularly (Stark et al., 1993), additional cleavages generating the structural proteins are catalised by cellular signalases in the ER or Golgi (Rümenapf et al., 1993), the remaining cleavages generating the NS proteins are catalised by the serine protease residing in NS2-3 with NS4A as cofactor (Xu et al., 1997).

The nascent viral NS proteins and cellular components associate with the 3' terminus of the genomic RNA to form replication complexes. The first step of viral RNA replication is initiated by synthesizing a full-length negative-strand RNA complementary to the genomic positive-strand RNA. Then the 5'UTR and the 3' end of the negative-strand intermediate contribute to the formation of a positive-strand promoter that catalyses the second replication step in which the negative strand serves as the template for synthesis of additional positive-strand RNA molecules (Behrens et al., 1998). The BVDV genome is not used simultaneously for translation and replication, translation and replication are tightly regulated during the BVDV life cycle (Gong et al., 1996). A translation to replication switch mechanism might occur after the accumulation of NS5A and NS5B proteins, which can inhibit the BVDV IRES-dependent translation *in vitro* (Li and McNally, 2001).

RNA synthesis of the members of *Flaviviridae* is localised to ER membranes in the perinuclear site of infected cells (Lubiniecki and Henry, 1974), but little is known about the regulation and viral/cellular components of the replication complexes. The C-terminal domain of NS3 contains both helicase and ATPase activities that are believed to be essential for RNA replication (Tamura et al., 1993; Warrener and Collett, 1995). NS5B is thought to function as the viral RNA polymerase, as it contains conserved motifs found in all positive-strand viral RNA polymerases and it has recently been shown to possess RNA-dependent RNA polymerase activity (Zhong et al., 1998). The exact function of NS4B and NS5A in BVDV replication is unknown. Experimental results indicate that NS3, NS4B, and NS5A are associated as components of a multiprotein complex (Qu et al., 2001).

Recently, a new group of cellular proteins was identified as components of the viral replication complex (Isken et al., 2003). These proteins associate specifically with both termini of the viral RNA genome involving regulatory elements in the 5' and 3'UTR regions. The possible role of this association is mediating the circular conformation of the viral genome that may be important for the coordination of translation and replication.

BVDV assembly takes place in the ER or the Golgi (Bielefeldt-Ohman, 1987). As described for members of the genus *Flavivirus*, release of *pestivirus*es occurs likely by budding of nascent virions through intracellular membranes into cytoplasmic vesicles. Virions reach the extracellular compartment by vesicular transport (exocytosis) as early as 10 h p.i.. The p7 protein is essential for the final assembly of the virions, and being a viroporin, by destabilising the membrane, facilitates virus release from the plasma membrane of mammalian cells (Harada et al., 2000).

1.5 General functions of the proteins of BVDV

N^{pro} has no counterpart among the other members of the *Flaviviridae*. N^{pro} is a nonstructural protein (Wiskerchen et al., 1991; Thiel et al., 1991). The function of N^{pro} is its proteolytic activity, which leads to cleavage at its C terminus (Stark et al., 1993; Rümenapf et al., 1998). In CSFV, it was shown that the N-terminal protease is not required for viral replication in cell cultures (Tratschin et al., 1998), but deletion of N^{pro} leads to attenuation (Mayer et al., 2004). The role of N^{pro} in interfering with the cellular innate immune system, inhibiting dsRNA-induced type I interferon (INF type I) production has also been demonstrated (Ruggli et al., 2003).

C: The function of this protein is the packaging of the genomic RNA and providing necessary interactions with the envelope glycoproteins to form the enveloped virion (Collett et al., 1988b).

E^{rns} is an envelope glycoprotein that has no counterpart in other *flavivirus*es. Unlike the other two envelope proteins E1 and E2, E^{rns} lacks a transmembrane domain and a vast quantity is secreted into the medium of infected cells (Rümenapf et al., 1993), but the protein is also attached to the virus envelope via direct interaction with E2 (Lazar et al., 2003). E^{rns} has been identified as an RNase, and this enzymatic activity is inhibited by virus-neutralising antibodies (Schneider et al., 1993; Hulst et al., 1994; Windisch et al., 1996). E^{rns} inhibits lymphocyte proliferation and protein synthesis due to selective induction of apoptosis in the lymphocytes of several species, leading to immunosuppression (Bruschke et al., 1997). Abrogating of RNase activity of E^{rns} of CSFV (Meyers et al., 1999) and BVDV (Meyer et al., 2002) lead to attenuation of the respective viruses. E^{rns} is also involved in the initial interaction between the virion and host cells (Iqbal et al, 2000; Iqbal et al., 2002). Furthermore, the E^{rns} glycoprotein of the BVDV can act as an inhibitor of dsRNA-induced INF type I production (Iqbal et al., 2004). E^{rns} induces considerable levels of non-neutralising antibodies in BVDV infected cattle (Weiland et al., 1992).

E1 is a glycoprotein that contains two hydrophobic domains that serve to anchor the protein into the membrane (Rümenapf et al., 1993). E1 forms heterodimers with E2. Convalescent cattle serum does not contain significant levels of antibody against E1 (Donis and Dubovi, 1987a), suggesting that this protein is deeply embedded into the viral envelope.

E2 is the major glycoprotein of BVDV that forms homodimers as well as heterodimers with E1 that are anchored into the lipid envelope via a transmembrane region (Weiland et al., 1990; Rümenapf et al., 1993; Weiland et al., 1999). E2 plays key role in initial virus attachment to the cell surface receptors (Donis et al., 1988; Xue and Minocha, 1993; Flores et al., 1996), membrane fusion (Schelp et al., 1995; Schelp et al., 2000), virus assembly and maturation. E2 is highly antigenic and elicits the production of neutralising antibodies. (Bolin et al., 1988; Toth et al., 1999). One of the three hypervariable sequence regions found in the BVDV genome is present in this polypeptide. The hypervariability may reflect immunologic selective pressure (Donis et al., 1991; Paton, 1995).

p7 forms the junction between the structural and the NS genes in *pestivirus*es. The feature of p7, E2p7 and the peculiar processing of E2/p7/NS2 region are well conserved among *pestivirus*es and

HCV, which is indicative of a common function for these proteins. The spatial proximity of E2 and p7 in the polyprotein and the formation of an E2p7 fusion protein suggest a role in glycoprotein maturation and viral morphogenesis. A cytoplasmic domain of p7 may interact with the capsid protein and initiate the budding process (Elbers et al., 1996). The p7 protein has a very similar structure to virosporins and is thougt to function in membrane permeabilisation and releasing of infectious progeny virus (Harada et al., 2000).

NS2-3 is the first cleavage product downstream of p7 that in most *pestivirus*es is partially processed to yield NS2 and NS3 (Collett et al., 1988b; Meyers et al., 1991). The degree of processing at the NS2/3 site shows remarkable variation among different *pestivirus* species and strains (reviewed in Meyers and Thiel, 1996). In the case of BVDV, the ncp biotype expresses NS2-3, the while cp biotype expresses also detectable level of NS3, therefore NS3 is considered the marker protein of cytopathogenicity. NS2-3 and NS3 represent multifunctional proteins with different activities: RNA binding (Deng and Brock, 1992), cysteine protease for the processing at the NS2/3 site (Lackner et al., 2004), serine protease for processing of the NS proteins of the replication complex (Wiskerchen and Collett, 1991; Tautz et al., 1997), *cis*-acting NTPase and helicase essential for viral replication (Warrener and Collett, 1995; Grassmann et al., 1999). Additional putative role of the uncleaved NS2-3 is the processing at the carboxi-terminus of the C protein, which contributes to the viral morphogenesis (Agapov et al., 2004). NS3 is the most conserved protein in *pestivirus*es (Collett, 1992), and induces high titre of antibodies lacking neutralising capacity after natural infection or vaccination with attenuated live vaccines (Bolin and Ridpath, 1989; Donis and Dubovi, 1987a).

NS4A is a cofactor for proteolitic activity of NS3 (Tautz et al., 2000), and required for cleavage at sites NS4B/NS5A and NS5A/NS5B (Xu et al., 1997).

NS4B is a transmembrane protein and membrane cytoplasmic domain of the protein may interact with viral proteins, such as NS3 and cellular factors that are involved in viral replication (Li and McNally, 2001; Qu et al., 2001). This protein also plays role in *pestivirus* cytopathogenicity. Mutations in this protein can suppress CPE, regardless of NS3 production and high level RNA accumulation.

NS5A is involved in the recruitment of essential components of the replication complex (Reed et al., 1998; Neddermann et al., 1999), as was described in the case of the *pestivirus*-related HCV (Grassmann et al., 2001). The NS5A can also interact with the translational elongation factor-1 (Johnson et al., 2001), which suggests the role of this protein during RNA replication.

NS5B has been identified as RNA-dependent RNA polymerase (Collett et al., 1988b; Zhong et al. 1998) that can direct RNA replication via both primer-dependent (elongative) and primer independent (*de novo*) mechanisms (Lai et al. 1999). The role of this protein in viral morphogenesis is also suspected (Ansari et al., 2004).

1.6 Biotypes of BVDV

According to their ability to cause CPE in cell cultures, BVDV strains are classified as cp or ncp biotypes (Gillespie et al., 1960; McClurkin et al., 1985). Ncp is the most common naturally occurring biotype including BVDV 1 and BVDV 2 strains, and is the only biotype that can lead to persistent infections of BVDV. The cp biotype occurs much less frequently, cp BVDV strains were isolated almost exclusively from MD cases. Both a cp and a persisting ncp biotype can be simultaneously isolated from animals succumbing to MD (Moennig and Plagemann, 1992). These isolates are called a " virus pair".

1.7 Cytopathogenicity of BVDV

The molecular analysis of different BVDV pairs elucidated that cp BVDV strains evolved *in vivo* from ncp BVDV by genomic alterations in cattle persistently infected with ncp BVDV (Meyers and

Thiel, 1996; Tautz et al., 1998). These alterations comprise of cellular insertions, duplications, rearrangements or deletions of viral genome sequences (reviewed in Meyers and Thiel, 1996; Kümmerer et al., 2000; Becher et al., 2002; Müller et al., 2003) and in some cases simple point mutations (Qi et al., 1992; Pellerin et al., 1995; Kümmerer et al., 1998; Kümmerer and Meyers, 2000). These changes in the viral genomes of cp BVDV strains lead to the cleavage of the nonstructural protein NS2-3 and the subsequent expression of the nonstructural protein NS3 that is responsible for the development CPE, and is considered the marker protein of cytopathogenicity (Donis and Dubovi, 1987b). However, most recently, the possible role of the NS4B protein in the cytopathogenicity of *pestivirus*es was also described. Mutations in this protein were shown to suppress CPE, regardless of NS3 production (Qu et al., 2001).

Cleavage at the N-terminus of NS3 can be the result of different mechanisms. In the majority of cp BVDV strains the genomic alterations occur at two points, the so-called positions A and B (Meyers et al., 1998). Position A is located at nucleotide position 4992 (amino acid position 1535), whereas position B is located at nucleotide position 5153 (amino acid position 1589) in the BVDV polyprotein (all the nucleotide and amino acid positions refer to BVDV SD-1, described by Deng and Brock, 1992). The exact location of position A is not so conserved, and appears approximately 50 bases upstream of position B. However, in cp BVDV strains containing insertion at position A, the NS2-3 cleavage also occurs at amino acid position 1589, indicating that the insertion generates the cleavage of the polyprotein *in trans*. A cell-derived insert Jiv, previously termed cIns, can be found at position A in the genome of several cp BVDV and BDV strains, (Meyers et al., 1990; Becher et al., 1996; Ridpath and Neill, 2000; Vilcek et al., 2000). About the role of Jiv in the BVDV cytopathogenicity, the following explanation was suggested: since NS2 of BVDV protein can be detected in the same perinuclear compartment as Jiv, it is possible that Jiv interacts via its domains with NS2 and induces conformational change of NS2-3. A change of conformation of NS2-3 could activate an intrinsic protease locating in the NS2-3 of BVDV (Rinck et al., 2001). In the closely related hepatitis C virus (HCV), NS2-3 cleavage is catalyzed by a viral metalloprotease, residing in the NS2-3 (Santolini et al., 1995).

The result of the cellular or viral insertions at position B is the proteolytic cleavage at the position B, indicating this mechanism acts *in cis*. Ubiquitin insertions at position B result in processing of NS2-3 by introducing a new cleavage site for UCHs at the carboxy terminus of the insertion. Ubiquitin-like proteins, like NEDD8, SMT3B (Qi et al., 1998; Baroth et al., 2000), as well as other cell derived insertions like LC3 and related sequences of GABA (A) RAP and GATE-16 (Meyers et al., 1998; Becher et al., 2002) also generate specific cleavage sites for cellular proteases. N^{pro} insertions at position B in the BVDV genome result in autocatalytic activity that acts at the carboxy terminus of the insertion (Meyers and Thiel, 1996).

In other cp BVDV strains analysed so far, point mutations in the NS2 or in one strain, BVDV CP7, a small insertion of viral origin at nucleotide position 4355 in the NS2 are responsible for the cp phenotype (Kümmerer and Meyers, 2000; Tautz et al., 1996). These genetic alterations may block the inhibitors of a newly found cysteine protease that is responsible for cleavage at the NS2/3 junction in both biotypes of BVDV (Lackner et al., 2004).

In cp BVDV infected cells, CPE that is characterised by condensation and margination of chromatin, cell shrinkage and generation of apoptotic bodies, is triggered by apoptosis (Zhang et al., 1996; Hoff and Donis, 1997). CPE induced by apoptosis can be the direct effect of the structural difference and subcellular localisation between NS2-3 and NS3 (Mendez et al., 1998; Zhang et al., 2003), or NS3 may upregulate BVDV RNA replication to a deleterious level for the cell (Mendez et al., 1998). The latter assumption was confirmed by Vassilev and Donis (2000) who showed that cp BVDV induced apoptosis correlates with increased intracellular viral RNA accumulation causing oxidative stress (Schweizer and Peterhans, 1999). Grummer et al (2002) proved the role of intrinsic apoptotic pathway in cells infected with cp BVDV. Recently, it was shown that cp BVDV strains induce endoplasmatic reticulum (ER) stress that lead to apoptosis of the infected cells (Jordan et al, 2002): ER stress is caused by both viral envelope glycoprotein accumulation in the lumen

(Rümenapf et al., 1993) and assembly of the viral replicase at the cytosolic side of the ER (Xu et al., 1997). Apart from the ncp BVDV isolates, in case of the cp BVDV strains, the NS2-3 cleavage can result in an altered replicase formation that may lead to increased ER stress followed by apoptosis.

1.8 Genetic subdivision of BVDV

*Pestivirus*es have been typed using different genomic regions, including 5'UTR (Pellerin et al., 1994; Vilcek et al., 1994; Baule et al., 1997), N^{pro} (Becher et al., 1997), E2 (van Rijn et al., 1997; Becher et al., 1999) and NS5B-3'UTR (Vilcek et al., 1999a). Comparison of the phylogenetic analyses showed that BVDVs form defined clusters (Tajima et al., 2001; Vilcek et al., 2001).

Genetic typing of BVDV 1 isolates from the USA and Canada revealed two distinct genetic groups, namely groups BVDV 1a and BVDV 1b (Ridpath et al., 1994; Ridpath and Bolin, 1998). Further analyses of isolates from different continents and countries demonstrated that there is considerably more genetic heterogenicity, including 3-12 subgroups (Baule et al., 1997; Vilcek et al., 2001 and 2004). Most of the Hungarian BVDV isolates belong to the 1b subgroup (unpublished result).

Appearance of BVDV 2 in North America initiated a search for the virus in other countries. BVDV 2 was detected in Japan (Nagai et al., 2001) and South America (Jones et al., 2001). In Europe, BVDV 2 isolates seem to appear only sporadically, namely in Belgium (Letellier et al., 1999), France (Vilcek et al., 2001), Germany (Wolfmeyer et al., 1997), Italy (Pratelli et al., 2001), the UK (Vilcek et al., 1997) and in Austria (Vilcek et al., 2003). The phylogenetic surveys of BVDV 1 and BVDV 2 isolates reveal similar levels of sequence variation in the 5'UTR sequences. This lends to support that BVDV 2 are not newly arising viruses, but newly recognised ones (Ridpath et al., 2000). So far, the BVDV 2 strains are classified into 4 subgroups (Giangaspero and Harasawa, 2000).

1.9 Clinical manifestation of BVDV

Infection with BVDV can result in a wide spectrum of clinical diseases ranging from subclinical infections to a highly fatal form known as MD. The clinical response to BVDV infection depends on multiple interactive factors. Host factors that influence the clinical outcome of BVDV infection include immunocompetency or immunotolerancy to BVDV, pregnancy status, gestational age of the foetus, immune status (passively derived or actively derived from previous infection or vaccination) and concurrent level of environmental stress. Genetic diversity as well as differences in virulence and cytopathogenicity among isolates may account for differences in the clinical response to infection (Fulton et al., 2003).

BVDV infections in immunocompetent non-pregnant cattle

In cattle seronegative and immunocompetent to BVDV, the majority (70-90%) of BVDV infections are subclinical. The likely source of these BVDV infections are cattle that are immunotolerant and PI with ncp BVDV (Bolin, 1990). Cattle with subclinical infection may show leukopenia and mild fever. Decrease in milk production can also be observed (Moerman et al., 1994). BVDV-specific antibody develops in response to the infection. When infection becomes clinical, the disease is termed as BVD. BVD can occur in animals ranging in age from 6 months to 1 year and is characterised by high morbidity, but low or non-existent mortality. The incubation period is approximately 5 to 7 days, and is followed by a transient fever and leukopenia (Duffell and Harkness, 1985). Viraemia occurs 4 to 5 days after infection and may persist for up to 15 days (Brownlie et al., 1987). Clinical symptoms include depression, anorexia, oculonasal discharge, occasionally oral lesions characterised by erosions and ulcerations, diarrhoea, and decrease in milk production in lactating cattle. BVDV has the ability to induce immunosuppression by impairing the cellular immune response (Bruschke et al., 1997), which can lead to secondary or concurrent infections, such as BHV-1, BRSV, PI-3, or *Pasteurella haemolytica* (Elvander et al., 1998; Fulton et

al., 2002). Virus is shed in low concentrations from cattle with this form of infection compared with cattle PI with BVDV.

Semen from immunocompetent bulls undergoing acute infection with BVDV only transiently contains virus (Paton et al., 1989), whereas semen from bulls that are PI with BVDV, permanently contains virus (Coria and McClurkin, 1978). Reduced conception rates have been reported in seronegative as well as in seropositive cattle bred or inseminated with semen from PI bulls (McClurkin et al., 1979; Paton et al., 1990). BVDV has been associated with ovaritis in infertile heifers (Ssentongo et al., 1980). BVDV has also been associated with a condition similar in appearance to a disease caused by BHV-1 known pustular vulvovaginitis (Stober, M., 1980).

Haemorrhagic syndrome has been reported in association with acute ncp BVDV 2 infection in North America (Rebhun et al., 1989; Corapi et al., 1990) in Belgium (Broes et al., 1992) in the United Kingdom (David et al., 1994) in Germany (Wolfmeyer et al., 1997) and in Japan (Nagai et al., 1998). The disease is characterised by marked thrombocytopenia, which results in bloody diarrhoea, epistaxis, petechial and ecchymotic hemorrhages on mucous membranes, and bleeding from injection sites. Additional findings include pyrexia and leukopenia. Not all BVDV 2 isolates cause clinically severe disease. Avirulent BVDV 2 isolates do exist and predominate over virulent BVDV 2 in nature. Infection with avirulent BVDV 2 results in a reduction of leukocytes that may be accompanied by a low-grade fever (Ridpath et al., 2000).

BVDV infections in immunocompetent pregnant cattle

BVDV infection of pregnant cattle may result in the transplacental spread of the virus to the foetus (Duffell and Harkness, 1985). The principle determinant of the outcome of foetal infection is the gestational age at the transplacental infection occurs. Both cp and ncp BVDV can cause transplacental infection and fetal loss, but non-cytopathogenic infection is more common (Dubovi, 1992). Ncp BVDV is the predominant biotype of the virus in cattle population, and only ncp BVDV transplacental infection of the foetus can lead to the development of immunotolerance (Brownlie et al., 1989).

Transplacental infection of the foetus from 50 to 100 days of gestation may result in foetal death. Expulsion of the foetus may occur from days up to several months after fetal infection. Fetal infections in the later period of gestation do not result in abortions, but late-term abortions have been reported (Bolin, 1990). The rate of abortions caused by transplacental infection is low (2-7%).

Transplacental infection of the foetus between 100 and 150 days of gestation, before the development of foetal immunocompetence, can result in numerous congenital defects (Duffell and Harkness, 1985) due to the effect of BVDV on cellular growth inhibition, cell differentation, or cell lysis. The congenital defects associated with BVDV are the following: microencephalopathy, cerebellar hypoplasia, hypomyelinogenesis, retinal atrophy and dysplasia, microphtalmia, thymic hypoplasia, alopecia, brachygnatism and skeletal defects (Brownlie, 1985).

Congenital defects are rare in calves infected with BVDV during the later stages of gestation, when the foetus becomes immunocompetent. Calves infected with BVDV after the 150. day of gestation period are normal at birth but are seropositive to BVDV (Duffell and Harkness, 1985).

Ncp BVDV infection before the development of immunocompetence of the fetus may result in the birth of calves that are immunotolerant to and PI with BVDV. The precise stage of fetal development during which infection must occur to cause immunotolerance is unknown, but it is uncommon after 100 days of gestation, although it still can occur at 125. days of gestation (Liess et al., 1984).

BVDV infections in immunotolerant cattle

Cattle that are immunotolerant to and persistently infected with ncp BVDV are permanently viraemic, continuously shed virus and may appear healthy (Cutlip et al., 1980). Although PI cattle

are immunotolerant to BVDV, they are immunocompetent with respect to other antigens (McClurkin et al., 1984; Houe and Heron, 1993). Immunotolerance is specific to the infecting ncp BVDV strain, but the PI cattle can develop immune response against heterologous strains of BVDV (Bolin et al., 1985a). The prevalence of persistent infection in the cattle population is low (0.1-1%) (Bolin et al., 1985b). PI cattle are an important source of viral transmission to susceptible cattle. PI females produce PI offspring (McClurkin et al., 1979), resulting in PI family lines that may provide a means to maintain the virus in a herd.

PI cattle seem to be a risk for other diseases and have decreased survivorship (Houe et al., 1993). PI calves may have a mortality rate of 50% in the first year of life. Some PI calves may be undersized and have slower rate of growth. Subclinical disease in the form of glomerulonephritis and encephalitis has been described in PI, but otherwise normal-appearing cattle (Cutlip et al., 1980). PI calves are predisposed to infections by other microorganisms, resulting often in pneumonia and enteritis. The increased susceptibility to disease may be associated with immunosuppression induced by persistent infection with BVDV (Potgieter et al., 1988).

Mucosal disease

MD develops when cattle that are PI with ncp BVDV become superinfected with a cp strain of BVDV. The origin of the cp virus can be external, however, the cytopathogenic strain may occur more commonly *de novo* from the ncp persistently infecting BVDV by molecular rearrangement (Meyers and Thiel, 1996).

MD is a sporadic form of BVDV infection and usually occurs in cattle ranging from 6 months to 2 years of age. Morbidity is less than 5%, but mortality rate approaches 100%. Acute MD is characterised by pyrexia, depression, weakness and anorexia. Heart and respiratory rates are elevated. Dehydration with acidosis develops as the disease progresses. In lactating dairy cattle milk production decreases. The course of the disease ranges from 2 to 3 days to 3 weeks with the final outcome of death. Leasions in the oral cavity involve the lips, gingival margins, tongue, and the posterior palate. Erosive lesions can develop on the external nares, and in the nasal cavity, on the vulva and teats. Mucopurulent ocular discharge is often observed with eccessive lacrimation and corneal oedema. Profuse, watery diarrhoea generally develops 2 to 3 days after the onset of clinical signs. In peracute cases, death may occur before the onset of diarrhoea. Severe leukopenia may be recognised in the early stages of the disease. Secondary bacterial infections are common (Brownlie, 1985).

Chronic MD is characterised by inappetence and weight loss. Diarrhoea may be continuous or intermittent. Nasal discharge and persistent ocular discharge are frequent findings. Alopecia, and hyperkeratinisation may develop. Long-term erosive lesions are found in the mouth and on the skin. Long-term lameness may develop because of laminitis, interdigital necrosis and hoof deformities. Secondary bacterial infections are common. Complete blood cell counts show pancytopenia characterised by anemia, leukopenia, neutropenia and lymphopenia. Cattle with chronic MD may survive up to 18 months and ultimately die of severe debiliation. In some cases MD with recovery is possible (Edwards et al., 1991).

Calves persistently infected with ncp BVDV can develop MD by two different pathogenic mechanisms. The first possibility is that the unaltered superinfecting cp BVDV is antigenically identical or closely related to the persistently infecting ncp BVDV strain. Under these conditions, the onset of MD occurs after a short incubation time of 2 to 3 weeks p.i. (early onset MD), and the animal succumbs before neutralising antibodies and activation of the cellular immunity become detectable. Cp BVDV has a particular tropism for gut-associated lymphoid tissues and Peyer's patches (Bielefeldt-Ohmann, 1988). The lesions that develop after the destruction of the lymphoid tissue in Peyer's patches and the collapse of their overlying intestinal mucosa due to the direct effect of cp BVDV (Liebler et al., 1991).

When the superinfecting cp BVDV strain is antigenically different from the persistently infecting ncp BVDV strain, a different pathogenic mechanism develops involving a recombinational event between the persistent ncp and the superinfecting cp virus. In this case, the incubation period is prolonged to several months (late onset MD), and high titres of neutralising antibodies against the superinfecting cp BVDV are generated. Furthermore, the cp BVDV strain responsible for the pathological lesions is not identical with the original superinfecting virus. The explanation of this phenomenon is that the original cp BVDV is cleared from the blood by the humoral immune response, but the clearance of the virus from the infected cells is incomplete, since the persinstently infecting ncp BVDV may interfere with the cellular immunity. Residual cp BVDV in infected cells could undergo recombination with the persistent ncp BVDV and new subpopulations emerge which are cleared again by the immune system, until subpopulations arise that are immunologically very similar or even identical to the persistent virus and therefore evade immune surveillance. These subpopulations can spread and induce MD (Fritzmeier et al., 1997; Fricke et al., 2001). The lesions characteristic to the late onset MD are not only the result of the direct cp effect of cp BVDV, but the virus-specific immune complexes that are associated with the affected tissues can likely induce lymphocyte migration and other immunologic reactions and promote the MD (Sentsui et al., 2001).

1.10 Immunology of the two biotypes of BVDV

Cp BVDV strains induce the synthesis of INF type I *in vitro* in infected macrophages, and kill their host cells by apoptosis in response to lipopolysaccharides (Adler et al., 1997). This phenomenon can explain the pathogenesis of MD, in which the characteristic lesions occur in the oral cavity and the gastrointestinal tract that contain high concentration of lipopolysaccharide endotoxin. By contrast, ncp biotypes of BVDV do not induce the synthesis of INF type I *in vitro*, and cells show no signs of viral infection, even though the viral titres produced by cp and ncp virus pairs may be similar. The explanation of this phenomenon is that ncp BVDV inhibits ds RNA-induced apoptosis and IFN type I synthesis (Schweizer and Peterhans, 2001).

This inhibition *in vivo* contributes to the different pathogenesis of the ncp and cp biotypes. The failure of both biotypes of BVDV to establish persistent infection in the conceptus during the first 40 days of intrauterine development may be due to INF-τ. This type of IFN is produced in very high concentration by the bovine trophoblast during the earliest stage of gestation. Its main function is believed to be in the maintenance of the gestation by preventing luteolysis in the ovary of the cow. However, INF-τ is known to have antiviral activity similar to the other type I INFs, and it may well act to prevent infection of the embryo (Peterhans et al., 2003).

From the 40. day of gestation to the development of the foetal immuncompetence only ncp viruses are able to establish persistent infection (Brownlie et al., 1989). Both biotypes were found to replicate in the foetus but replication of the cp biotype was more limited. IFN type I was found in the amnionic fluid of fetuses infected with cp BVDV, but was undetectable in the amnionic fluid originating from infection with the ncp variant. The foetal immune response (INF type I production) can either eliminate cp BVDV on its own, or preventing the development of immunotolereance, elimination of cp BVDV occurs when the foetus becomes immunolologically competent. Therefore, failure of ncp BVDV to induce IFN type I may have evolved to enable the virus to establish persistent infection in the early foetus (Charleston et al., 2001).

In immunocompetent cattle CD4+ cells play an essential role in the immunity to BVDV (Howard et al., 1992). Infection of immunocompetent calves with ncp BVDV results in transient viraemia and nasal excretion of the virus, with resolution of infection about 12-14 days after infection, while infection of calves with homologous cp BVDV results in lower titres of virus in nasal secretions and undetectable viraemia (Lambot et al., 1998). Virus-specific antibody is first detected shortly after viral clearance of both biotypes.

However, there is a significant difference in the kinetics of the development of a specific T-cell response after ncp and cp BVDV infection. T-cell proliferative responses are not detectable until 6-8

weeks after ncp infection, but were detectable by 3-4 weeks after cp BVDV infection (Collen and Morrison, 2000). The possible explanation of these findings is that intranasal cp BVDV infections that confined to the mucosal and submucosal tissues result in rapid and potent induction of IFN type I, which induce primary immune response and restritcts viral growth. Furthermore, since cp BVDV does not compromise the antigen-presenting cells in their ability to present viral antigens (Glew et al., 2003), these cells can migrate and stimulate proliferative responses in CD4+ T cells in local lymph nodes that coordinate the systemic immune response.

Since ncp BVDV inhibits IFN type I synthesis at the primary site of infection (Schweizer and Peterhans, 2001), and causes immunosuppression by the tropism of the virus to the antigen-presenting cells, ncp BVDV does not stimulate the innate immune response at the mucosal surface. As innate immunity is not stimulated at the local site, antigen-presenting cells will not become activated and viral growth is not restricted. However, when free virus enters the lymph node, interaction with plasmacytoid antigen-presenting cells would result stimulating primary immune response (Howard et al., 1999). As the elevation of tissue INF type I levels is slower by 24-36 h after ncp infection compared to cp infection, ncp virus will become more readily disseminated, and the onset of protective immune response delayed. The establishment of viraemia for a period of days after acute infection is the prerequisite for the survival of ncp BVDV, i.e., the development of PI animals (Niskanen et al., 2002).

Understanding the early event in the immune response to BVDV will aid the rational design of new vaccines. The quality and onset of the immune response after vaccination will depend in part on how the innate immune response is stimulated.

1.11 Diagnosis of BVDV infection

The direct methods of the diagnosis of BVDV include virus isolation and detection in cell culture, detection of viral antigens and detection of viral nucleic acid. Virus isolation is performed by incubating samples on low-passage cultures of bovine kidney, testis or turbinate cells, followed by using fluorochrome- (IFA) or enzyme-linked (IPX) antibodies that can detect the presence of BVDV. Virus isolation is the reference for virological diagnostics, but the presence of toxic substances or antibodies can lead to false negative diagnosis. Furthermore, ncp BVDV infected cell line or foetal calf serum can lead to fals positive results. Several methods of the detection of viral antigen by ELISA are available. These tests are rapid, independent of using cell cultures, but in genereal, their sensitivity is relatively low. Most of the ELISAs are sandwich type, some rely on the extraction of viral antigen, but new assays are being developed that do not need preliminary treatment of clinical samples. Immunohistochemistry can detect intracellular viral antigen and is the test of choice for detection of BVDV in tissues (Sandvik, 1999).

For detection of BVDV RNA, RT-PCR techniques are used, most of them are nested type (Belák and Ballagi-Pordány, 1991; Elvander et al., 1998). These methods have the advantage of being resistant to toxic substances and interfering antibodies, but being extremely sensitive (mostly the nested type), sample contamination can lead to false positive results. This problem can be circumvented by the use of real-time PCR assays that are even more sensitive, can easily be automated, furthermore using them in multiplex combination, additional viral agents can be assayed in the same sample (Belák and Thorén, 2001). The high-degree diversity of *pestivirus*es makes the direct diagnosis of BVDV complicated. The only method that can reliably differentiate among the different species and strains of the genus is genetic sequencing.

The indirect methods of BVDV detection include virus neutralisation and ELISA tests. VN is the gold standard for antibody detection, sensitive and specific, but cell-culture dependent. Therefore, the two types of ELISAs, namely indirect and competitive are used when large sample throughput is required. Since the four species of the genus *Pestivirus* are antigenically close related, cross reactions may occur in the case of the indirect methods, but the antibody titres against the hetrologous species are much lower than those against the homologous ones (Becher et al., 2003).

1.12 Molecular epizootology of BVDV

Identification of BVDV isolates with the tools of molecular virology allows the investigation of the distribution and movement of the virus within a country or in broader view, between continents. Following the course of an epidemic is also possible. In Sweden, where the cattle population is scattered into distinct farms situated long distance from each other, it was shown that each herd has its own predominating single BVDV strain. However, geographical clustering of the virus was not possible, indicating that BVDV can travel long distances most likely via movement of infected animals (Vilcek et al., 1999b). In contrast, in countries, where cattle population is dense and animal purchase is frequent, more than one BVDV strain can be detected simultaneously in a single herd (Vilcek et al., 2003). Spreading of BVDV 2 worldwide by contamination in foetal calf serum into countries where different BVDV profile is present has been also detected (Vilcek et al., 1998). This example shows that biological contaminations may have a transboundary character, endangering animal and human health worldwide.

1.13 Control of BVDV

The strategy of control or eradication BVDV depends on national regulations, farmer's organisations and financial resources. Control and eradication of BVDV are possible with or without using vaccines. The basis of eradication without using vaccines is the recognition and removal of PI animals from the herds. The Scandinavian countries and also a few other regions in Europe are aiming towards complete eradication of BVDV without use of vaccines (Lindberg, 2003).

In many countries, vaccines are used to control BVDV infections. Classical BVDV vaccines are of two different types: live attenuated and inactivated (van Oirschot et al., 1999). The modified live vaccines contain one more or less attenuated cp BVDV strain. The main advantage of live attenuated vaccines is their low price and high efficacy. A single vaccination gives strong immune response and antibodies are present in high concentration for one year, and then can persist in relatively high titres for several further years. The attenuated vaccines can clinically protect immunocompetent animals against viral challenge (Cortese et al., 1996, 1998). However, even live attenuated vaccines did not protect completely against congenital infection, and vaccination of PI cattle did not protect from developing MD after superinfection with a cp strain (Bolin et al., 1985a). The live BVDV vaccines cause several adverse effects after vaccination. Passing the placental barrier, the foetus is infected, and severe clinical signs, including congenital defects are generated (Liess et al., 1984). Furthermore, if the vaccine strain is closely related to the ncp BVDV strain in the PI animal, early onset of postvaccional MD can occur, as it was observed in several cases (Fuller, 1965; Bittle and House, 1973; Bálint et al., 2005a). If the vaccine strain is not closely related to the persistently infecting ncp strain, during replication, the live vaccine strain might recombine in the PI animal with the respective ncp wild type strains of BVDV, and this recombination can lead to the development of the delayed onset of MD (Ridpath and Bolin, 1995; Fritzmeier et al., 1995; Becher et al., 2001). A further disadvantage of the attenuated live virus vaccines is their immunosuppressive effect (Roth and Kaeberle, 1983).

To generate inactivated BVDV vaccines, the virus strains are grown in high titres, followed by a subsequent inactivation, which is performed mainly by chemical treatment. The advantage of these vaccines that they are safe, the original strains and the possible other agents are completely inactivated, thus reversion to virulence and recombination after vaccination with the field virus strain is impossible. The inactivated vaccines are not immunosuppressive, and do not infect the foetus. The drawback of these vaccines is that they are expensive and during inactivation immunogenic activity can decrease (van Oirschot et al., 1999). Further disadvantage of these vaccines is that booster vaccination is required to reach protective immunity and the immunity

generated by these vaccines is much shorter than that of the attenuated vaccines. In addition, the inactivated vaccines can interfere with the maternal immunity (Shultz, 1993).

Using molecular methods in the development of vaccines against BVDV opened new perspectives. Subunit BVDV vaccines containing recombinant, baculovirus-expressed BVDV proteins have been described (Bolin and Ridpath, 1996). Development of DNA vaccines against BVDV is also in progress (Harpin et al., 1999; Nobiron et al., 2003; Bálint et al., manuscript in preparation). Full-length infectious cDNA clones were also constructed and used for immunisation (Vassilev et al., 2001). Their availability enables to perform reverse genetic engineering to develop attenuated strains of BVDV. Using these novel techniques, an RNase-negative attenuated BVDV 2 was also constructed and used for immunisation (Meyer et al., 2002). Deleting parts of the 5´UTR also lead to the development of attenuated vaccine candidates (Makoschey et al., 2004).

The genetically engineered vaccines also provide the possibility to differentiate between vaccinates and naturally infected animals, and may therefore be the basis for a so-called marker vaccine. Most recently, using chimeric cDNA construct that contained BVDV backbone and CSFV E2 gene, an innocuous and efficacious marker vaccine was developed agains CSFV (Reimann et al., 2004).

Surprisingly, none of the vaccines take genetic and antigenic variability of BVDV isolates into account. A difference of 35-fold in the neutralising activity of defined, genotype-monospecific sera versus heterologous BVDV genotype was detected (Wolfmeyer et al., 1997). Therefore, the development of novel BVDV vaccines has to aim at solving also this probleme in the future.

2 Aims of the studies

2.1 General aims

The main goal of the following studies was to investigate the molecular biology of BVDV, with special regard to viral recombinations and cytopathogenicity. Cytopathogenicity markers of the Hungarian cp BVDV strains have not been characterised yet. Since many of these strains were connected with the use of a live attenuated vaccine BVDV-X, our further goal was to elucidate the scenario around the application of the live attenuated vaccine by using methods of molecular biology in a retrospective study.

2.2 Specific aims

1) To elucidate the molecular basis of cytopathogenicity of six Hungarian cp BVDV strains isolated in the early 1970s from various forms of BVD (i.e. respiratory disease, enteritis and MD).
2) To clarify the genetic factors contributing to the cytopathogenicity of two recently isolated Hungarian cp BVDV strains.
3) To clarify the observation that different BVDV strain-derived sequences are present in different batches of BVDV-X by determining the full-length genomic sequence of the marketed BVDV-X and the pre-registration BVDV-Xpre vaccine batches.
4) To detect possible attenuation markers in the genome of BVDV-X.
5) To confirm the role of the 45-nucleotide insertion found in BVDV-X in the processing of the NS2-3 protein by transient expression studies and Western blot analysis.
6) To give a final proof about the crutial role of the 45-nucleotide insetion in the cytopathogenicity of BVDV-X by reverse genetic methods, i.e., constructing a full-length infectious cDNA clone of BVDV-X and generating an insertion-negative mutant BVDV-XINS-.

3 Comments on methods

The detailed descriptions of the methods used in the different studies of this thesis are given in the Materials and Methods sections of the individual papers. Comments referring to the selection and modification of methods are added here.

Cell cultures, infection, transfection, virus titration

The experiments were performed according to standard procedures (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004). Secondary cultures of BT cells were applied that are in use in our institutes for BVDV diagnosis and research. The BVDV-free status of the cell line was assessed according to our routine screening procedures. For transfection of BT cells, the electroporation method proved to be the most successful (papers I and IV, not shown).

Extraction of RNA

Since the BVDV genome does not contain poly(A) tail, total RNA was extracted from the lysates of BVDV-infected BT cells. For this purpose using the TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) was found to be the most suitable method, even in case of generating long PCR products to assemble full-length genomic cDNA.

Synthesis of cDNA

For the amplification of small targets, cDNA was synthesized with M-MLV RT (Invitrogen, Carlsbad, CA, USA), from RNA primed with random hexanucleotides pd(N)6 (Amersham Biosciences, Piscataway, NY, USA). This method was applied for the detection of insertions and duplications (paper I).

For generation of long cDNA copies, the Superscript II RT (Invitrogen, Carlsbad, CA, USA) and an antisense primer VDAS1, complementary to the 3'UTR of the genome were used (papers I, II, III and IV). Using double-stranded cDNA did not increase the signal from amplification of large fragments (not shown).

PCR

Single PCR assays using the Expand High Fidelity Kit (paper I) and Expand Long Template Kit (Roche Diagnostics, Basel, Switzerland) (papers II and III) were performed to generate amplicons for sequencing. For cloning the whole NS2-3 region of BVDV-X and assembling the full-length clone of BVDV-X, the newly developed KOD HiFi DNA polymerase (NOVAGEN, Darmstadt, Germany) was used, since its efficacy and proof-reading activity was found to be better than those of other DNA polymerase enzymes.

Cloning and nucleotide sequencing

Cloning and subcloning procedures used in papers I and IV were performed according to standard procedures (Sambrook et al., 1989). For cloning the whole NS2-3 region of BVDV-X, the pCI mammalian expression vector (Promega, Madison, WI, USA) proved to be better than the pCDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). To avoid the instability problems of the full-length cDNA clone of BVDV-X, a low-copy-number plasmid pANCR1180 (Ruggli et al., 1996) and ElectroTen Blue recombinant *E. coli* competent cells (Stratagene, La Jolla, CA, USA) were used. Sequencing work in papers I and II were intitiated with the ALF Express sequencer (Amersham Biosciences, Piscataway, NJ, USA), but due to the poor results sequencing was continued with the ABI Prism sequencer (Model 377), using the Big Dye Terminator V3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA). The obtained sequences were edited and analysed with the multiple programs of the DNAstar software package (Lasergene, Madison, WI, USA).

Western blot analysis

Since the NS2-3 protein of BVDV is attached to the ER via its transmembrane domain and the NS3 in spite of its hydrophylic characteristics is also membrane associated, furthermore, since the transient expression of these proteins resulted in low amounts, vast quantity of the intracellular membranes of BT cells were used for the Western blot analyses. To reach this goal, all the transfected cells were scraped from the bottom of the wells of the tissue culture plates, centrifuged at 10 000 g for 10 minutes to pellet all the cell-debris, washed with ice-cold PBS to remove the proteins interfering with the assay, and resuspended in 50 µl PBS. This concentrated suspension was then used to carry out SDS-PAGE and Western blot as described in Paper I.

Immunoperoxidase staining

This method was performed as described by Meyling (1984), in experiments of paper IV. It is the method for detection of BVDV specific antigens in serum samples of persistently infected animals and in preparations containing ncp virus. In the experiment in paper IV this technique was used to demonstrate the presence of viral antigen in cells transfected with RNA recovered from the full-length cDNA clones pBVDV-XR and pBVDV-XR-INS-.

4 Results and discussion

4.1 Detection of cytopathogenicity markers in Hungarian cp BVDV strains isolated in the 1970s (I)

In the past one and a half decade, using methods of molecular virology, the cytopathogenicity markers of several cp BVDV strains have been identified. In Hungary, the numerous cp BVDV isolates have not been molecularly characterised so far. In the first study (paper I), we analysed cytopathogenicity markers in the genomes of six cp BVDV strains isolated in the 1970s, representing various forms of BVD infection, i.e., MD, enteritis and respiratory symptoms (Table 1., paper I). Four of these strains originated from MD (H6379, H6712 and H-BVD MD) and enteritis (H8427) cases, following vaccination with a live attenuated vaccine, termed here for ethical reasons BVDV-X. The additional two cp BVDV strains included in this study (H3887, H3142) were isolated from respiratory disease some years earlier, before the widespread use of the BVDV-X vaccine in the country.

The different cp BVDV isolates and the vaccine strain were propagated and plaque purified. Using the routine diagnostic PCR assay mentioned in the Materials and methods of paper I, we excluded the presence of mixed virus strains in the individual isolates. The most common cytopathogenicity markers of BVDV are insertions at position A and B, in the latter case together with large genome duplications containing an additional copy of NS3. To screen for these genetic cp markers of the Hungarian cp BVDV strains, we first used an RT-PCR assay that detects presence of insertions near to (position A) or at the NS2/3 junction region (position B). An additional PCR assay was carried out to detect duplications of the NS3 gene (Greiser-Wilke et al., 1993). Amplicons indicating the presence of insertions at position A or B and large duplications of the BVDV genome were not detected, indicating that the most common cp markers are not the factors contributing to the cytopathogenicity of these isolates.

4.2 Characteristics of the cytopathogenicity markers (I)

To analyse further the factors possibly leading to cytopathogenicity, the complete NS2-3 coding region of the six cp BVDV isolates and of the vaccine virus were sequenced. The analysis showed that the complete NS2-3 region of the vaccine virus and the three MD and the one enteritis isolates were identical (Figs. 1 and 2, paper I). This strongly suggests that the observed MD and enteritis cases were caused by BVDV-X vaccine superinfection of animals persistently infected with an ncp BVDV strain closely related to the vaccine strain, leading to early onset of MD, as it was described previously in several cases (Brownlie et al., 1984; Bolin et al., 1985a). Sequencing of the 5'UTR and E2 regions showed that in these regions the sequences of the above-mentioned isolates and of the vaccine strain are identical, this further confirmed that after MD and enteritis cases, the vaccine virus was re-isolated. Unfortunately, at the time of the isolation of the above-mentioned cp BVDV strains, the exact pathogenesis of MD was not elucidated, thus recovery of the ncp counterparts of these isolates was not attempted, therefore they were not available for genetic comparisons.

Sequencing the whole NS2-3 gene of the above-mentioned four strains and the vaccine showed that the genetic factor that possibly contributes to their cytopathogenicity is a 45-nucleotide-long insertion at nucleotide position 4355, further upstream of position A. Searches for similarities in the GenBank showed that this insertion was located originally at the NS4B/NS5A junction region, and surprisingly was inserted in the NS2 gene in-frame. The found novel cytopathogenicity marker represents, to our knowledge, the first viral insertion of the NS4B/NS5A junction region described in the NS2-coding region of a cp genome (Figs. 1 and 3, paper I). So far, only in one cp BVDV strain, BVDV CP7 (Tautz et al., 1996) was viral sequence duplication identified at position 4355,

but this duplication proved to be a non-in-frame 27-nucleotide-long viral sequence deriving from the NS2. The 45-nucleotide duplication includes the NS4B/NS5A junction, which is surprising, since in its normal context, this junction site is cleaved by the NS3 serine protease. In all cp BVDV strains, the NS2-3 cleavage has been established to occur at the N-terminus of the NS3. Whether the NS4B/NS5A junction in this insertion introduces an additional cleavage site within the NS2 was elucidated in further studies on expression of NS3 in these strains.

The insertions found in the two respiratory cp BVDV isolates proved to be different from the above-mentioned viral duplications. So far, insertions described at position 4355 consisted of small duplications of sequences of viral origin (Tautz et al., 1996). In contrast, in strain H3887, a 21-nucleotide insertion was found that represents the first non-viral insertion identified at this position of NS2 (Figs. 1 and 2, paper I). The inserted sequence showed the closest homology to a murine interferon-induced guanylate-binding protein 1 gene, but the bovine counterpart of this gene was not found in the GenBank. Cellular (Jiv) insertions in the NS2 genomic region, characterised so far, are presumed to induce conformational changes in this protein, thereby activating viral protease (Mendez et al., 1998). The possible role of this foreign insertion in cytopathogenicity of isolate H3887 is presently being determined.

A third type of cytopathogenicity markers was found in the NS2 gene of H3142 strain. The 42-nucleotide-long insertion was found six nucleotides downstream, but nevertheless in the vicinity of position 4355. The in-frame inserted sequence comprised 42 nucleotides deriving from the middle part of the NS5B gene. Also, one codon was deleted 93 nucleotides downstream of this insertion, but whether this deletion takes part in the mechanism of cytopathogenicity of this virus (in combination with the insertion) is not known (Figs. 1, 2 and 3, paper I). The finding is without precedent in studies of cp BVDV genomes.

All the above-described insertions concerned the same area of the NS2 gene. Since an increasing number of insertions of viral origin are found at this location of cp BVDV genomes, position 4355 may represent a hot spot for recombination of viral and cellular sequences. Maybe, if more cp BVDV strains are identified with cytopathogenicity markers at this position, this genomic region can be considered as the third position (putative position C) where recombinations leading to cp phenotype of BVDV can occur. Nevertheless, the obtained results showed the occurrence of different types of viral sequence rearrangements and novel cellular insertions in the genomes of cp isolates of BVDV, revealing more sides of the complex issue of cytopathogenicity in this virus.

4.3 Detection of cytopathogenicity markers in recently isolated Hungarian cp BVDV strains (II)

The widespread use of the BVDV-X vaccine from the early 1970s to the mid 1990s contributed to the “positive effect” of causing MD in PI animals, reducing the natural source of BVDV. This may explain the phenomenon that cp BVDV strains were not isolated for nearly 30 years. After characterising the cytopathogenicity markers of all the archive cp BVDV strains, we focused on the newly emerged cp BVDV strains. Therefore, the genetic alterations, which can contribute to the cytopathogenicity of two cp BVDV strains (H4956 and H115/PCR), isolated recently from MD cases in Hungary, were examined. Since the regulations do not allow the use of live attenuated vaccines against BVDV, the role of vaccination to induce MD in these cases was excluded. Unfortunately, efforts to isolate the ncp counterpart of these cp viruses failed. Ncp BVDV strains isolated at the same herds proved to be antigenically different from the cp variants (Bálint, unpublished), which can be the consequence of frequent cattle purchase. A further goal was to compare the genetic alterations with those observed in the genome of the cp strains previously described in paper I. The isolates were propagated, plaque-purified, checked by RT-PCR to detect the possible presence of mixed populations, as described earlier. To screen for genetic cp markers, we first used the RT-PCR assay that detects presence of large insertions in the NS2-3 junction region, or of duplications of the NS3 gene that was mentioned in Paper I. PCR detecting insertions

resulted in a product from strain H4956 that showed a size shift of 801 bp, whereas amplification of strain H115/PCR resulted in the product size of 402 bp, characteristic either of the ncp or cp BVDV isolates without sequence alteration in this region (Fig. 1, paper II). However, PCR detecting duplications resulted in product of 1212 bp from strain H115/PCR, indicating the duplication of the NS3 gene, whereas strain H4956 did not show signs of NS3 duplication (Fig. 1, paper II).

4.4 Characteristics of the cytopathogenicity markers (II)

In the genome of cp BVDV strain H4956, a 133-amino-acid-long sequence of a cell-derived insert Jiv, previously termed cIns, was identified at position A (Figs 2 and 4, paper II), that was similar to the insertions found in several cp BVDV and BDV strains, (Becher et al., 1996; Ridpath and Neill, 2000; Vilcek et al., 2000; Nagai et al., 2003). The Jiv transcript is expressed at low level in bovine cells, while the expression level increases following infection with a ncp strain of BVDV. The increased amount of the transcript can serve as a template of recombination with the genome of ncp BVDV. The cellular insert of BVDV strain NADL located 9 bases downstream of that of strain H4956. In the majority of Jiv-containing BVDV 1 isolates, the insertion is located further downstream. The location of the insert of H4956 was the closest to the insert positions of Southern African isolates Mo6 and SA1 (Baule, unpublished result), where the insertion resided one basis upstream of that of the H4956 strain (Fig. 3, paper II). The extreme heterogeneity of termini of the inserts as well as that of the flanking viral sequences, described so far, suggest the possible role of the secondary RNA structure in recombination. Our finding confirms this observation, since the ends of the insert and the flanking viral sequences are different from those the other published sequences (Fig. 3, paper II). A Jiv insertion with a length of 90 codons was shown to be essential for NS2-3 cleavage (Mendez et al., 1998). The 133-amino-acid-long insertion of strain H4956 fulfilled this requirement, containing all the 90 amino acids of the Jiv90. The amino acid identity with the cellular Jiv protein was found to be 96%. One base substitution of valine instead of methionine at position corresponding to amino acid 546 of the cellular Jiv protein (Rinck et al., 2001) was found, but this substitution is present in many cp BVDV strains. However, two additional amino acid substitutions were found: isoleucine instead of phenylalanine at position 570 and proline instead of leucine at position 579 (Fig. 2, paper II). These substitutions are not present in the so-far sequenced Jiv-containing cp BVDV and BDV isolates. The reason for the amino acid substitutions not present in other Jiv containing cp BVDV strains needs further elucidation.

In strain H115/PCR, large sequence duplication, containing an ubiquitin monomer inserted at position B was found. The large duplication started from nucleotide position 7978 in the NS4B gene. In cp BVDV strains with ubiquitin containing sequence duplications, isolated so far, the positions of the 5' border of insertions of ubiquitin are located exclusively in the NS4B gene ranging from 7483 (TGAC) to 7813 (Mo1). Since the 5' border of the duplication in strain H115/PCR is further downstream, our findings further confirmed the observation that the 5' position of recombination is not conserved in this region (Table 2, Fig. 4, paper II), excluding the role of the site-specific recombination as in case of Jiv.

Additional difference besides the 5' border of the inserted ubiquitin of strain H115/PCR is that the duplication contains only one ubiquitin monomer. So far, only in reference strain cp BVDV Osloss was one ubiquitin monomer described as cytopathogenicity marker, but without any sequence duplication. In other related strains, the duplications contain additional complete or truncated ubiquitin monomers or fragments of the NS2 gene (Table 2, paper II).

The reason for the presence of the large insertions in the genome of cp BVDV strains that can increase the length of the genome even by 30%, has been elucidated recently (Agapov et al., 2004). The viral morphogenesis needs the uncleaved form of NS2-3 in the context of NS4A. In the viral life cycle, after translation, the N^{pro} autoprotease cleaves itself from the polyprotein, then the translocation signal sequence of E^{rns} translocates the structural protein-coding region to the lumen of the ER, where the individual proteins are cleaved by cellular signalases. However, the capsid

protein remains to be anchored to the membrane via the translocation signal sequence. The role of the NS2-3 with the NS4A as cofactor is the proteolytic cleavage at the C-terminus of the capsid resulting in the release of this protein. The uncleaved NS2-3 is a membrane-anchored protein, while the cleaved NS3 is less associated to the intracellular membranes, thus it cannot perform this cleavage. Insertions at position B generate 100% cleavage and NS3 release, thus these strains must carry an additional uncleaved NS2-3 protein together with NS4A. This latter phenomenon explains the observations that the duplications start from the NS4B gene.

The results of this study elucidated that there is no connection between the formerly described and the recently isolated Hungarian cp BVDV strains, concerning cp markers. The insertions and duplication of the latter strains further confirm that recombinations occurring at positions A and B are the most common mechanisms leading to the development of BVDV cytopathogenicity, and insertions in position B coexsist with large duplications of the BVDV genome.

4.5 Identification of different viral sequences in different batches of BVDV-X (III)

To control BVDV infections, a live vaccine BVDV-X, was used in Central Europe from the end of the 1960s to the beginning of the 1990s. The vaccine was prepared from a seed stock of the cp BVDV reference strain Oregon C24V that was serially passaged in cell cultures and in animals. Using this vaccine for more than two decades, the vaccination provided sufficient protection against postnatal infections, but from field observations and laboratory diagnoses it was suspected that vaccination of PI animals might have lead to the development of early onset of MD.

In paper I, four cp viruses originating from MD and enteritis cases were analysed for possible cytopathogenicity markers. Nucleotide sequence analysis of the 5'UTR, E2 and the complete NS2-3 region of the cp field isolates and the BVDV-X vaccine strain confirmed the theory of early onset of MD and re-isolation of the vaccine virus in vaccinated animals. Surprisingly, the sequence of the NS2-3 gene of the vaccine virus was found to be different from that of the Oregon C24V strain, the original strain for BVDV-X vaccine development.

To clarify the identity and the origin of the BVDV-X strain, three vaccine batches (one prior to and two after the registration process) were obtained and examined. Analysis of the 5'UTR of an original batch prior to registration (named here as BVDV-Xpre) proved that this genomic region of the master stock was identical with BVDV Oregon C24V. However both batches of the marketed vaccine virus had Osloss-like 5'UTR sequences. To further elucidate the raised questions, the complete nucleotide sequences of the pre-registration batch (BVDV-Xpre) and of one of the marketed vaccine batches (termed BVDV-X) have been determined and compared with published full-length sequences of BVDV strains, including Oregon C24V. Since at the time of the registration of BVDV-X the methods of identifying different BVDV strains were not so developed as at present, our basic goals were the followings: (i) verifying, whether the Oregon C24V strain was the starting point of the BVDV-X vaccine production; (ii) investigating the presence and ratio of Oregon C24V nucleotide sequences in the genome of the marketed vaccine BVDV-X. Our hypothesis was that finding both Oregon C24V and Osloss-like nucleotide sequences in the different batches of the BVDV-X vaccine could reveal that recombination events occurred between the original Oregon C24V strain and a heterologous BVDV virus strain during the numerous passages *in vitro* and *in vivo.*

4.6 Differences between the complete genome of BVDV-Xpre and BVDV-X (III)

The BVDV-Xpre virus proved to be a derivate of the Oregon C24V strain. There were differences at 10 nucleotide positions, but only four of them caused amino acid substitutions (Table II, paper III),

affecting the E1, E2 and NS5A regions. Both the 5' and 3'UTRs were found to be identical to those of the published sequence of Oregon C24V strain. Since the molecular base of attenuation is not clear in the case of BVDV, these data are very useful, because, this is the first full-length sequence of a live BVDV vaccine, and its identical virulent counterpart can be found in the GenBank.

The entire genomic sequence of the live cp BVDV vaccine strain BVDV-X is composed of 12 308 nucleotides. The 5'UTR is 381 nucleotides long, whereas the 3'UTR comprises of 182 nucleotides. The ORF begins at position 382 and ends at position 12126, consists of 11745 nucleotides, which encode a polyprotein of 3915 amino acids.

The nucleotide sequence of the 5'UTR of BVDV-X compared with that of the published sequences of BVDV 1 and BVDV 2 strains revealed nucleotide sequence identity of 83%-98% with the BVDV 1 strains and 65% with the BVDV 2 reference strain 890 (Table III, paper III). The phylogenetic tree derived from the 5'UTR of the vaccine virus and of the published sequences revealed that BVDV-X belongs to subgroup 1b of BVDV. The closest nucleotide similarity, of 98%, was found with strain Osloss, whereas similarity with BVDV Oregon C24V was only of 83% (Fig. 1, paper III).

The nucleotide sequence of the 3'UTR of BVDV-X compared with that of the published sequences of BVDV 1 and BVDV 2 strains revealed nucleotide sequence identity of 64%-95% with BVDV 1 strains and of 49% with the BVDV 2 reference strain 890 (Table III, paper III). The closest nucleotide similarity, of 95%, was found with the 3'UTR of strain Osloss, whereas similarity with BVDV Oregon C24V was only 64%.

The deduced amino acid sequence of the polyprotein of BVDV-X was compared with published sequences of the polyproteins of BVDV 1 and BVDV 2 (Table IV, paper III). The highest amino acid variability was found in the E2 region, 84-91% amino acid identity with BVDV 1 strains and 62% amino acid identity with BVDV 2 reference strain 890. The lowest amino acid variability was found in the NS3 and NS5B regions, 94-98% and 84-97% amino acid identity with BVDV 1 strains and 91% and 79% amino acid identity with BVDV 2 reference strain 890.

To determine whether the genome of BVDV-X contains Oregon C24V-derived sequences, the complete nucleotide sequence of the BVDV-X genome was compared to that of BVDV Oregon C24V, in 200-nucleotide sections. The results showed that there is no region where the nucleotide similarity between the two viruses reaches 100% (Fig. 2, paper III). The highest nucleotide similarity, of 89%, was found in the NS3 region, whereas the lowest similarities of 62.5% and 63.5% were found in the N-terminal part of E2 and in the variable region 3'V of the 3'UTR, respectively. The average nucleotide similarity was found to be of 77.4% between BVDV-X and BVDV Oregon, whereas it was significantly higher, of 93.7%, between BVDV-X and BVDV strain Osloss. The genome scanning also revealed that cytopathogenicity markers, except for the 45-nucleotide insertion, are not present in the genome of BVDV-X.

Summarising the data of the full-length sequence of BVDV-Xpre proved that the original strain used for development of BVDV-X was Oregon C24V. Since correct documentation of this batch is not available, it is not known, how many *in vitro* and *in vivo* passages it has undergone, thus the role of the detected sequence differences in viral attenuation -mainly in the E1 and E2 regions- cannot be easily determined.

In contrast, sequence data of the genomic sequence of BVDV-X revealed that this virus is a BVDV 1b variant, that showed the highest average sequence identity of 93.7% to reference BVDV strain Osloss, whereas the average sequence identity with BVDV Oregon C24V was sigificantly lower, of 77.4%, and the detailed genome scanning revealed that the genome of BVDV-X did not contain Oregon C24V sequence. This indicates that BVDV-X did not derive from Oregon C24V, but it might have originated from a pickup of an exogenous cp BVDV strain from the *in vitro* or *in vivo* steps of passaging. It is possible that an animal used for the *in vivo* passaging was infected with exogenous cp BVDV, which was later re-isolated and used for vaccine production. A miss-labeling of tubes is another possibility to be taken into account. It has to be emphasised that in the historic years, when BVDV-X was used as vaccine, unequivocal methods for strain identification had not

still been developed, neither panels of monoclonal antibodies, nor molecular tools, like nucleotide or amino acid sequencing were available. Thus, the means to detect a virus-switch or a virus pickup were limited at that time. The comparative genome analysis in the present study revealed the surprising fact that BVDV-X contained a virus very different from BVDV-Xpre. Considering the major differences in the genomes of the two viruses, it is likely that not viral recombination, but a pickup of a foreign virus occurred in this case. The vaccine control data indicate that similarly to the attenuated Oregon C24V, the uptaken foreign virus was also attenuated, harmless and immunogenic. This could be the reason that despite the virus-switch, the BVDV-X vaccine continuously maintained its innocuity and efficacy, as proven by regular quality testings, and the presence of the foreign virus remained unnoticed over many years.

The unchanged safety and efficacy of the vaccine can be considered a lucky scenario, since picking up of a new virus during passaging could have lead to very serious consequences. Contamination of products, like vaccines or FCS, is a severe risk in the biological industry (Vilcek et al., 1998). Besides the above listed dangers, it is interesting to note that in the present case the strain-switch may probably have had some “positive” practical effects. In a recent study we found that the Osloss-like virus of the BVDV-X vaccine was closely related to the cp BVDV strains isolated in the region of vaccination during the early 1970s (paper I). The closer relation emphasises that the BVDV-X immunisation presumably gave a stronger protection against the local Central European field isolates than the American Oregon C24V variant would have provided.

The results of this study emphasise the necessity of more precise quality testing of commercially available live virus vaccines, including monoclonal antibody analysis and sequencing, to verify their authenticity. A further outcome of this work is that the full-length nucleotide sequence of a BVDV strain, BVDV-X has been determined, and this information will hopefully provide additional data to the panel of *pestivirus* biology and vaccinology.

4.7 Expression studies on the NS2-3 gene of BVDV-X (I)

Sequencing of the whole genome of the BVDV-X vaccine confirmed that further genetic patterns besides the 45-nucleotide insertion possibly contributing to cytopathogenicity of this strain are not present in the genome, therefore the role of the 45-nucleotide insertion in the NS2/3 processing was examined. The whole NS2-3 gene of the BVDV-X vaccine was cloned into the pCI mammalian expression vector (Promega, Madison, WI, USA), and an insertion-negative mutant was also constructed by using PCR-directed mutagenesis. BT cells were transfected with both constructs, and the processing of NS2-3 was examined with Western blot. Transient expression studies with the complete NS2-3 from BVDV-X construct revealed that the small insertion alone can contribute to partial cleavage of NS2-3, leading to the expression of the 80 KDa NS3, the marker protein of cp BVDV strains, while the insertion-negative mutant resulted in the expression of the uncleaved 125 kDa NS2-3 protein (Fig. 4, paper I). The studies also revealed that the insertion generated partial cleavage of the NS2-3 protein, allowing the uncleaved NS2-3 to act in viral morphogenesis. Furthermore, the NS2-3 of BVDV-X is not cleaved at the NS4B/NS5A junction site, this further confirms that processing at the exact N-terminal of NS3 is the prerequisite of efficient BVDV replication (Tautz and Thiel, 2003).

4.8 Reverse genetic studies for confirmation of the cytopathogenicity marker of BVDV-X (IV)

Reverse genetic techniques are invaluable tools in specific manipulating of viral genomes, which contribute to the better understanding of the biology of different viruses. In the next study these reverse genetic tecniques were used to give a final proof that the 45-nucleotide insertion is not only responsible for NS2-3 cleavage, but also for the cytopathogenicity of BVDV-X.

The selected strategy for recovery of the recombinant BVDV-X virus based on the transfection of BT cells with the complete, genomic-like RNA of BVDV-X, that was generated *in vitro* by run-off transcription from full-length cDNA construct with T7 RNA polymerase described originally by Boyer and Haenni (1994). Clarifying the molecular basis of cytopathogenicity of different cp BVDV strains have been performed in several laboratories by using full-length infectious cDNA clones (Meyers et al., 1996; Vassilev et al., 1997; Mendez et al., 1998; Kümmerer and Meyers, 2000). By using such a cDNA clone of BVDV reference strain BVDV CP7, the crucial role of the 27-nucleotide insertion in cytopathogenicity of BVDV CP7 has been demonstrated (Meyers et al., 1996), thus following similar molecular approaches, two main aims were set up in the next study: i) construction of a full-length infectious cDNA clone of BVDV-X and characterisation of the recovered virus; ii) deletion of the insertion in context of the full-length clone to prove that the insertion-negative rescued virus switches to the ncp phenotype.

The details of construction of the full-length clone are described in the Methods chapter of paper IV. Briefly: in the first step long PCR products overlapping the whole BVDV-X genome were amplified and cloned separately into a low copy number plasmid (Fig. 1A, paper IV). The clones were subsequenly joined to obtain the full-length cDNA clone of the virus, pBVDV-XR (Fig. 1B, paper IV). A T7 RNA polymerase promoter site was inserted upstream of the 5' end of the genome, and a *SmaI* restriction enzyme site was included at the 3' end of the genome to linearise the plasmid and form the exact 3' end of the genome. A very careful cloning and subcloning strategy was selected to avoid the instability problems of the large clones: a highly proofreading enzyme was applied in the PCR assays, a widely used low-copy-number plasmid, pANCR1180 (Ruggli et al., 1996) and ElectroTen Blue recombinant *E. coli* competent cells (Stratagene, La Jolla, CA, USA), specially designed for the propagation of large plasmids were used.

4.9 Characterisation of the infectious virus recovered from the transcribed RNA of pBVDV-XR (IV)

Since BVDV-X is a cp BVDV strain, transfection of infectious viral RNA should result in the formation of plaques. In BT cells transfected with pBVDV-XR derived RNA, CPE was first observed at about 48 h post-transfection, and became advanced by 72 h post-transfection (not shown). Plaque assay of the first passage of the recovered virus, BVDV-XR showed that the recovered virus has cp phenotype (Fig. 2A, paper IV).

Identification of the recombinant virus was carried out by using antigen and nucleic acid detection assays. The IPX staining showed advanced cytoplasmic staining in BT cells infected with the first passage of BVDV-XR at 48 h p.i. (Fig. 2B, paper IV), showing that the recombinant virus is BVDV. In contrast, non-transfected BT cells did not show staining.

Two different RT-PCR assays targeting the 5'UTR-E^{rns} and NS2 regions confirmed that BVDV-XR contains of BVDV-X-specific nucleic acid (Fig. 3, paper IV). To exclude the possibility of carryover of plasmid DNA, the same PCR reactions were repeated without reverse transcription, and no PCR product was obtained. In addition, no RT-PCR products were obtained from the uninfected cell control (not shown).

Analysis of the growth characteristics of the recovered virus and the parent BVDV-X virus by plaque assay revealed that the first signs of CPE occurred in both cases at 24 h p.i, and became became advanced by 72 h. The recombinant virus reached a titre of $4x10^6$ PFU/ml, while the titre of the parent virus was $2x10^7$ PFU/ml at the same time point as shown in the growth curve in Fig. 4, paper IV.

The reason for this finding is probably the appearance of four amino acid substitutions in the polyprotein of BVDV-XR (E2, NS4B, NS5A, NS5B) found after sequencing of the full-length clone. This phenomenon is in accordance with the data of previous full-length cDNA clones, as the

amplification and cloning steps may result in introducing mutations (Meyers et al., 1996; Vassilev et al., 1997), but the recombinant virus is appropriate for the further reverse genetic studies.

4.10 *In vitro* generation of noncytopathogenic BVDV (IV)

Unfortunately, the natural ncp counterpart of the vaccine virus, necessary for the further examinations, was not available. Therefore an insertion-negative mutant full-length infectious clone, namely pBVDV-XR-INS-, was constructed from the clone pBVDV-XR by using PCR-directed mutagenesis. The only difference at genomic level between the original infectious clone and the inserion-negative mutant was the absence of the 45-nucleotide insertion in the latter one. The absence of the insertion in pBVDV-XR-INS- was demonstrated by a PCR amplification reaction with primers flanking the insertion (Fig. 3, paper IV). After transfection of BT cells with RNA transcribed from the insertion-negative mutant, CPE was not detected (Fig 2A, Paper IV). The mutant virus, BVDV-XR-INS- kept its ncp characteristics after the third passage. However, IPX analysis with polyclonal antibodies clearly showed foci of positive cells (Fig. 2B, paper IV). These data confirmed that removal of the 45-nucleotide insertion switched the originally cp BVDV-X to an ncp virus. Furthermore, BVDV-XR-INS- showed the same growth characteristics as its cp counterpart (Fig. 4, paper IV). These data confirmed that the removal of the 45-nucleotide insertion switched the originally cp BVDV-X to an ncp virus. This is indicating that the 45-nucleotide insertion is responsible for the cytopathogenicity of BVDV-X.

So far, together with the results of this thesis, one cellular and four viral insertions have been found in the genomes of different cp BVDV isolates, located at nucleotide 4355. The development of these cytopathogenicity markers has not yet been elucidated. One possible exlplanation is that position 4355 may represent a hot spot for recombination of viral and cellular sequences. On the other hand, a functional selection of the BVDV genomes containing insertions at this particular position as a result of “productive recombinations” cannot be excluded. The cytopathogenic variant of BVDV generated by recombination events at nucleotide position 4355 may have considerable advantage for replication in the animal, therefore may be selected from the pool of other recombinants.

At present, the exact role of insertions at position 4355 in the NS2 gene of cp BVDV strains is not known. A recently found cysteine protease generates efficient NS3-3 cleavage in both cp and ncp BVDV, but the autoproteolysis is downregulated at 9 h p.i. It is hypothesised that the insertion found in CP7 interferes with the downregulation process by making the autoproteolysis less sensitive to cofactors (Lackner et al., 2004).

In summary, together with the results of this study, by using full-length infectious cDNA clones, the crucial role of two different viral insertions at position 4355 in cytopathogenicity of BVDV strains CP7 and BVDV-X has been established, respectively. The pBVDV-XR plasmid backbone provides available tool to examine the possible role of the additional two insertions at position 4355 in the genomes of Hungarian cp BVDV isolates originating from respiratory disease cases, being genetically very close to these strains. All the insertions found at position 4355, although vary in size and nucleotide composition, very likely act in a similar way. By the use of full-length cDNA clones, further experiments will determine their common mechanism that contributes to the maintaining of the cp phenotype of BVDV.

5 New scientific results

1. The studies revealed that three different, previously unknown small insertions of viral and cellular origin can possibly contribute to the cytopathogenicity of cp BVDV strains isolated in the 1970s and of the live attenuated vaccine BVDV-X.
2. In the genome of two cp BVDV strains isolated recently from MD cases, two different, but similar to the earlier described cytopathogenicity markers were found, and proved that recombinations leading to cytopathogenicity of BVDV frequently occur at positions A and B.
3. The whole genome of the pre-registration vaccine batch BVDV-Xpre was fully sequenced, which is the first fully sequenced attenuated BVDV vaccine, and its virulent counterpart can be found in the GenBank. Furthermore, it was proven that the strain used for vaccine production was Oregon C24V.
4. The whole genome of BVDV-X was sequenced, and thorough genome scanning revealed that the marketed vaccine did not contain Oregon C24V sequences, indicating the strain-switch during vaccine production. Furthermore, the complete genome sequencing proved that the marketed vaccine belongs to BVDV 1b subgroup and has the closest similarity to reference BVDV strain Osloss.
5. Other possible factors contributing to cytopathogenicity were not found in the genome of BVDV-X. Expression studies of the NS2-3 gene of BVDV-X revealed that the possible cytopathogenicity marker induced expression of NS3, the marker protein of BVDV cytopathogenicity, and the NS4B/NS5A junction region included in the 45-nucleotide insertion is not cleaved in this case.
6. Construction and mutation of the full-length infectious clone of BVDV-X proved that the 45-nucleotide viral insertion is responsible for the cytopathogenicity of BVDV-X. The backbone of the cDNA clone will be a useful tool in examining the role of the remaining two newly found viral and cellular insertions in BVDV cytopathogenicity.

General conclusion

The tools of molecular virology provided novel means to perform retrospective study on the scenario around the application of a live BVDV vaccine several decades ago. In addition, new aspects were obtained concerning the genetic background of cytopathogenicity of BVDV, and a full-length infectious clone has been constructed. These achievements are useful for the characterisation of *pestivirus*es, and for the development of more effective means against BVDV infections.

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Scientific publications of the thesis

I. Bálint, Á., Pálfi, V., Belák, S., Baule, C., 2005. Viral sequence insertions and a novel cellular insertion in the NS2 gene of cytopathic isolates of bovine viral diarrhea virus as potential cytopathogenicity markers. *Virus Genes* **30:** 49-58.

II. Bálint, Á., Baule, C., Kiss, I., Kecskeméti, S., Belák, S., 2005. Cytopathogenicity markers in the genome of Hungarian cytopathic isolates of bovine viral diarrhoea virus. *Acta Veterinaria Hungarica* **53:** 125-136.

III. Bálint, Á., Baule, C., Pálfi, V., Belák, S., 2005. Retrospective genome analysis of a live vaccine strain of bovine viral diarrhea virus. *Veterinary Research* **36:** 89-99.

IV. Bálint, Á., Baule, C., Pálfi, V., Belák, S. A 45-bp insertion in the NS2 gene is responsible for the cytopathogenicity of bovine viral diarrhoea virus. *Virus Genes*, accepted for publication.

V. Bálint, Á., Kiss, I., Belák, S. New data on the molecular biology of bovine viral diarrhoea virus (BVDV). *Magy. Áo. Lapja,* accepted for publication.

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I

Viral sequence insertions and a novel cellular insertion in the NS2 gene of cytopathic isolates of bovine viral diarrhea virus as potential cytopathogenicity markers

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Running Title: Insertions in the NS2 gene of BVDV

Key words: Bovine viral diarrhea virus, cytopathogenicity, markers, NS2-3, NS3, vaccine

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Abstract.

Cytopathogenicity of bovine viral diarrhea virus (BVDV) has been shown to correlate with the presence of insertions of cellular sequences, duplication of viral sequences with or without insertions, deletions and point mutations in the genomes of cytopathogenic (cp) strains. In the present study we have investigated cytopathogenicity markers in the genomes of six cp BVDV isolates. The viruses were selected as representatives of various forms of BVDV infection, presumably induced by vaccination with a live attenuated vaccine. The complete NS2-3 coding region of the six isolates and of the vaccine virus were amplified by reverse transcription-polymerase chain reaction (RT-PCR) and sequenced. In the genomes of four isolates (H6379, H6712, H8427 and H-BVD MD) and of the vaccine virus, a 45-nucleotide viral insertion was found at nucleotide position 4355. The insertion encompassed nucleotides 8402-8446, encoding 15 amino acids of the NS4B/NS5A junction region in a normal BVDV genome. Isolate H3887 had a 21-nucleotide insertion of non-viral origin, also located at nucleotide position 4355. The insertion has a high homology with a gene coding for murine interferon-induced guanylate-binding protein 1, and represents the first non-viral insertion identified at this position of the NS2 coding region. Isolate H3142 carries a 42-nucleotide insertion at nucleotide position 4361, which is identical to a part of the NS5B gene mapping to position 11078-11119. Besides the insertion, this isolate also has a deletion of three nucleotides (position 4448-4450). The role of the 45-nucleotide insertion in expression of NS3 was investigated for the vaccine virus. The NS2-3 gene of this virus, and a generated insertion-negative variant were cloned in pCI and expressed in bovine turbinate cells. Western blot analysis revealed that the insertion contributed to a partial cleavage of NS2-3 generating NS3. The genome rearrangements found in these isolates occurred preferentially at position 4355, suggesting that this part of the genome could represent a potential hot spot for recombination events in ncp BVDV. Insertions at this position lead to generation of NS3, the marker protein for cytopathogenicity, as has already been demonstrated for cp BVDV strain CP7. The molecular mechanism underlying this phenomenon remains to be elucidated.

Bovine viral diarrhea virus (BVDV) is a member of the *Pestivirus* genus, which together with the genera *Flavivirus* and *Hepacivirus*, forms the *Flaviviridae* family [1, 2, 3]. The genome is a positive-stranded RNA of about 12.5 kb in size, which comprises a single open reading frame (ORF) flanked at the 5´ and 3´ ends by untranslated regions (UTRs) [4, 5, 6]. The 5´UTR functions as an internal ribosome entry site (IRES) that promotes cap-independent translation initiation [7, 8]. The ORF encodes a large polyprotein of approximately 4000 amino acids that is co- and post-translationally processed into 11 or 12 mature viral proteins: NH_2-N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A, NS5B-COOH [2, 3].

According to their ability to cause cytopathic effect (CPE) in cell cultures, BVDV strains are classified as cytopathogenic (cp) or noncytopathogenic (ncp) biotypes [9, 10]. Depending on the stage of gestation, infection of pregnant cattle with ncp BVDV, may result in embryonic death, foetal resorption, abortions, stillbirths, or in birth of persistently infected (PI) calves. PI animals are immunotolerant to the persisting ncp virus and are predisposed to develop mucosal disease (MD), a fatal form of infection [11, 12, 13]. MD can be induced either by superinfection of the PI animal with an antigenically closely related cp BVDV or generated *de novo* from a ncp virus in a PI animal [11, 12, 14]. Both a cp and the persisting ncp biotypes can be co-isolated from animals succumbing to MD [15]. Such isolates are called a ” virus pair”.

The molecular analysis of different BVDV pairs elucidated that cp BVDV strains evolved *in vivo* from ncp BVDV by RNA recombination in cattle persistently infected with ncp BVDV [14, 16]. These recombination events result in cellular insertions, duplications, rearrangements or deletions of viral genome sequences [16, 17, 18, 19]. However, some cp BVDV strains show no recombination-induced genome alterations [20, 21]. Analysis of such a strain, BVDV Oregon, demonstrated a relation between point mutations in the NS2 region and cytopathogenicity [22, 23]. These changes in the viral genomes of cp BVDV strains lead to expression of the nonstructural protein NS3 that is responsible for the development CPE [24]. Most recently, the possible role of the NS4B protein in pestivirus cytopathogenicity was also described. Mutations in this protein were shown to suppress CPE, regardless of NS3 production [25]. In the majority of cp BVDV strains, the mechanism leading to production of NS3 has been identified [16]. However, for a number of strains containing point mutations [22, 23], cellular or viral [27] insertions in the NS2 gene upstream of the NS2/3 junction, the exact mechanisms leading to cytopathogenicity is still elusive and deserves further investigations.

The aim of the present study was to investigate cytopathogenicity markers in the genomes of six cp BVDV strains isolated in Hungary in the 1970s, representing various forms of BVDV infection, i.e., MD, enteritis and respiratory symptoms (Pálfi, personal communication). Four of these strains (H6379, H6712, H8427 and H-BVD MD) originated from MD and enteritis cases, following vaccination with a live attenuated vaccine, termed here for ethical reasons as BVDV-X. This vaccine was widely used in large cattle farms in Hungary from the end of the 1960s for nearly 30 years to control BVD. The vaccine provided sufficient protection against congenital and postnatal infections [28], but it was suspected to have led to the development of MD upon vaccination of persistently infected animals. Such a phenomenon, i.e., recombination between a live vaccine strain and the persisting BVDV strain(s) leading to early [29, 30] or late onset of MD [31] has been described in several cases in the USA and in Germany. The additional two cp BVDV stains included in this study (H3887, H3142) were isolated from respiratory disease, some years earlier, before the widespread use of the BVDV-X vaccine in Hungary.

Materials and methods

Cells and viruses

Secondary bovine turbinate (BT) cells were cultured in Eagle´s Minimum Essential Medium (EMEM) supplemented with 2 mM of L-glutamine and 10% foetal calf serum (FCS). Following the routine procedures of our institute, cells and each batch of FCS were tested regularly for the absence of pestiviruses by reverse transcription-PCR (RT-PCR) [32] and immunoperoxidase assay (IPX) using BVDV polyclonal antiserum (VLA, Addlestone, UK). For FCS, the absence of anti-pestivirus antibodies was shown by lack of virus neutralization using the standard procedures of our institutes.

The BVDV-X modified live vaccine was purchased from the supplier. Six cp BVDV isolates from cattle, listed in Table 1, were selected as representatives of various forms of BVDV-induced disease, i.e. enteric and respiratory syndromes, as well as MD, listed in Table 1. Isolates H8427 was isolated from an animal with enteritis and isolates H6379, H6712 and H-BVD MD were isolated from cattle that developed MD following vaccination with the BVDV-X vaccine. Isolates H3887 and H3142 originated from cattle with respiratory disease.

Preparation of virus stocks

To obtain pure virus stocks, each isolate and two different batches of the vaccine virus were plaque purified by the standard methods. Briefly, 80% confluent cell monolayers in 6 well tissue culture plates were inoculated with ten-fold dilutions of the respective virus isolates. After one hour adsorption, the cell monolayers were washed and then overlaid with low melting agarose (Sigma) containing EMEM and 2% FCS, followed by incubation at 37˚C in an atmosphere of 5% CO_2 for 72 hours. Five plaques of each isolate were picked and propagated in fresh BT cells. Virus particles of the pure stocks were pelleted from supernatants of infected BT cells by ultracentrifugation (Airfuge; Beckman Instruments), at 55 000 rpm for three hours, and resuspended in PBS without Ca^{++} and Mg^{++}. Virus stocks were kept at -70˚C until use.

Reverse-transcription polymerase chain reaction (RT-PCR) for detection of insertions and duplications

Total RNA was extracted using the TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. The cDNA synthesis was carried out with M-MLV RT enzyme (Invitrogen) and random hexanucleotides (Amersham Biosciences). PCR amplification was performed as described [33], using primer sets for detection of insertions (Primers A and C) and duplications (Primers B and C). PCR products were separated on 2% agarose gels stained with ethidium bromide and visualised under UV light (UVP). The amplicons were purified using microcolums (QIAquick gel extraction kit, Qiagen), according to the manufacturer´s instructions. The purified DNA products were sequenced from both directions with the same primers as for amplification reactions with an ABI Prism sequencer (Applied Biosystems, Model 377), using the Big Dye Terminator V3.0 sequencing kit (Applied Biosystems).

RT-PCR for generation of amplicons encompassing the complete NS2-3 coding region

To obtain long cDNA copies of the viral genome, the Superscript II reverse transcriptase system (Invitrogen) and an antisense primer VDAS1 ($5'^{12242}$- CTGTGTGCA TTRARTGTAGTGTT-$3'^{12220}$), located in the 3´UTR of BVDV, was used. The transcription was performed in 20 µl reaction mixtures containing 250 ng RNA, 20 pmole primer, 200 µM of each dNTP, 24 U of RNAse inhibitor (Amersham Biosciences) and 200 U of Superscript II.

For the amplification of the whole NS2-3 region, primers 125AF (5’^{3320}-GAGGGG CCGGTAGAAAAGAC-3’^{3339}) and 125BR (5’^{7323}-GCATAYTGGAGGTGGGTK GTGT-3’^{7302}) were used (all nucleotide positions refer to the genome of the non-cytopathogenic strain SD-1). The reactions were prepared in a 50 µl total volume, containing 1X buffer for a final 2.25 mM $MgCl_2$ concentration, 200 µM of each dNTP, 20 pmole of each primer, 5 µl of cDNA and 2.5 U of Expand polymerase mix (Roche). After a 2 min initial denaturation, 35 cycles were performed with the following parameters: 45 sec at 94˚C, 45 sec at 52˚C and 4 min at 68˚C. A final extension at 68˚C for 10 min was included.

The 4 Kb PCR products were separated on 0.8% agarose gels stained with ethidium bromide and visualised under UV light (UVP). Purification and sequencing of PCR products were carried out as described above. Initial sequencing was performed with the same primers used to generate the amplicons. Further oligonucleotides were designed based on these sequence data to complete the whole NS2-3 sequence. The sequences of oligonucleotides used are available upon request. For each isolate, two independent PCR products were sequenced. Where differences were found, a third independent PCR product was generated and also sequenced to resolve the consensus sequence.

Sequence analysis

Nucleotide sequences of the PCR products were edited and analyzed with multiple programs of the DNASTAR software package (Lasergene). Search for sequence similarities in GenBank were performed using the BLAST programs (The National Center for Biotechnology Information).

Construction of plasmids expressing the NS2-3 and NS2-3INS$^-$ genes of BVDV-X

Based on the determined sequence of the NS2-3 gene of the BVDV-X, primers were designed to amplify the NS2-3 gene for cloning. The forward primer NS23V1F (5’^{3770}-AT*CTCGAG*ATGGTTGGAGGAATGGCAAG-3’^{3789}) contained an *Xho*I site, while the reverse primer NS23V1R (5’^{7201}-GC*TCTAGA*TCACAGTCCTACCACTTGCT-3’^{7182}) included an *Xba*I restriction site extension at the 5’end. The PCR fragment was generated with the Expand High Fidelity PCR System, using the reaction and cycling conditions described above. The DNA fragment was purified, cleaved with *Xho*I and *Xba*I (New England Biolabs) and cloned into a similarly cleaved pCI mammalian expression vector (Promega) according to standard procedures [34]. The resulting clone was designated pNS2-3. Since the ncp variant of the BVDV-X virus was not available, we generated an expression vector where the insertion in the NS2-3 gene was deleted to mimic the ncp equivalent NS2-3. For this purpose, the pNS2-3 clone was used as template to amplify a PCR product with primers NS23V1F and INSR (5’^{4374}-TTGTAGTCCACCCCCCCTAGCCAACTCTTTTCCACATCAG-3’^{4335}). The INSR primer contained at the 5’ end 20 nucleotides directly downstream of the insertion, followed by the 20 nucleotides upstream of the insertion. An additional PCR product was generated with INSF (5’^{4335}-CTGATGTGGAAAAGAGTTGGC TAGGGGGGGTGGACTACAA-3’^{4374}) and NS23R primers using the same template. The INSF primer was the reverse complement of the INSR primer. A final joining PCR was then carried out using primers NS3V1F and NS3V1R, and the two above-mentioned PCR fragments as templates (Fig. 4A). The PCR product was subsequently cloned as described above, and was designated pNS2-3INS$^-$.

Transfection of cells with pNS2-3 and pNS2-3INS$^-$, SDS-PAGE and Western blot analysis

Bovine turbinate cells were trypsinised and washed with ice-cold HEPES buffered saline (HeBS). $2x10^6$ cells were suspended in 400 µl HeBS and subsequently mixed with 2 µg of the respective plasmids, followed by an incubation on ice for 5 min. Cell transfection was done by electroporation in 0.4 cm cuvettes at 260V and 500 µF using a Gene Pulser II electroporator (Bio-Rad). The cells were resuspended in 3 ml EMEM containing 5% FCS, seeded in 6 well tissue culture plates and

incubated for 48 hours at 37ºC in 5% CO_2. The cells were scraped from the bottom of the wells, centrifuged at 10 000 rpm for 10 minutes, washed with ice-cold PBS and resuspended in 50 µl PBS. The suspension was mixed with 50 µl of 2x sample loading buffer (0.1M Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 1M DTT) and boiled for 5 min. Samples of 20 µl were run in 10% SDS-PAGE gel. Western blotting was carried out with standard methods [34], using monoclonal antibodies against the NS3 protein as primary antibodies (WB435, WB440, Addlestone, UK), and a rabbit anti-mouse serum (Dako-Cytomation) as secondary antibody.

Results

Analysis of the NS2-3 gene for insertions and duplications, using RT-PCR

Primers A and C amplify a 402 base pair (bp) product in the NS2-3 gene of ncp strains and in that of cp strains without insertions, whereas a shift in product size is seen if the strain contains an insertion in the NS2-3 junction [33]. The PCR products from all the analyzed isolates had an estimated size of 402 bp. Size alterations indicative of large insertions in the NS2-3 junction region of the viral genomes were not observed. Primers B and C generate a PCR product exclusively when a duplicated NS3 sequence is present downstream the first NS3. PCR products were not obtained from the analyzed isolates, indicating the lack of duplications of the NS3 region of their viral genomes.

Sequence analysis of the complete NS2-3 gene of cp BVDV isolates

The complete NS2-3 gene regions of the six cp BVDV isolates and of the vaccine strain were analyzed for sequence alterations that could be responsible for the cp phenotype. The nucleotide as well as the deduced amino acid (aa) sequences of the vaccine strain and of four strains isolated from cattle after vaccination, namely H6379, H6712, H8427 and H-BVD MD proved to be identical in the whole NS2-3 gene (Figs. 1A and 2). Similarity searches revealed 100% homology between the variable C-terminal part of the NS2 gene of the Changchun 184 cp BVDV strain [35] and the above-mentioned Hungarian isolates (not shown). Furthermore, in these strains, a 45-nucleotide insertion was found in the NS2 gene at nucleotide position 4355, that was identical a the viral sequence of the NS4B-NS5A junction region encompassing nucleotides 8402-8446 (Figs. 1B and 3). The deduced amino acid sequence of the insertion showed that the small duplication was inserted into the same frame as at its original position (Figs. 1B and 2).

The NS2 genes of strains H3887 and H3142 were different from those of BVDV-X and the related MD and enteritis isolates, and showed 82% nucleotide and 94% amino acid similarity with the sequence of cp BVDV strain ILLC, respectively [36] (not shown). Isolate H3887 contained a 21-nucleotide insertion at the same position as the previous isolates (Fig. 1A). Searches in the GenBank revealed that this insertion has highest nucleic acid homology (81%) with a gene coding for murine interferon-induced guanylate-binding protein 1 (Accession number: XM 143605) (Fig. 1C). In isolate H3142, a 42-nucleotide insertion was found six bases downstream of position 4355 (Fig. 1A). This insertion was identical to the sequence spanning positions 11078- 11119 in the NS5B gene (Figs. 1D and 3). This sequence, encoding 14 amino acids, was inserted in the same reading frame as at its original position (Figs. 1D and 2). In addition, 93 nt downstream of this insertion, at nucleotide position 4448, three nucleotides were absent (Fig. 1A), resulting in a deletion of one amino acid at position 1355 in the NS2 gene of isolate H3142 (Fig. 2).

The role of the insertion in the production of NS3, the marker protein of cytopathogenicity of BVDV

To investigate the role of the small insertion in the NS2 gene of the vaccine strain in cytopathogenicity, the complete NS2-3 gene of the BVDV-X vaccine was cloned into pCI mammalian expression vector to obtain pNS2-3, and an insertion-minus variant pNS2-3INS^{-} was also constructed. BT cells were transfected with the construct followed by SDS-PAGE and Western blot analysis with anti-NS3 monoclonal antibodies. An 80 and a 125 kDa bands were obtained from the pNS2-3 construct, corresponding to the cleaved and uncleaved form of the NS2-3 protein. Only one band, running at around 125 kDa was detected with the INS^{-} variant, corresponding to an uncleaved NS2-3(Fig. 4B).

Discussion

Cytopathogenicity in BVDV correlates with the expression of the non-structural protein NS3. In ncp strains, NS3 is colinear with the C-terminal part of the NS2-3, which is expressed as a fusion protein. Invariably, the cp strain is found to have generated *de novo* by genetic changes in the ncp counterpart, or by recombination between an exogenous cp virus and an antigenically related ncp counterpart in a PI animal [11, 12, 14]. In this study we analysed cp isolates representing different BVDV-induced syndromes: Four strains originated from cattle enteric and MD forms, presumably induced by vaccination with a live attenuated vaccine BVDV-X, whereas two additional strains were isolated from cattle with respiratory disease. To screen for genetic cp markers, we first used an RT-PCR assay that promptly detects presence of large insertions in the NS2-3 junction region, or of duplications of the NS3 gene [32]. Amplicons of shifted size (from 402 bp) or indicative of large duplications were not detected, establishing that common cp markers like insertions of cellular sequences in the NS2-3 junction region, and duplication of the NS3 gene were not the factor of cytopathogenicity in these isolates. Since the BVDV-X vaccine presumed to have caused the MD and enteric disease outbreaks was marketed as Oregon C24V derivate, sequence comparisons were made with the NS2-coding region of this strain. For Oregon C24V, it is established that point mutations in the NS2 region correlate with the cp phenotype [22, 23]. The NS2-3 junction regions of the three MD isolates, of the enteritis isolate, and curiously, also of the vaccine virus, do not carry any of the point mutations described for Oregon C24V, and show completely different sequence patterns, indicating a distinct cp virus origin.

To analyse further the factors possibly leading to the cytopathogenicity, the complete NS2-3 coding region of the six cp BVDV isolates and of the vaccine virus were sequenced. The analysis showed that the complete NS2-3 region of the vaccine virus and of three MD isolates (H6379, H6712, H-BVD MD) and of the enteritis isolate (H8427) were identical, strongly suggesting that the observed MD and enteritis cases were caused by BVDV-X superinfection of animals persistently infected with a closely related ncp BVDV strain to BVDV-X, leading to early onset of MD, as it was described previously in several cases [29, 30]. Sequencing of the 5'UTR and E2 regions showed that in these regions the sequences of the above-mentioned virus strains are identical, that further confirmed the re-isolation of the vaccine virus (Bálint, unpublished result). Unfortunately, at the time of the isolation of the above-mentioned cp BVDV strains, the exact pathogenesis of MD was not elucidated, thus recovery of the ncp counterparts of these isolates was not attempted, therefore they were not available for genetic comparisons.

The 45-nucleotide insertion found in the three MD, the enteritis isolates and in the vaccine virus represents, to our knowledge, the first viral insertion of the NS4B-NS5A junction region to be described in the NS2-coding region of a cp genome. Strain BVDV CP7 [26] has a 27-nucleotide insertion of a viral sequence deriving from the NS2. The location of these viral insertions, however, is similar in the two cases, i.e., at nucleotide position 4355. This could indicate that position 4355 is a preferential spot for viral sequence rearrangements of the type found in these cp BVDV isolates. It

is surprising, however, that the duplication includes the NS4B-NS5A junction that, in its normal context, is cleaved by the NS3 serine protease. In all cp BVDV strains the NS2-3 cleavage has been established to occur at the N-terminus of the NS3. Whether the NS4B-NS5A junction in this insertion introduces an additional cleavage site within the NS2 is an interesting question that will be addressed in further studies on expression of NS3 in these strains.

Sequencing of the whole genome of the BVDV-X vaccine confirmed that further genetic patterns besides the above-mentioned insertion possibly contributing to cytopathogenicity of this strain are not present in the genome, therefore the role of the 45-nucleotide insertion in the NS2 gene was examined. Expression studies with the complete NS2-3 from BVDV-X and with the artificial NS2-3INS^{-} construct revealed that the small insertion alone can contribute to partial cleavage of NS2-3, leading to the expression of NS3, the marker protein of cp BVDV strains.

The insertions found in the two respiratory cp BVDV isolates proved to be different from the above-mentioned viral duplications. So far, insertions described at position 4355 consisted of small duplications of sequences of viral origin [26]. The 21-nucleotide insertion found in strain H3887 represents the first non-viral insertion identified at this position of NS2. The inserted sequence showed the closest homology to a murine interferon-induced guanylate-binding protein 1 gene, and a bovine counterpart of this gene was not found in the GenBank. Foreign (cellular) insertions in the NS2 genomic region are presumed to induce conformational changes in this protein, thereby introducing a cleavage site specific for a cellular (or viral) protease [16]. The possible role of this foreign insertion in cytopathogenicity of isolate H3887 is presently being determined.

The insertion in isolate H3142 is present six nucleotides downstream, but nevertheless in the vicinity of position 4355. The inserted sequence was different from the previous ones in that it comprised 42-nucleotides deriving from the middle part of the NS5B gene. Also, one codon was deleted 93 nucleotides downstream this insertion, but whether this deletion takes part in the mechanism of cytopathogenicity of this virus (in combination with the insertion) is not known. The finding is without precedent in studies of cp BVDV genomes.

All the insertions described in this study and found in cp BVDV CP7, concerned the same area of the NS2 gene. In case of CP7 the 27-nucleotide insertion has been demonstrated crucial for cytopathogenicity by the use of an infectious cDNA clone [37].

In conclusion, this study showed the occurrence of different types of viral sequence rearrangements and novel cellular insertions in the genomes of cp isolates of BVDV, revealing more sides of the complex issue of cytopathogenicity in this virus. Position 4355 may represent a hot spot for recombination of viral sequences, as an increasing number of insertions of viral origin are found at this location of cp BVDV genomes.

The nucleotide sequences of the whole NS2-3 gene of isolates H3142, H3887 and the BVDV-X vaccine strain have been deposited in GenBank under accession numbers AJ544865, AJ544864 and AJ544866, respectively.

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Table 1. List of the cp BVDV isolates used in this study

Isolate	Country of origin	Year of isolation	Sample of origin	Clinical signs
H3887	Hungary	1972	Lungs	Respiratory
H6379	Hungary	1976	Spleen	Mucosal disease
H3142	Hungary	1971	Lungs	Respiratory
H6712	Hungary	1976	Spleen	Mucosal disease
H8427	Hungary	1978	Intestines	Enteritis
H-BVD MD	Hungary	1977	Peyer patches	Mucosal disease

Fig. 1. (A): comparison of the nucleotide sequences of the insertions in the NS2 gene and the flanking regions of BVDV CP7 reference strain and of the cp BVDV strains included in this study: H3887, H6379, H3142, H6712 and BVDV-X. The insertions found in each isolate and the three-nucleotide deletion in the NS2 coding region of isolate H3142 at nucleotide position 4448 are shaded. The insertions were found at nucleotide position 4355, except in isolate H3142 where it is located at position 4361. All the nucleotide positions refer to the genome of the non-cytopathogenic strain SD-1.

(B): comparison of the 45-nucleotide in-frame insertion in the NS2 gene of BVDV-X as representative of cp isolates H6379, H6712, H8427 and H-BVD MD, and the sequence at its original location in the NS4B/NS5A junction at nucleotide as well as at amino acid levels. The NS4B/NS5A cleavage site is indicated by an arrow in the deduced amino acid sequence, displayed at the bottom.

(C): similarity analysis of the 21-nucleotide insertion in the NS2 gene of isolate H3887. A complete homology was found with a stretch of the sequence of the murine interferon-induced guanylate-binding protein 1 (Acession number: XM 143605) gene.

(D): comparison of the 42-nucleotide in-frame insertion in the NS2 gene of isolate H3142 and the sequence at its original location in the middle part of NS5B gene at nucleotide as well as at amino acid level.

(A)

```
                         4355                                                                  4374

CP7     : CGTGGAGAAGAGTTGG------GGTGTTCCTTATCCGGACCCTCAAACG------------------CTGGGGGGTTAGACTACAG
H3887   : T..A..A.........------..GTGGAGCAG.GA.A.AGAA------------------------T.A..A....G..TC...A
H6379   : T.....A.........------..GA.GGAC.C.GAA.GGAAGAT..G.AACCTGTCTGGGAATTAT..A.......G.......A
H3142   : TA.A..A.........TTGGGA.AG..G..AGT.ATAACAG.AG.TGGTGAAGTATACATAAGA---------....G..T....A
H6712   : T.....A.........------..GA.GGAC.C.GAA.GGAAGAT..G.AACCTGTCTGGGAATTAT..A.......G.......A
BVDV-X  : T.....A.........------..GA.GGAC.C.GAA.GGAAGAT..G.AACCTGTCTGGGAATTAT..A.......G.......A

                                                                                    4448        4460

CP7      : GACAATTGACTCTGTCTATGATGTGGATGAAAGTGGAGAAGGCGTGTACCTCTTCCCGTCCAGACAGAAGAAAAATAAGAATATCAG
H3887    : ..GGG..A....C.....C..C........G...........T..A..T.....T..A..T..G......GC.C.C..A...T.TG.
H6379    : .............A.T......A.........T.A....G.................T.....G.....TGGC..G..A...G....
H3142    : ..GGG..A.......T.....C........G...........T..A..T.........A..T..G.T....GC.---..A...T.TG.
H6712    : .............A.T......A.........T.A....G.................T.....G.....TGGC..G..A...G....
BVDV-X   : .............A.T......A.........T.A....G.................T.....G.....TGGC..G..A...G....
```

(B)

```
1    GGGATGGACTCTGAAGGGAAGATAAGGAACCTGTCTGGGAATTAT 45

8402 GGGATGGACTCTGAAGGGAAGATAAGGAACCTGTCTGGGAATTAT 8446

1    GMDSEGKIRNLSGNY 15

2673 GMDSEGKIRNLSGNY 2687
                ↑
```

(C)

```
5    GGAGCAGTGAGAAAGAA 21

1277 GGAGCAGTGAGAAAGAA 1293
```

(D)

```
1      GAGGTGCCAGTTATAACAGCAGATGGTGAAGTATACATAAGA 42

11078 GAGGTGCCAGTTATAACAGCAGATGGTGAAGTATACATAAGA 11119


1    EVPVITADGEVYIR 14

3565 EVPVITADGEVYIR 3578
```

Fig. 2. Comparison of the amino acid sequences of the complete NS2 genes of reference strain BVDV CP7 and of the cp BVDV strains included in this study: H3887, H6379, H3142, H6712 and BVDV-X. The sequences of the insertions and the one amino acid deletion found in the NS2 gene of strain H3142 at amino acid position 1355 are shaded. The insertions were found at amino acid position 1324 except in isolate H3142 where it is located at position 1326. All the amino acid positions refer to the genome of the non-cytopathogenic strain SD-1.

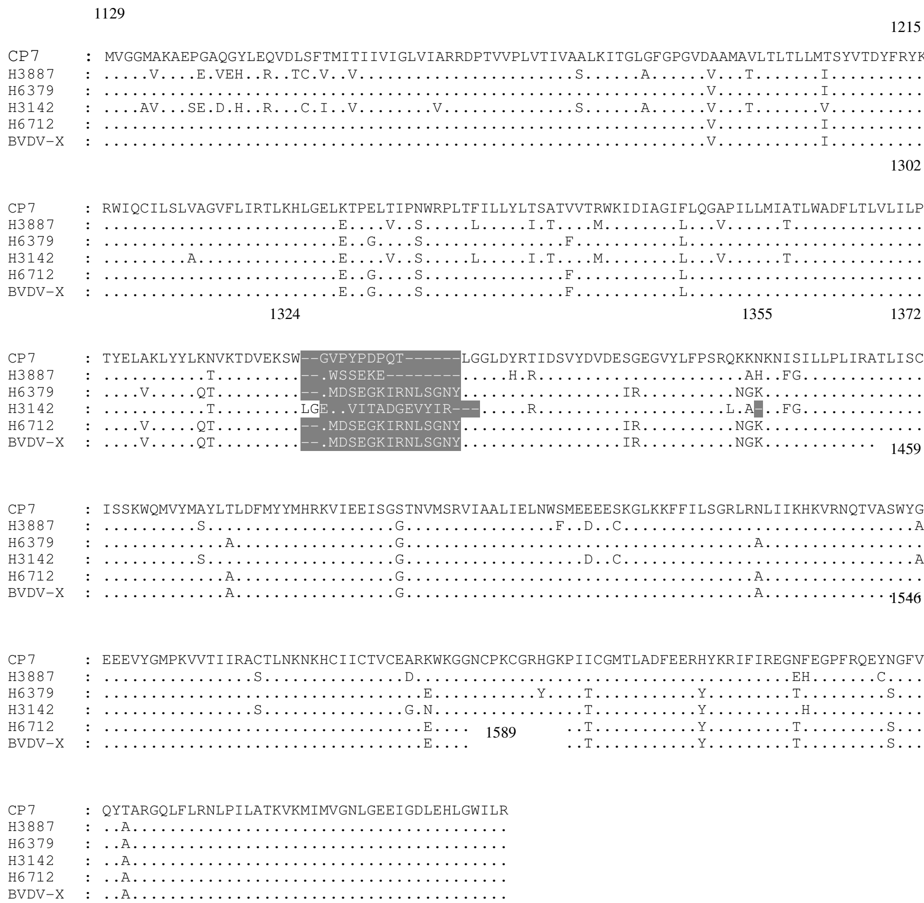


Fig. 3. (A): schematic representation of the non-structural part of the genome of the BVDV-X strain, as representative of cp isolates H6379, H6712, H8427 and H-BVD MD. The insertion in the NS2 and the original position of this sequence at the NS4B/NS5A junction site in the genome are indicated with black boxes and arrows. The sequence positions of the nonstructural genes are shown below.

(B): schematic representation of the non-structural part of the genome of isolate H3142. The insertion in the NS2 and the original position of this sequence at nucleotide position 8402-8446 in the middle part of the NS5B in the genome are indicated with black boxes and arrows. The sequence positions of the nonstructural genes are shown below.

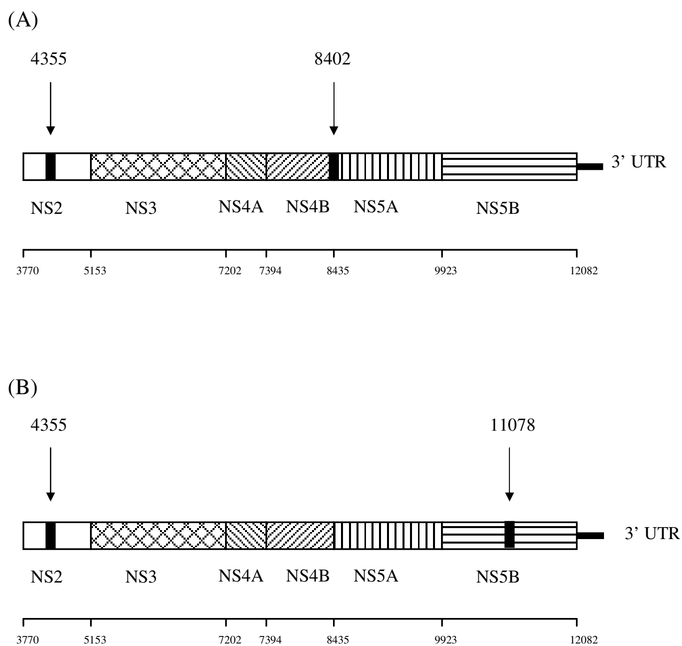
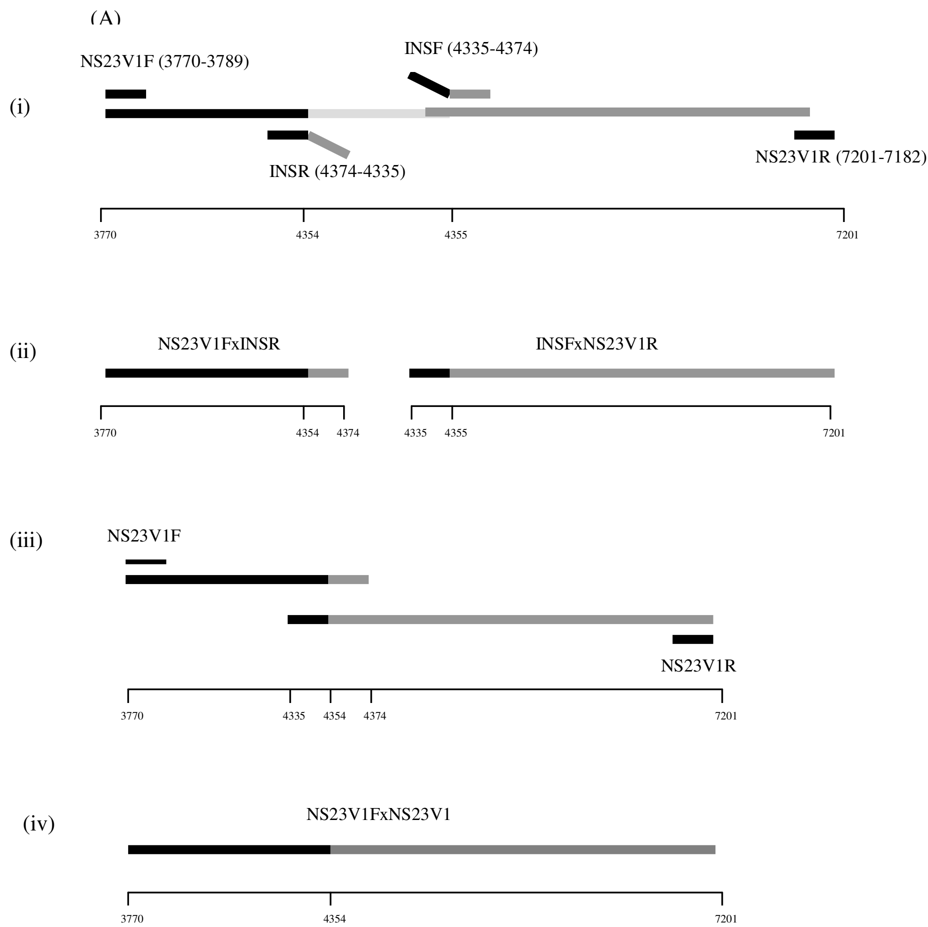
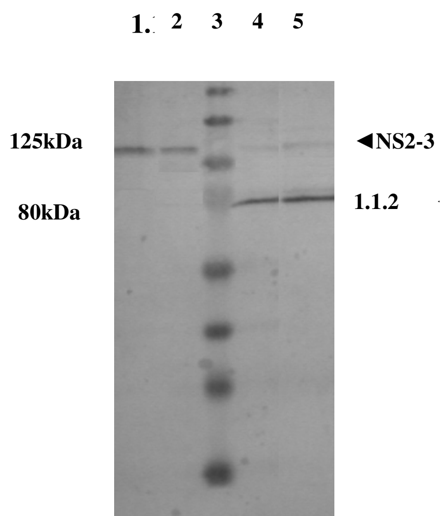


Fig 4. (A): strategy of deleting the 45-nucleotide insertion from pNS23 to generate pNS23INS⁻. The details of the procedure are described in the Materials and Methods. The black and dark grey lines represent the sequences flanking the insertion upstream and downstream, respectively. The light grey box indicates the insertion. The 5' and 3' sequence positions of primers are indicated in brackets and the location of the PCR fragments are shown below. Two initial PCRs were performed using primers NS23V1F with INSR and INSF with NS23V1R (i) to obtain PCR fragments NS23V1FxINSR and INSFxNS23V1R, respectively (ii). In the second step, the two PCR fragments were joined by a PCR using NS23V1F and NS23V1R primers (iii) to obtain the final PCR product NS23V1FxNS23V1R (iv). The PCR fragment was subsequently cloned into pCI mammalian expression vector to obtain pNS23INS⁻.

(B): immunoblot analysis of the proteins expressed by pNS2-3 (lane 4) and pNS2-3INS⁻ (lane 2). Lanes 1 and 5 represent as positive controls: a Hungarian ncp BVDV strain H2840 and the BVDV-X vaccine, respectively. Lane 3 is the Prestained Protein Ladder (Fermentas). Numbers on the left indicate the molecular masses (in kilodaltons [kDa]). The positions of NS2-3 and NS3 proteins are marked by arrowheads.



(B)



II

Cytopathogenicity markers in the genome of Hungarian cytopathic isolates of bovine viral diarrhoea virus

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Abstract

Since genetic recombination is a major factor in the evolution of the cytopathogenic (cp) bovine viral diarrhoea virus (BVDV) biotypes, in this study the cytopathogenicity markers were investigated in the genome of two cp BVDV strains recently isolated from mucosal disease (MD) cases in Hungary. In the genome of strain H4956, a Jiv-like insertion was found similar to those described in reference strain NADL and in other BVDV 1, BVDV 2 and border disease virus (BDV) strains. The 133 amino acid Jiv-like sequence is inserted at nucleotide position 4984 (amino acid position 1533), 9 nucleotides upstream of that of strain NADL. The insertion showed 96% amino acid sequence identity with the cellular Jiv protein. In the genome of cp BVDV strain H115/PCR, an ubiquitin-containing duplication was found. The duplicated sequence started at nucleotide position 7978 (amino acid 2531) in the NS4B gene. The duplication contained a complete ubiquitin monomer of 76 amino acids and the complete NS3 gene starting at nucleotide position 5153 (amino acid 1589), that corresponds to the first N-terminal amino acid of NS3. The duplication located further downstream of the known ubiquitin-containing genomic regions of cp BVDV strains, and it consisted of the shortest inserted nucleotide sequence. The insertions and duplication of strains H4956 and H115/PCR further confirmed that recombinations occurring at positions A and B are the most common mechanisms leading to the development of BVDV cytopathogenicity.

Introduction

Bovine viral diarrhoea virus (BVDV), along with border disease virus (BDV) and classical swine fever virus (CSFV), belong to the *Pestivirus* genus, which together with the genera *Flavivirus* and *Hepacivirus*, comprises the *Flaviviridae* family (Rice, 1996; Thiel et al., 1996). BVDV is an enveloped virus with a positive-stranded RNA genome of about 12.5 kb, which comprises a single open reading frame (ORF) flanked at the 5´ and 3´ ends by untranslated regions (UTRs) (Collett et al., 1988, Deng and Brock, 1992). The ORF encodes a polyprotein composed of approximately 4000 amino acids that is co- and post-translationally processed by both viral and cellular proteases into 11 or 12 mature viral proteins: NH_2-N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A, NS5B-COOH (Rice et al., 1996; Thiel et al., 1996).

At present, two biotypes of BVDV, namely cytopathogenic (cp) and noncytopathogenic (ncp) are recognized, and the viruses are classified according to their ability to cause cytopathic effect (CPE) in cell cultures (McClurkin et al., 1985). In the pathogenesis of mucosal disease (MD), both cp and ncp biotypes are required. MD can be induced either by superinfection of the PI animal with cp BVDV or by *de novo* generation of a cp mutant from the persisting ncp virus (Bolin et al., 1985; Tautz et al., 1998). Both a cp and a persisting ncp biotype can be simultaneously isolated from animals succumbing to MD. These isolates are called a ” virus pair”.

The molecular characterization of different BVDV pairs showed that cp BVDV strains evolved *in vivo* from ncp BVDV by genomic alterations in cattle persistently infected with ncp BVDV. These alterations include cellular insertions, duplications, rearrangements or deletions of viral genome sequences (Meyers and Thiel, 1996; Tautz et al., 1998) and in some cases simple point mutations (Kümmerer et al., 2000). The different mechanisms involved in the generation of cp BVDV strains lead to the cleavage of the nonstructural protein NS2-3 and the subsequent expression of the nonstructural protein NS3 that is responsible for the development CPE and is considered the marker protein of cp BVDV strains.

In a recent study the cytopathogenicity markers in the genome of cp BVDV strains isolated in the 1970s from MD, enteric and respiratory forms of BVD were examined. In the genome of these strains small insertions of cellular origin and small duplications of viral origin were found (Bálint et al., submitted). Interestingly, further cp BVDV strains were not isolated in Hungary until 2003. The aim of this study was to examine which genetic alterations contribute to the cytopathogenicity of two cp BVDV strains isolated recently from MD cases in Hungary. A further goal was to compare these changes with those observed in the genome of the cp strains previously detected in Hungary.

Materials and methods

Cells and viruses

The cp BVDV strains H4956 and H115/PCR were isolated from cattle showing the symptoms of MD. Secondary bovine turbinate (BT) cells were cultured in Eagle´s Minimum Essential Medium (EMEM) supplemented with 2 mM of L- glutamine and 10% foetal calf serum (FCS). Following the routine procedures of our institute, cells and each batch of FCS were tested regularly for the absence of *pestiviruses* by reverse transcription-PCR (RT-PCR) and immunoperoxidase assay (IPX) using BVDV polyclonal antiserum (VLA, Addlestone, UK). For FCS, the absence of anti-*pestivirus* antibodies was shown by lack of virus neutralization.

Infection of cells

To obtain pure virus stocks, each isolate was plaque purified by the standard methods. Briefly: for propagation of the viruses, 80% confluent cell monolayers in 6 well tissue culture plates were inoculated with different dilutions of the respective virus strains. After one hour adsorption, the cell layers were washed three times with EMEM, then were overlaid with low melting agarose (Sigma) containing EMEM and 2% FCS, followed by incubation at 37˚C in an atmosphere of 5% CO_2 for 72 hours. Five plaques of each isolate were picked and BT cells grown in 25 cm^2 tissue culture flasks were infected and incubated as described above. The plaque-purified viruses were examined by the above-described RT-PCR followed by sequencing. Virus particles of the pure stocks were pelleted from supernatants of infected BT cells by ultracentrifugation (Airfuge; Beckman Instruments), at 55 000 rpm for three hours. Virus stocks were resuspended in PBS without Ca^{++} and Mg^{++} and kept at -70˚C.

RT-PCR for detection of insertions and duplications

Total RNA was extracted using the TRIzol LS reagent (Gibco BRL) according to the manufacturer's instructions. The cDNA synthesis was carried out with M-MLV RT enzyme (Gibco BRL) and VDAS1 reverse primer locating in the 3'UTR (Baule, 2000). For PCR amplification the Expand High Fidelity Kit (Roche) was used with different primer sets for detection of insertions (Primers A and C, Table 1) and duplications (Primers B and C, Table 1), as it was previously described (Greiser-Wilke et al., 1993). The reactions were prepared in a 50 µl total volume, containing 1X buffer for a final 2.25 mM $MgCl_2$ concentration, 200µM of dNTP, 20 pmole of each primer, 5 µl of cDNA and 2.5 U of Expand polymerase mix. After a 2 min initial denaturation, 36 cycles were performed with the following parameters: 45 sec at 94˚C, 45 sec at 52ºC, 2 min at 68˚C. After cycling, a final extension was applied for 10 min at 68˚C.

PCR products were separated on 1.5% agarose gels stained with ethidium bromide and visualized under UV light (UVP). The amplicons were purified with microcolumns (QIAquick Gel Extraction Kit, Qiagen) according to the manufacturer's instructions. The pure DNA products were sequenced from both directions with the same primers as for amplification reactions with an ABI Prism sequencer (Applied Biosystems, Model 377), using the Big Dye Terminator V3.0 sequencing kit (Applied Biosystems). Two cDNA batches were sequenced and compared, where difference was found, a third amplification and sequencing was carried out.

Sequence analysis

Nucleotide sequences of the PCR products were edited and analyzed with multiple programs of the DNASTAR software package (Lasergene). Search for sequence similarities in GenBank were performed using the BLAST programs (The National Center for Biotechnology Information).

Results

Search for insertions and duplications by RT- PCR

Primers A (located in the C-terminal part of the NS2) and C (located in the N-terminal part of NS3) amplify a 402 base pair (bp) product in the NS2-3 gene of ncp strains and in that of cp strains without insertions, whereas a shift in product size is seen if the strain contains an insertion in the NS2-3 (Greiser-Wilke et al., 1993). PCR product from strain H4956 showed a size shift of 801 bp,

whereas amplification of strain H115/PCR resulted in the product size of 402 bp, characteristic either of the ncp or cp BVDV isolates without sequence alteration in this region (Fig. 1).

Primers B (locating in the C-terminal part of NS3) and C generate PCR product exclusively when a duplicated NS3 sequence is present downstream of the first NS3. Using these primers, PCR product from H4956 was not obtained, but a 1212 bp product was amplified from strain H115/PCR, indicating the duplication of the NS3 gene (Fig. 1).

Data of the above-described two RT-PCR assays suggest that the genetic markers of cytopathogenicity of strains H4956 and H115/PCR are different from those of the recently examined Hungarian cp BVDV strains, where no products were obtained using PCRs to detect insertions or duplications.

Type of the sequence insertion of strain H4956

The inserted sequence of H4956 cp BVDV strain started at position corresponding to nucleotide 4984 (amino acid 1533) of the NS2 gene of ncp BVDV strain SD-1 (Deng and Brock, 1992). Figs. 2 and 4 show that the insertion was similar to the Jiv (formerly cINS) sequence found in reference strain NADL and in a number of BVDV 1, BVDV 2 strains (Collett et al., 1988; Ridpath and Neill, 2000; Vilcek et al., 2000; Rinck et al., 2001) and BDV isolates (Becher et al., 1996). The insertion proved to be 133 amino acids long, indicating that it belongs to the longer Jiv insertions, and it contained the 90 amino acids of Jiv90, the part of Jiv essential for developing cp phenotype (Fig. 2). The amino acid identity with the cellular Jiv protein was found to be 96%. One base substitution of valine instead of methionine at position corresponding to amino acid 546 of the cellular Jiv protein (Rinck et al., 2001) was found, but this substitution is present in many cp BVDV strains. However, two additional amino acid substitutions were found: isoleucine instead of phenylalanine at position 570 and proline instead of leucine at position 579 (Fig. 2). These substitutions are not present in the so-far sequenced Jiv-containing cp BVDV and BDV isolates. The cellular insert of strain NADL located 9 bases downstream of that of strain H4956. In the majority of Jiv-containing BVDV 1 isolates, the insertion is located further downstream. The location of the insert of H4956 was the closest to the insert positions of Southern African isolates Mo6 and SA1 (Baule, 2000) where the insertion resided one basis upstream of that of the H4956 strain (Fig. 3).

Type of the sequence duplication in strain H115/PCR

The duplicated sequence of cp BVDV strain 115/PCR started at position corresponding to nucleotide 7978 (amino acid 2531) in the NS4B gene of ncp BVDV strain SD-1. The duplication contained a complete ubiquitin monomer of 76 amino acids and the complete NS3 gene starting from nucleotide position 5153 (amino acid 1589), that corresponds to the first N-terminal amino acid of NS3 (Figs. 2 and 4, Table 2). The duplication located further downstream of the known ubiquitin-containing duplications of cp BVDV strains and consisted of the shortest nucleotide sequence of only one ubiquitin monomer without N-terminally truncated ubiquitin and NS2 coding region as found in other cp BVDV and BDV strains. The amino acid sequence of the ubiquitin of the insert was found to be identical to that of the cellular ubiquitin (Fig. 2).

Discussion

In the majority of cp BVDV strains the genomic alterations occur at two points, the so-called positions A and B (Meyers et al., 1998). Position A is located at amino acid 1535, whereas position B is located at amino acid 1589 in the region coding the viral polyprotein. Insertions at position B result in processing of NS2-3 by introducing a new cleavage site for cellular and viral proteases at the carboxy terminus of the insertion, making Gly_{1590} the N-terminal amino acid of NS3. The exact location of position A is not so conserved; it appears approximately 50 bases upstream of Gly_{1590}. However, in cp BVDV strains containing insertion at position A, the NS2-3 cleavage also occurs at

position 1589, indicating that the insertion generates the cleavage of the polyprotein *in trans* (Meyers and Thiel, 1996).

In a recent study, six Hungarian cp BVDV strains, isolated between 1971 and 1978, representing various forms of BVD, i.e., enteric and respiratory symptoms, as well as MD were examined for their cytopathogenicity markers (Bálint et al., submitted). Interestingly, none of these strains contained genetic alterations at positions A or B, but small insertions of viral (NS4B/NS5A junction region and a part of NS5B gene) and cellular (part of the murine interferon-induced guanylate-binding protein 1) origin was detected at nucleotide position 4355, representing a so far seldom observed cytopathogenicity marker (Tautz et al., 1996; Baule, 2000). In the years of isolation of these cp viruses cattle were regularly immunized with a live attenuated vaccine (Bálint et al., submitted). The widespread use of the vaccine presumably reduced the number of the persistently infected animals, so the frequency of generating new cp BVDV strains occurred at a very low rate. The application of the live vaccine stopped in Hungary in the middle of the 1990s, and only inactivated vaccines have been used to control BVD. The change in vaccination regimes might be the reason that recently the occurrence of cp BVDV strains has become more frequent again. Two of the new cp BVDV isolates were analyzed in the present work to determine cytopathogenicity markers in the newly emerging cp variants.

In the genome of cp BVDV strain H4956, a cell-derived insert Jiv, previously termed cIns, was identified, that was similar to the insertions found in several cp BVDV and BDV strains, (Becher et al., 1996; Ridpath and Neill, 2000; Vilcek et al., 2000; Nagai et al., 2003). The Jiv transcript is expressed at low level in bovine cells, while the expression level increases following infection with a ncp strain of BVDV. The increased amount of the transcript can serve as a template of recombination with the genome of ncp BVDV. The extreme heterogeneity of termini of the inserts as well as that of the flanking viral sequences suggests the possible role of the secondary RNA structure in recombination. Our finding confirms this observation, since the ends of the insert and the flanking viral sequences are different from those the other published sequences. A jiv insertion with a length of 90 codons was shown to be essential for NS2-3 cleavage (Mendez et al., 1998). The 133 amino acid long insertion of strain H4956 fulfilled this requirement, containing the 90 amino acids of the Jiv90. The reason for the amino acid substitutions not present in other Jiv containing cp BVDV strains needs further elucidation.

In cp BVDV isolates with ubiquitin containing sequence duplications, the positions of the 5' border of insertions of ubiquitin are located exclusively in the NS4B gene ranging from 7483 (TGAC) to 7813 (Mo1). In strain H115/PCR the duplication locates further downstream at nucleotide position 7978, confirming the observation that the 5' position of recombination is not conserved in this region (Table 2), excluding the role of the site-specific recombination as in case of Jiv.

Additional difference besides the 5' border of the inserted ubiquitin of strain H115/PCR is that the duplication contains only one ubiquitin monomer. So far, only in reference strain cp BVDV Osloss was one ubiquitin monomer described as cytopathogenicity marker, but without any sequence duplication. In other related strains, the duplications contain additional complete or truncated ubiquitin monomers or fragments of the NS2 gene (Table 2.).

Summarizing the results of this study, it was elucidated that there is no connection between the formerly described and the recently isolated Hungarian cp BVDV strains, concerning cp markers. The insertions and duplication of the latter strains further confirm that recombinations occurring at positions A and B are the most common mechanisms leading to the development of BVDV cytopathogenicity.

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TABLE 1.

Sequence and location of the primers used in the RT-PCR amplifications searching for insertions and duplications in the genomes of cp BVDV strains H4956 and H115/PCR.

Primer	Sequence (5´-3´)[1]	5´ position[2]
VDAS1[3]	CTG TGT GCA TTR ART GTA GTG TT	12242
A[4]	GCA GAT TTT GAA GAA AGA CAC TA	4934
B[4]	GTG GAG ACT GGG AAA GCA CT	7163
C[4]	TTG GTG TGT GTA AGC CCA	5318

[1] K = G/T; R = A/G; Y = C/T

[2] Nucleotide position corresponding to BVDV SD-1

[3] Baule, 2000

[4] Greiser-Wilke et al., 1993

TABLE 2.

Localization and content of the ubiquitin-containing duplications in the genomes of cp BVDV strains analyzed so far. Data of the strain investigated in this study are shaded.

BVDV strain	Beginning of the duplication	Content of the duplication
CP1[1]	NS4B, nt 7537	one N-terminally truncated and one complete ubiquitin monomer of 366 nt and the complete NS3 gene
TGAC[2]	NS4B, nt 7483	NS2 gene from nt 5129-5153, one N-terminally truncated and one complete ubiquitin monomer of 270 nt and the complete NS3 gene
Ill-C[2]	NS4B, nt 7530	NS2 gene from nt 4535-5153, one complete ubiquitin monomer of 240 nt and the complete NS3 gene
190[2]	NS4B, nt 7687	one N-terminally truncated and one complete ubiquitin monomer of 276 nt and the complete NS3 gene
Mo14 and Mo20[3]	NS4B, nt 7584	one N-terminally truncated and one complete ubiquitin monomer and the complete NS3 gene
Mo2[3]	NS4B, nt 7537	one N-terminally truncated and one complete ubiquitin monomer of 444 nt and the complete NS3 gene
Mo8[3]	NS4B, nt 7537	two complete ubiquitin monomers of 456 nt and the complete NS3 gene
Mo1[3]	NS4B, nt 7813	NS2 gene from nt 4548-4823, 15 nt of unknown origin one N-terminally truncated and one complete ubiquitin monomer and the complete NS3 gene
H115/PCR	NS4B, nt 7978	one complete ubiquitin monomer of 228 nt and the complete NS3 gene

[1] Meyers et al., 1991

[2] Qi et al., 1992

[3] Baule, 2000

FIG. 1.

Results of the RT-PCR amplifications searching for insertions and duplications in the genomes of cp BVDV strains H4956 and H115/PCR. Lanes (from the left): 1 and 5, 1 kb molecular weight markers (Fermentas); 2, 801 bp product of the PCR of H4956 with primers A and C; 3, 402 bp product of the PCR of H115/PCR with primers A and C; 4, no product was obtained from the PCR of H4956 with primers B and C; 1212 bp product of the PCR of H115/PCR with primers B and C.

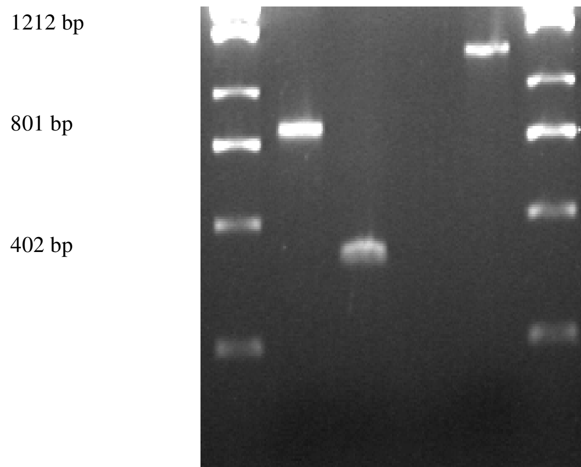


FIG. 2. (A): comparison of the amino acid sequences of the theoretical ncp strain (INS-H4956) and cp BVDV strain H4956 at the flanking region of the Jiv-like insertion, and comparison of the amino acid sequences of the insertion and that of the cellular Jiv protein. The amino acid differences are shaded. The upper numbers indicate the amino acids at the 5' and 3' borders of the insertion corresponding to the amino acid positions in BVDV SD-1 (Deng and Brock,

1992). The lower numbers indicate the amino acids at the 5' and 3' borders of the insertion corresponding to the amino acid positions in JIV (Rinck et al., 2001). The 90 amino acids of Jiv90, necessary for establishing cytopathogenicity are underlined. (B): comparison of the amino acid sequences of the theoretical ncp strain (Ins-115/PCR) and cp BVDV strain H115/PCR at the flanking region of the ubiquitin insertion, and comparison of the amino acid sequence of the insertion and that of the cellular ubiquitin protein. The numbers indicate the amino acids at the 5' and 3' borders of the insertion corresponding to the amino acid positions in BVDV SD-1.

(A)

```
                     1533
INS-H4956  : RHYKRIFIREGE------------------------------------------------
H4956      : RHYKRIFIREGERSVNEFLSKLQEAMNTMMCSRCQGKHRRFEVDREPKSARYCAECNRLH
JIV        : ------------RSVNEFLSKLQEAMNTMMCSRCQGKHRRFEMDREPKSARYCAECNRLH
                        516

INS-H4956  : ------------------------------------------------------------
H4956      : PAEEGDIWAESSMLGPKITYFALMDGKVYDITEWAGCQRVGISPDTHRVPYHISFGSRMP
JIV        : PAEEGDFWAESSMLGLKITYFALMDGKVYDITEWAGCQRVGISPDTHRVPYHISFGSRMP

                                      1534
INS-H4956  : -------------------------HEGPYRQECNGF
H4956      : GTSGRQRATPDAPPADLQDFLSRIFHEGPYRQECNGF
JIV        : GTSGRQRATPDAPPADLQDFLSRIF------------
                                    649
```

(B)

```
                     2531
H115/PCR     : ESVVILSTTMMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQL
UBIQUITIN    : ----------MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQL INS-H115/PCR:
ESVVILSTTM--------------------------------------------------

                                       1590
H115/PCR     : EDGRTLSDYNIQKESTLHLVLRLRGGGPAVCKKITE
UBIQUITIN    : EDGRTLSDYNIQKESTLHLVLRLRGG----------
INS-H115/PCR: --------------------------GPAVCKKITE
```

FIG. 3.
Amino acid sequences and positions of the flanking region of the Jiv-like insertions in the genome of different cp BVDV and BDV strains. The numbers indicate the neighboring amino acids in BVDV SD-1. The data of the strain investigated in this study are shaded.

	1536		1537
BVDV1-SD1 ncp[1]	FIREGNFEG	No insertion	PFRQEYNGF
BVDV1-NADL[2]	FIREGNFEG	Insertion (90 aa)	PFRQEYNGF
BVDV1-Indiana[3]	FIREGNFEGP	Insertion (105 aa)	FRGEYNGF
BVDV-1 cp 2324_94[3]	FIREGNFEGPF	Insertion (140 aa)	RQEYNGF
BVDV1 cp 5.19516[3]	FIREGNFDGPF	Insertion (132 aa)	RQEYNGF
BVDV1cp 5569[3]	FIREGNFEGPFRQEC	Insertion (115 aa)	SGF
BVDV1 cp 869[3]	FIREGNFEAPL	Insertion (101 aa)	RQEYNGF
BVDV1cp 7923[3]	FIREGNFEGP	Insertion (104 aa)	FRQEYNGF
BVDV1 cp 88055[3]	FIREGNFEG	Insertion (90 aa)	PFRQEYNGF
BVDV1cp A2146[3]	FIREGNFEGPF	Insertion (97 aa)	RQEYNGF
BVDV1MD1[4]	FIREGNFEGPFR	Insertion (104 aa)	QEYNGF
BVDV1-Mo6[4]	FIREG	Insertion (98 aa)	NHEGPFRQEYNGF
BVDV1-SA1[4]	FIREG	Insertion (99 aa)	NFEGPFRQEYNGF
BVDV1-H4956	FIREGE	Insertion(133 aa)	HEGRYRQECNGF
BVDV2-125c[5]	FIREGNFEG	Insertion (91 aa)	FRQEYNGF
BVDV2-296c[5]	FIREG	Insertion (91 aa)	CHDGPFREEYKGY
BVDV2-5912c[5]	F-	Insertion (104 aa)	REGCHDGPFREEYKGY
BVDV2-6082c[5]	KGYVQYT	Insertion (136 aa)	ARGQLFLRNL
BVDV2-Galena[5]	FIREGCPDGPL	Insertion (151 aa)	REEYKGY
BVDV2-ND8799c[5]	FIREGCHDGPF	Insertion (100 aa)	REEYKGY
BVDV2-297c[5]	FIREGCLDGP	Insertion (124 aa)	REEYKGY
BVDV2-SD1630c[5]	FIREGCHDGPF	Insertion (147 aa)	REEYKGY
BDV-Cumnock[6]	FIRE	Insertion (133 aa)	GHFREEYKGY
BDV-Moredun[6]	FIREA	Insertion (114 aa)	GPFREEYKGY

[1] Deng and Brock, 1992

[2] Collett et al., 1988

[3] Vilcek et al., 2000

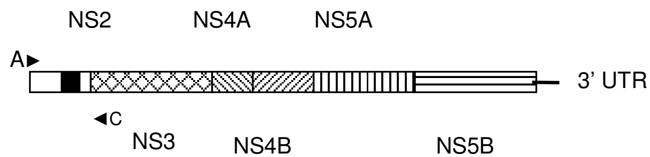
[4] Baule, 2000

[5] Ridpath and Neill, 2001

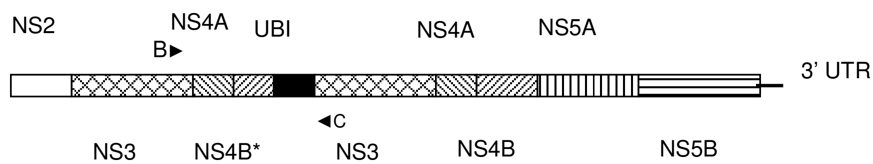
[6] Becher et al., 1996

FIG. 4. (A): Schematic representation of the organization of the nonstructural proteins of cp BVDV strain H4956. The Jiv-like insertion is indicated with black box. The primers used for generation of the PCR product are marked with arrowheads. (B): Schematic representation of the organization of the rearranged nonstructural proteins of cp BVDV strain H115/PCR. NS4B* indicates the truncated NS4B gene in the duplication. The ubiquitin-like insertion is indicated with black box. The primers used for generation of the PCR product are marked with arrowheads.

(A)



(B)



III

Retrospective genome analysis of a live vaccine strain of bovine viral diarrhea virus

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Running Title: The genome of a BVDV vaccine

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Abstract – A live bovine viral diarrhea (BVDV) vaccine, marketed as a derivate of the Oregon C24V strain, was used between the end of the 1960s and the beginning of the 1990s in Central Europe. Since laboratory investigations of mucosal disease cases in vaccinated animals suggested recombinations between the vaccine and wild type variants of BVDV, and recombinational nucleotide sequences seemed distinct from BVDV Oregon C24V, the aim of the present retrospective study was to analyze the genomes of a pre-registration (termed here BVDV-Xpre) and of a marketed (BVDV-X) batch of the vaccine. Results of the complete genome analysis of BVDV-Xpre confirmed that the original virus strain used at the start of the vaccine production was Oregon C24V. Surprisingly, the analysis of the complete nucleotide sequence of the BVDV-X marketed vaccine revealed that this strain belongs to the BVDV 1b subgroup, with a 93.7% nucleotide sequence homology with BVDV reference strain Osloss. The homology to BVDV Oregon C24V was significantly lower (77.4%), and a thorough sequence scanning showed that the genome of BVDV-X had not derived from Oregon C24V. These data indicate the very likely scenario that a strain different to Oregon C24V was picked up during the *in vitro* or *in vivo* passages for vaccine development. Despite of the virus-switch, the BVDV-X vaccine continuously maintained its innocuity and efficacy, as proven by the regular quality testing data, and the presence of the foreign virus remained unnoticed over many years. The results of this work emphasize that the contamination of commercially available live vaccines with exogenous BVDV strains is a real risk factor, and a unequivocal analysis, including molecular methods, is needed to verify their authenticity.

Keywords: bovine viral diarrhea virus / vaccine / strain switch / control

INTRODUCTION

Bovine viral diarrhea virus (BVDV), a major pathogen of cattle, is a member of P*estivirus* genus that together with the genera F*lavivirus* and *Hepacivirus* belongs to the *Flaviviridae* family [39; 41]. BVDV is an enveloped virus with an uncapped and non-polyadenylated positive-stranded RNA genome of about 12.5 kb in size. The genome comprises a single open reading frame (ORF) that is flanked by 5' and 3' untranslated regions (UTRs) [11; 12; 14]. The 5´UTR functions as a so-called internal ribosome entry site (IRES) that promotes cap-independent translation initiation [30; 31]. The ORF encodes a polyprotein of about 4000 amino acids that is co- and post-translationally processed by viral and cellular proteases into 11 or 12 mature viral proteins: NH_2-N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A, NS5B-COOH [34].

According to their ability to cause cytopathic effect (CPE) in cell cultures, BVDV strains are differentiated as cytopathogenic (cp) or noncytopathogenic (ncp) biotypes [18; 22]. Both cp and ncp biotypes of BVDV are involved in the pathogenesis of mucosal disease (MD), a fatal clinical manifestation of BVDV [reviewed in 23]. Infection of pregnant cattle with ncp BVDV in the first trimester of gestation results in the birth of persistently infected (PI) calves. PI animals are immunotolerant against the respective ncp virus. After superinfection with an antigenically closely related cp strain, which is either of exogenous origin or develops by genetic alterations of the resident ncp virus, the PI animals are predisposed to develop early onset of MD [6; 8], whereas superinfection of PI animals with an antigenically different cp BVDV can result in late onset of MD [16; 35].

A cp and an ncp BVDV strain, isolated from the same animal succumbing to MD, are termed a “virus pair”. Molecular analysis of different BVDV pairs indicated that cp BVDV strains evolved in vivo from ncp BVDV by RNA recombination in cattle persistently infected with ncp BVDV [reviewed in 23; 38]. These recombination events result in insertions of cellular sequences, duplications, rearrangements or deletions of viral sequences, affecting the NS2-3 region [2; 4; 20; 23] or the N-terminal region of the BVDV genome [24; 25]. These changes in the genomes of cp BVDV strains lead to expression of the non-structural protein NS3, that is considered the marker protein for cytopathogenicity. However, mutations in the NS4B were shown to abolish cytopathogenicity despite the NS3 production [33]. Interestingly, some cp BVDV strains show no recombination-induced genome alterations [21; 29; 32].

To protect cattle against the various forms of BVDV infections, both modified live and inactivated vaccines are in use. A live vaccine, termed here for ethical reasons as vaccine BVDV-X, was used in Central Europe from the end of the 1960s to the beginning of the 1990s. The vaccine was prepared from a seed stock of the cp BVDV reference strain Oregon C24V that was serially passaged in cell cultures and in animals. After registration, the vaccine has undergone regular quality testing. During the use of the vaccine in more than two decades, the following main observations were made: a) vaccination provided sufficient protection against postnatal infections; b) from field observations and laboratory diagnosis it was suspected that vaccination of PI animals might have lead to the development of early onset of MD (Pálfi, personal observation).

In a recent study, four cp viruses originating from these MD cases were investigated [1]. Nucleotide sequence analysis of the complete NS2-3 region of the cp field isolates and the BVDV-X vaccine strain confirmed the theory of early onset of MD in vaccinated animals. Surprisingly, the sequence of the NS2-3 gene of the vaccine virus was found to be different from that of the Oregon C24V strain, and included a viral insertion located at the same position as the insertion found in BVDV strain CP7 [37].

To clarify the identity and the origin of the BVDV-X strain, three vaccine batches (one prior to and two after to the registration process) were obtained and examined. Analysis of the 5'UTR of an original batch prior to registration (named here as BVDV-Xpre), proved that this genomic region of

Törölt: ¶

the master stock was identical with BVDV Oregon C24V. However both batches of the virus from the marketed vaccine had Osloss-like 5'UTR sequences. To further elucidate the raised questions, the complete nucleotide sequences of the pre-registration batch (BVDV-Xpre) and of one of the marketed vaccine batches (termedBVDV-X) were determined and compared with published full-length sequences of BVDV strains, including Oregon C24V. Since at the time of the registration of BVDV-X the methods of identifying different BVDV strains were not so developed as at present, our basic goals were the followings: (i) verifying, whether the Oregon C24V strain was the starting point of the BVDV-X vaccine production; (ii) investigating the presence and ratio of Oregon C24V nucleotide sequences in the genome of the marketed vaccine BVDV-X. Our hypothesis was that finding both Oregon C24V and Osloss-like nucleotide sequences in the different batches of the BVDV-X vaccine could reveal that recombination events occurred between the original Oregon C24V strain and a heterologous BVDV virus strain during the numerous passages *in vitro and in vivo.*

2. MATERIALS AND METHODS

2.1. Cells and viruses

Secondary bovine turbinate (BT) cells were cultured in Eagle´s Minimum Essential Medium (EMEM) supplemented with 2 mM of L-glutamine and 10% foetal calf serum (FCS). By following routine procedures used at our institute, cells and FCS were tested regularly for the absence of *pestiviruses* by a reverse transcription-PCR (RT-PCR) targeting the 5'UTR [15] and by a routine immunoperoxidase assay (IPX) using BVDV polyclonal antiserum (VLA, Addlestone, UK). For FCS, the absence of anti-*pestivirus* antibodies was shown by lack of virus neutralization following standard procedures used at our institute. The BVDV-Xpre and two different batches of the marketed vaccine originating from the 1980s and 1990s were obtained from the manufacturer.

2.2. Infection of cells

To obtain pure virus stocks, the three batches were plaque purified by the standard methods. Briefly: for propagation of the viruses, 80% confluent cell monolayers in 6-well tissue culture plates were inoculated with ten-fold dilutions of the respective virus strains. After one hour adsorption, the cells were washed with EMEM, then overlaid with low melting agarose (Sigma-Aldrich, St. Louis, MO, USA) containing EMEM and 2% FCS. The cells were incubated at 37˚C in an atmosphere of 5% CO_2 for 72 hours. Five plaques of each batch were picked and used to infect fresh BT cells. The plaques were examined by the above-described routine RT-PCR. The PCR products were sequenced and compared in order to rule out that the vaccine batches did not contain mixed virus populations. Virus particles were pelleted from supernatants of infected BT cells by ultracentrifugation (Airfuge; Beckman Instruments, Fullerton, CA, USA), at 55 000 x g for three hours. Virus stocks were resuspended in PBS without Ca^{++} and Mg^{++} and kept at -70˚C.

2.3. RNA extraction and cDNA synthesis

RNA was extracted from virus stocks using the TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. To obtain long cDNA copies of the viral genomes, the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and an antisense primer (VDAS1 located in the 3´ UTR of BVDV, see Table II) were used. The transcription was performed in 20 µl reaction mixtures containing 500 ng RNA, 40 pmole primer, 200 µM of each dNTP, 24 U of RNAse inhibitor (Amersham Biosciences, Piscataway, NY, USA) and 200 U of Superscript II.

2.4. PCR amplification and sequencing

For the amplification of overlapping fragments of the whole viral genome, the Expand Long Template Kit (Roche Diagnostics, Basel, Switzerland) was used. The sequences and positions of oligonucleotides used in the initial PCR reactions for amplification of BVDV-X are shown in Table I. For the initial PCR reactions and the subsequent sequencing of the genome of BVDV-Xpre, a set of 32 primers designed based on the published sequence of BVDV Oregon C24V was used. The sequences of these primers are available upon request. The reactions were prepared in a 50 µl total volume, containing 1X buffer for a final $MgCl_2$ concentration of 2.25 mM, 200 µM of each dNTP, 20 pmole of each primer, 5 µl of cDNA and 2.5 U of Expand polymerase mix. After 2 min initial denaturation, 36 cycles were performed with the following parameters: 45 sec at 94˚C, 45 sec at the annealing temperature of the corresponding primer, 1 min/kilobases of the sequence at 68˚C. After cycling, a final extension was applied for 10 min at 68˚C.

PCR products were analyzed on 0.8% agarose gels. The amplicons were purified by using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The purified DNA products were sequenced from both directions first with the same primers as used for the amplification reactions. Sequencing was performed in an ABI Prism sequencer (Model 377), using the Big Dye Terminator V3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA). After initial sequencing, further oligonucleotides were designed based on these data to complete the whole genomic sequence. The preliminary sequencing of the different plaques of the pre-registration batch BVDV-X-pre showed that the examined sequences of the plaques were identical. Furthermore recent results of sequencing the whole NS2-3 gene of the BVDV-X vaccine and cp BVDV field isolates [1], and preliminary results from sequence analysis of the plaque-purified stocks of BVDV-X showed that the virus was different from Oregon C24V. Therefore, two independent cDNAs originated from a single plaque of the pre-registration batch (BVDV-Xpre) as well as one batch of the marketed vaccine strain (BVDV-X) were amplified and fully sequenced. Where differences were found, a third PCR product was generated and sequenced to determine the consensus sequence.

2.5. Determination of the 5' and the 3' terminal sequences

To determine the 3' end of the BVDV-Xpre genome, RNA ligation was performed with T4 RNA ligase (New England Biolabs, Beverly, MA, USA), followed by RT-PCR. Viral RNA was reverse-transcribed using M-MLV RT (Invitrogen, Carlsbad, CA, USA) and a reverse primer 14A located in the 5'UTR region. Subsequently, amplification was performed using oligo Oregon32f, located in the NS5B gene, as upstream primer and oligo12A, located in the 5' UTR, as reverse primer (Table I). The same strategy was followed in case of BVDV-X, using a reverse primer BVD7R located in E^{RNS} region for reverse transcription. Subsequently, amplification was carried out using oligo BVD3CF, located at the end of the NS5B gene, as upstream primer and oligo14A, located in the 5' UTR, as reverse primer (Table I). For determination of the 5' end of the two genomes, the 5'RACE Version 2.0 system (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions. The PCR products were purified and sequenced. Sequences were compared to the 5' and 3' ends of published full-length BVDV genomes in order to determine the exact terminal sequences.

2.6. Sequence analysis

The nucleotide sequences were edited and analyzed with multiple programs of the DNASTAR software package (Lasergene, Madison, WI, USA). Search for sequence similarities in GenBank were performed using the BLAST programs (The National Center for Biotechnology Information, Bethesda, MD, USA).

2.7. Comparison with full-length sequences of BVDV strains

The complete nucleotide and deduced amino acid sequence of BVDV-Xpre was compared with the published full-length sequence of Oregon C24V. The nucleotide and deduced amino acid sequences of BVDV-X were compared with the published sequences of the complete genomes of type 1 strains Osloss, CP7, CP75A, ILLC, ILLCNCP, NADL, NADLNCP, SD-1, Oregon, Oregon C24V, and type 2 strains 1373, NY93, and 890.

3. RESULTS

3.1. Analysis of the full-length nucleotide sequence of the BVDV-Xpre genome

The BVDV-Xpre virus proved to be a derivate of the Oregon C24V strain. There were differences at 10 nucleotide positions, and only four of them caused amino acid (aa) changes (Table II), affecting the E1, E2 and NS5A regions. Both the 5' and 3' UTRs were found to be identical to those of the published sequence of Oregon C24V strain.

3.2. Analysis of the complete nucleotide sequence of the BVDV-X genome

The entire genomic sequence of the live cp BVDV vaccine strain BVDV-X is composed of 12 308 nucleotides. The 5'UTR is 381 nucleotides long, whereas the 3'UTR comprises 182 nucleotides. The ORF begins at position 382 and ends at position 12126, consists of 11745 nucleotides, which encode a polyprotein of 3915 amino acids.

The nucleotide sequence of the 5'UTR of BVDV-X compared with that of the published sequences of BVDV 1 and BVDV 2 strains revealed nucleotide sequence identity of 83%-98% compared with the BVDV 1 strains and 65% with the BVDV 2 reference strain 890 (Table III). The phylogenetic tree derived from the 5'UTR of the vaccine virus and of the published sequences revealed that BVDV-X belongs to subgroup 1b of BVDV. The closest nucleotide similarity, of 98%, was found with strain Osloss, whereas similarity with BVDV Oregon C24V was only of 83% (Fig. 1).

The nucleotide sequence of the 3'UTR of BVDV-X compared with that of the published sequences of BVDV 1 and BVDV 2 strains revealed nucleotide sequence identity of 64%-95% compared with BVDV 1 strains and of 49% with the BVDV 2 reference strain 890 (Table III). The closest nucleotide similarity, of 95%, was found with the 3'UTR of strain Osloss, whereas similarity with BVDV Oregon C24V was only 64%.

3.3. Analysis of the polyprotein of BVDV-X

The deduced amino acid sequence of the polyprotein of BVDV-X was compared with published sequences of the polyproteins of BVDV 1 and BVDV 2 (Table IV). The highest amino acid variability was found in the E2 region, 84-91% aa identity with BVDV 1 strains and 62% aa identity with BVDV 2 reference strain 890. The lowest amino acid variability was found in the NS3 and NS5B regions, 94-98% and 84-97% aa identity with BVDV 1 strains and 91% and 79% aa identity with BVDV 2 reference strain 890.

3.4. Complete sequence scanning of BVDV-X to determine sequence similarity with BVDV Oregon C24V

To determine whether the genome of BVDV-X contains Oregon C24V-derived sequences, the complete nucleotide sequence of the BVDV-X genome was compared to that of BVDV Oregon C24V, in 200-nucleotide sections. The results showed that there is no region where the nucleotide similarity between the two viruses reaches 100% (Fig. 2). The highest nucleotide similarity, of 89%, was found in the NS3 region, whereas the lowest similarities, of 62.5% and 63.5%, were found in the N-terminal part of E2 and in the variable region 3'V of the 3'UTR, respectively. The average nucleotide similarity was found to be of 77.4% between BVDV-X and BVDV Oregon, whereas it was significantly higher, of 93.7%, between BVDV-X and BVDV strain Osloss.

4. DISCUSSION

To control the various forms of diseases caused by BVDV, both modified live and inactivated vaccines have been commonly used since the early 1960s [10; 27]. The first vaccines aimed at reducing the severity of clinical symptoms of postnatal BVD. After elucidation of the pathogenesis of persistent infection and MD in the mid 1980s [6; 8], and the role of PI cattle in the disease, the main goal of modern BVDV vaccines is to prevent foetal infections.

Modified live vaccines contain cp BVDV strains, and generally give better immunological response than inactivated vaccines. Live vaccines can clinically protect immunocompetent animals against viral challenge [13]; however, they do not confer complete protection against congenital infection. Furthermore, vaccination of PI cattle may even trigger development of MD after superinfection with a cp strain [7]. A further disadvantage is that the live vaccines contain limited antigen mass and need the possibility to replicate in the host in order to develop the necessary immunity. During replication, the live vaccine strains might recombine in the PI animal with the resident ncp strains of BVDV and lead to early [1; 5; 17] or delayed onset of postvaccinal MD, as it was observed in several cases [3; 16; 35]. Such situation may cause considerable problems during control or eradication programs. The cp BVDV strains of the live vaccines can cross the placental barrier independent on the time of gestation and infect foetuses [28], but in contrast to the ncp BVDV strains, they do not induce persistent infections [9]. A further disadvantage of the live virus vaccines is their immunosuppressive effect [36].

The presence of foreign BVDV strains in permanent cell lines or in foetal calf serum batches may cause serious problems during the development and/or production of the live vaccines. If such cells or serum are used in vaccine production, the vaccine can be contaminated with ncp BVDV and become a source of spread of BVDV infections [26]. A further possible scenario is the accidental pickup of a cp strain and the continued production of the vaccine from this foreign cp virus.

To examine the questions raised in the introduction, the complete genomes of the BVDV-Xpre and one of the marketed batches (BVDV-X) have been sequenced. Data of the full-length sequence of BVDV-Xpre proved that the original strain used for development of BVDV-X was Oregon C24V. Since correct documentation of this batch is not available, it is not known, how many *in vitro* and *in vivo* passages it has undergone, thus the role of the detected sequence differences –mainly in the E1 and E2 regions- compared to the Oregon C24V strain cannot be determined.

The sequence data of the genomic sequence of BVDV-X revealed that this virus is a BVDV 1b variant, that showed the highest average sequence homology, of 93.7%, to reference BVDV strain Osloss. In contrast, the average sequence homology with BVDV Oregon C24V was sigificantly lower, of 77.4%, and the detailed genome scanning revealed that the genome of BVDV-X did not contain Oregon C24V sequence. This indicates that BVDV-X did not derive from Oregon C24V,

but it might have originated from a pickup of an exogenous cp BVDV strain from the *in vitro* or *in vivo* steps of passaging. It is possible that an animal used for the *in vivo* passaging was infected with exogenous cp BVDV, which was later re-isolated and used for vaccine production. A miss-labelling of tubes is another possibility to be taken into account. It has to be emphasized that in the historic years when BVDV-X was used as vaccine, unequivocal methods for strain identification were not still developed, neither panels of monoclonal antibodies, nor molecular tools, like nucleotide or aa sequencing were available. Thus, the means to detect a virus-switch or a virus pickup were limited at that time. The comparative genome analysis in the present study revealed the surprising fact that BVDV-X contained a virus very different from BVDV-Xpre. Considering the major differences in the genomes of the two viruses, it is likely that neither viral recombination, nor virus-switch, but a pickup of a foreign virus occurred in this case. It is likely that similarly to Oregon C24V, the uptaken foreign virus was also harmless and immunogenic. This could be the reason that despite the virus-switch, the BVDV-X vaccine continuously maintained its innocuity and efficacy, as proven by regular quality testings, and the presence of the foreign virus remained unnoticed over many years.

The unchanged safety and efficacy of the vaccine can be considered a lucky scenario, since picking up of a new virus during passaging could have lead to very serious consequences. Contamination of products, like vaccines or FCS, is a severe risk in the biological industry. A previous study from our group revealed for example BVDV 2 contamination in a FCS batch used in New Zealand [40]. Since BVDV 2 has not been observed in this country before, it was important to learn that the batch of the serum originated from the USA. This example shows that biological contaminations may have a transboundary character, as a risk factor to animal and human health worldwide.

Besides the above listed dangers, it is interesting to note that in the present case the strain-switch may probably have had some “positive” practical effects. In a recent study we found that the Osloss-like virus of the BVDV-X vaccine was closely related to the cp BVDV strains isolated in the region of vaccination during the early 1970s [1]. The closer relation emphasizes that the BVDV-X immunisation presumably gave a stronger protection against the local Central European field isolates than the American Oregon C24V variant would have provided.

In summary, the molecular approach was applied in the present retrospective study to reveal an interesting intermezzo in the history of veterinary vaccinology. The nucleotide sequence analysis proved that millions of animals were immunised over two decades with a “foreign” virus strain, which was presumably picked up during the series of passages for vaccine production. Fortunately, the new virus was of low pathogenicity, as shown by the regular vaccine quality control tests. The results of this study emphasize the necessity of more precise quality testing of commercially available live virus vaccines, including monoclonal antibody analysis and sequencing, to verify their authenticity.

A further outcome of this work is that the full-length nucleotide sequence of a BVDV strain, BVDV-X has been determined, and this information will hopefully provide additional data to the panel of pestivirus biology and vaccinology.

The nucleotide sequence of the whole genome of the BVDV-X vaccine strain has been deposited in GenBank under accession number AJ585412.

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Table I.

List of the primers used in this study for generating initial PCR products

Primer	Sequence (5´-3´)	5´ position*
13A[1]	GCT AGC CAT GCC CTT AGT AGG A	101
12A[1]	GGC CTT TGC AGC ACC CTA TCA G	325
14A[1]	ATC AAC TCC ATG TGC CAT GTA CAG C	372
16F	ACA ATG GAA CTT ACA A	1207
BVD7R	ACC AAC CAT GCT TGT TCC ACT	1419
C912F[2]	TTG ATA ACA GGG GTA CAA GG	2441
E100[2]	CAT ATG GTC TGC AAG GCA TAG G	3284
125AF	GAG GGG CCG GTA GAA AAG AC	3320
A[3]	GCA GAT TTT GAA GAA AGA CAC TA	4934
B[3]	TTG GTG TGT GTA AGC CCA	5318
C[3]	GTG GAG ACT GGG AAA GCA CT	7163
125BR	GCA TAY TGG AGG TGG GTK GTG T	7323
NS5BR1	AGG CTG GGT TGG GCT ATT GTG TG	10498
NS5BF3	TTG CCT ATA GGT TTG AGG ACA TAG	11382
OREGON32F	GCC TAT TGG TCC TCT CGC AG	11610
BVD3CF	GAG GAC GGC AAT TGG CTG GTA	11843
VDAS1	CTG TGT GCA TTR ART GTA GTG TT	12242

* Nucleotide position corresponding to BVDV SD-1

[1] See in reference [15]

[2] VLA

[3] See in reference [19]

Table II.

Nucleotide and deduced amino acid sequence differences between the complete genomes of Oregon C24V and BVDV-Xpre. Silent mutations are marked with dashes.

Nucleotide position	Nucleotide substitutions		Amino acid substitutions	
	Oregon C24V	BVDV-Xpre	Oregon C24V	BVDV-Xpre
2112	C	T	-	-
2283	C	T	Alanine	Valine
2533	A	G	Asparagine	Aspartic Acid
3883	A	T	-	-
8835	G	A	Glycine	Glutamic Acid
9411	G	A	Arginine	Histidine
9586	G	A	-	-
10372	T	A	-	-
10426	G	A	-	-
11458	C	T	-	-

Table III.

Nucleotide identity of the 5’UTR and 3’UTR of the BVDV-X vaccine strain and other BVDV 1 and BVDV 2 strains

Virus strain	Nucleotide identity	
	5UTR	3UTR
BVDV 1 Osloss	98%	95%
BVDV 1 CP7	90%	89%
BVDV 1 ILLC	92%	89%
BVDV 1 NADL	83%	70%
BVDV 1 Oregon	83%	64%
BVDV 2 890	65%	49%

Table IV.

Amino acid identity between proteins of the BVDV-X vaccine strain and other BVDV 1 and BVDV 2 strains

Virus strain	Amino acid identity											
	N^{pro}	C	E^{rns}	E1	E2	P7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
BVDV 1 Osloss	96%	97%	96%	94%	91%	95%	92%	98%	98%	94%	95%	97%
BVDV 1 CP7	92%	92%	94%	93%	86%	91%	89%	98%	98%	94%	92%	96%
BVDV 1 ILLC	89%	89%	95%	93%	84%	91%	84%	94%	96%	87%	90%	84%
BVDV 1 NADL	86%	89%	89%	85%	77%	77%	78%	97%	92%	92%	93%	91%
BVDV 1 Oregon	86%	87%	91%	88%	76%	83%	77%	97%	93%	91%	84%	92%
BVDV 2 890	72%	81%	78%	84%	62%	68%	59%	91%	85%	83%	69%	79%

Fig. 1.

Phylogenetic tree derived from the 5'UTR of the so-far completely sequenced BVDV 1 and BVDV 2 strains and the BVDV-X vaccine strain. The units at the bottom of the tree indicate the number of substitution events.

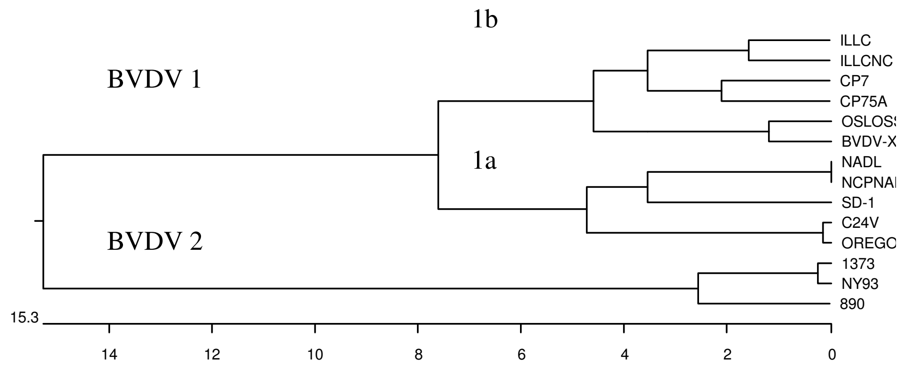
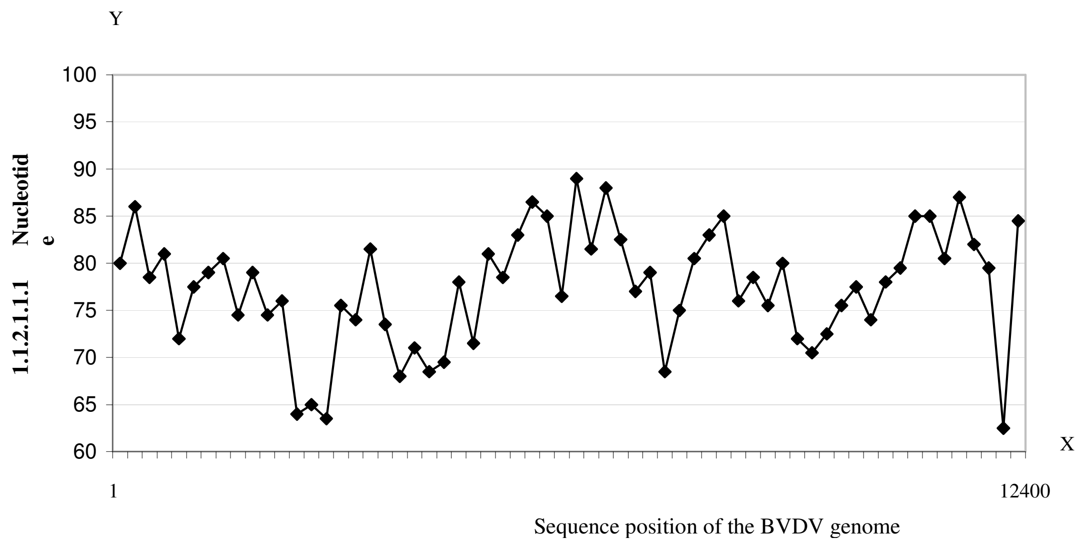


Fig. 2.

Graphical plot showing the nucleotide sequence similarity between BVDV-X and BVDV Oregon C24V. Axis Y shows the sequence similarity in %. Axis X shows the sequence positions. One section corresponds to 200 nucleotides.



IV

A 45-nucleotide insertion in the NS2 gene is responsible for the cytopathogenicity of a bovine viral diarrhoea virus strain

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Keywords: **bovine viral diarrhoea virus, vaccine, infectious clone, cytopathogenicity marker, NS2-3, NS3**

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Abstract. Cytopathogenicity (cp) markers have recently been investigated in the genomes of field isolates of bovine viral diarrhoea virus (BVDV). Most of the isolates originated from mucosal disease (MD) cases observed after vaccination with a live attenuated vaccine, termed here BVDV-X. The NS2-3 genes of these isolates and of the vaccine proved to be identical, including a 45-nucleotide (nt) viral insertion at nt position 4355. The insertion originated from the NS4B/5A junction region of the BVDV genome. Interestingly, in BVDV strain CP7 a 27-nt insertion originating from the NS2 is located exactly at the same position. Complete genome analysis of BVDV-X did not reveal further potential cp markers. Furthermore, expression studies indicated that the insertion promotes NS2-3 cleavage. In order to examine the possible role of the 45-nt insertion in viral cytopathogenicity in details, a full-length infectious cDNA clone of BVDV-X was generated, and bovine turbinate (BT) cells were transfected with RNA transcribed from the clone. The recovered virus, termed BVDV-XR, showed slight retardation in growth in comparison with the original BVDV-X, and induced cytopathogenic effect (CPE). Since the natural non-cytopathogenic (ncp) counterpart of the vaccine virus was not available, an insertion-negative mutant cDNA clone was generated from BVDV-XR by PCR-directed mutagenesis. The recovered virus, termed BVDV-XR-INS-, showed the same growth characteristics as its cp counterpart BVDV-XR, but caused no CPE. These findings provide a direct proof that the 45-nt insertion at position 4355 has a basic role in the cytopathogenic character of this BVDV strain.

Introduction

Bovine viral diarrhoea virus (*BVDV) is a member of Pestivirus genus that together with the genera Flavivirus and* Hepacivirus belongs to the *Flaviviridae* family [1]. The enveloped virions of BVDV contain uncapped and unpolyadenylated positive-stranded RNA genome of about 12.5 kb in size that comprises a single open reading frame (ORF) [2, 3], which is flanked with 5' and 3' untranslated regions (UTRs) [4, 5]. The ORF encodes a polyprotein of about 4000 amino acids that is co- and post-translationally processed by viral and cellular proteases to give rise to 11 or 12 mature viral proteins: NH_2-N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A, NS5B-COOH [6].

Due to their effect in cell cultures *in vitro*, cytopathogenic (cp) or noncytopathogenic (ncp) biotypes of BVDV are distinguished [7, 8]. *In vivo*, in animals persistently infected with ncp BVDV, superinfection with the cp biotype of BVDV causes the fatal mucosal disease (MD) [9, 10]. A cp and an ncp BVDV strain isolated from the same animal succumbing to MD are termed a virus pair.

The cp BVDV strains were shown to have evolved *in vivo* from ncp BVDV mainly by RNA recombination in cattle persistently infected with ncp BVDV [reviewed in 11, 12]. As a result of recombination, cellular insertions, duplications, rearrangements or deletions of viral genome sequences are found in the NS2-3 region [11, 13, 14, 15] or in the N-terminal region of the BVDV genome [16, 17]. In contrast, some cp BVDV strains show no recombination-induced genome alterations, but point mutations [18, 19, 20]. These different changes in the genome of cp BVDV strains lead to the expression of NS3, which is considered as the marker protein of cytopathogenicity. In ncp BVDV this protein is expressed exclusively in the context of an uncleaved fusion protein, NS2-3. However, mutations in NS4B were shown to abolish cytopathogenicity despite NS3 production [21].

In a recent study cytopathogenicity markers were investigated in the genomes of cp field isolates originating mostly from cases of MD. These cases occurred after vaccination with a live attenuated vaccine, termed here for ethical reasons BVDV-X [22]. The NS2-3 genes of the vaccine virus and of the MD isolates were found to be identical. Furthermore, a viral insertion was found at nucleotide (nt) position 4355 in the NS2 gene of BVDV-X, at the same position as the insertion was described in strain BVDV CP7 [23]. The in-frame insertion consists of 45 nucleotides originally located at the junction region of the NS4B/NS5A genomic region. Since the 5'UTR and E2 regions were also identical in the five viruses, it is likely that the vaccine itself caused the early MD cases, and was re-isolated from the succumbed animals. Therefore, research activity was focused on BVDV-X. The complete nucleotide sequence of the marketed vaccine virus BVDV-X was determined, and sequence analysis did not reveal further genetic changes contributing to the cytopathogenicity of BVDV-X [24]. Furthermore, expression studies revealed that the insertion induces NS2-3 cleavage [22]. In order to determine the role of this insertion in cytopathogenicity of BVDV-X, the aims of this study were: i) construction of a full-length infectious cDNA clone of BVDV-X and characterisation of the recovered virus; ii) deletion of the insertion in context of the full-length clone to prove that the insertion-negative mutant virus switches to the ncp phenotype.

Materials and Methods

Cells and Viruses

Secondary bovine turbinate (BT) cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 2 mM of L-glutamine and 10% foetal calf serum (FCS). Following the routine procedures, cells and FCS were tested regularly for the absence of pestiviruses with a

reverse transcription-PCR (RT-PCR) targeting the 5'UTR [25] and with routine immunoperoxidase assay (IPX) using BVDV polyclonal antiserum (VLA, Addlestone, UK). For FCS, the absence of anti-pestivirus antibodies was shown by lack of virus neutralization using the standard procedures. The completely sequenced batch of BVDV-X virus [24] was used in this study.

Infection of cells

An 80% confluent cell monolayer in 25 cm^2 tissue culture flask was inoculated with BVDV-X. After one hour adsorption the cell layer was washed with EMEM, then overlaid with EMEM and 2% FCS, followed by incubation at 37˚C in 5% CO_2 for 72 hours. Virus particles were pelleted from supernatants of lysed BT cells by ultracentrifugation (Airfuge; Beckman Instruments, Fullerton, CA, USA), at 55 000 g for three hours. The virus stock was resuspended in phosphate buffered saline (PBS) without Ca^{++} and Mg^{++} and kept at -70˚C. If not specified, a multiplicity of infection (MOI) of 0.1 was used.

RNA extraction and cDNA synthesis

RNA was extracted from virus stocks using the TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. To obtain long cDNA copies of the viral genome, the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and the 3EXTSMA antisense primer located in the 3'UTR of BVDV (Table 1) were used. The transcription was performed in 20 µl reaction mixture containing 500 ng RNA, 10 pmole primer, 200 µM of each dNTP, 24 U of RNAse inhibitor (Amersham Biosciences, Piscataway, NY, USA) and 200 U of Superscript II.

Construction of a full-length cDNA clone of BVDV-X

Based on the data of the full-length genomic sequence of BVDV-X primers were designed to amplify overlapping fragments of the whole genome (Table 1). Long-range PCR assays were performed using the KOD HiFi DNA polymerase (NOVAGEN, Darmstadt, Germany) to generate PCR products ranging between 1.5 and 5.2 kb (Fig. 1A). The reactions were prepared in a 50 µl total volume, containing 1X buffer#2 for a final 1.5 mM $MgCl_2$ concentration, 200 µM of each dNTP, 20 pmole of each primer, 5 µl of cDNA and 1 U of KOD HiFi DNA polymerase. After a 2 min initial denaturation 30 cycles were performed with the following parameters: 30 sec at 94˚C, 30 sec at annealing temperature of the corresponding primer, 30 sec at 68˚C. After cycling a final extension was applied for 1 min at 68˚C.

Clones generated from these PCR assays were used to construct the final full-length clone of the BVDV-X genome. Restriction enzyme cleavages and cloning steps were performed according to standard protocols [26]. Restriction endonucleases and DNA modifying enzymes were purchased from Fermentas (Vilnius, Lithuania) and New England Biolabs (NEB, Beverly, MA, USA). For all the subcloning steps and for the final cloning the pANCR1180 low-copy-number vector [27] was used. ElectoTenBlue competent *E. coli* cells (Stratagene,La Jolla, CA, USA) were used to propagate the respective plasmids. The schematic representation of the strategy of generating the full-length clone is shown in Fig. 1B. Briefly, a PCR product generated from amplification with 5EXTT7 and BVD7R primers was digested with *SalI* and *ApaI* and was cloned into pANCR1180 to construct pANCRI clone. A second PCR product obtained from amplification with 13A and V6R primers was digested with *ApaI* and was cloned into pANCR1180 to get pANCRII clone. A third PCR product generated with V5F and Cl2R primers was digested with *ApaI* and *NsiI*, and was cloned into pANCR1180 to obtain pANCRIII clone. A fourth PCR product obtained from amplification with AF3V1F and 3EXTSMA primers was digested with *NsiI* and *SacI*, and was cloned into pANCR1180 to create pANCRIV clone. All the clones were sequenced with an ABI Prism sequencer (Model 377) using the Big Dye Terminator V3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and the obtained sequences were compared with the published sequence of

BVDV-X. Clone pANCRIII was digested with *ApaI* and *NsiI*, and the fragment was cloned into pANCRIV to generate pANCRIII,IV clone. Clone pANCR I was digested with *SalI* and *ApaI*, and the fragment was cloned into pANCRIII,IV to construct pANCRI,III,IV clone. At the final step, the fragment from pANCRII was excised with *ApaI*, and was inserted into pANCRI,III,IV plasmid to obtain the full-length clone pBVDV-XR.

pBVDV-XR-INS- derivate

To construct pBVDV-XR-INS-, a mutated full-length clone of BVDV-X that lacks the 45-nt insertion in the NS2 gene, PCR-directed mutagenesis was carried out as previously described [22]. Briefly, two primers were designed: primer INSF contained at the 5' end 20 nucleotides directly upstream of the insertion, followed by the 20 nucleotides downstream of the insertion, while primer INSR was the reverse complement of INSF (Table 1). A PCR product was generated with primers 13A and INSR using pANCRBVDV-X as template. An additional PCR product was amplified with primers INSF and 4815R using the same template. These PCR fragments were finally pieced together with a joining PCR, using primers 13A and 4815R. This PCR fragment was cleaved with *ApaI* and was cloned into pANCRI,III,IV to obtain pBVDV-XR-INS-.

In vitro transcription

A *SmaI* site was included at the end of the BVDV-X sequence (in primer 3EXTSMA, see Table 1) for linearisation of the full-length cDNA clones at the exact 3' end of the BVDV-X genome and for run-off transcription of the full-length RNAs. Two µg of the plasmids pBVDV-XR and pBVDV-XR-INS- was digested with *SmaI* (Fermentas, Vilnius, Lithuania), treated with proteinase K (Sigma-Aldrich, St. Louis, MO, USA), extracted with phenol:chloroform and precipitated with ethanol. The pelleted DNA was finally resuspended in 20 µl of RNase-free water. The *in vitro* transcription was carried out with the Megascript T7 kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Briefly, the T7 transcription reaction mixture was incubated for three hours, followed by DNaseI treatment for 15 min. RNA was precipitated in 2.5 M LiCl overnight at -20°C. RNA was pelleted by centrifugation, washed with 70% ethanol and resuspended in 20 µl of RNase-free water.

Transfection of BT cells with the in vitro transcribed RNA

BT cells were trypsinised and washed with ice-cold RNase-free HEPES buffered saline (HeBS). $2x10^6$ cells were suspended in 400 µl HeBS, subsequently mixed with 2 µg of RNA, followed by incubation on ice for 5 min. Electroporation was performed in 0.4 cm cuvettes (Bio-Rad, Hercules, CA, USA) at 250V and 500 µF with a Gene Pulser II electroporator (Bio-Rad, Hercules, CA, USA). The cells were resuspended in 3 ml EMEM containing 2% FCS, and were seeded in a 6-well tissue culture plate and were incubated for 72 hours at 37ºC in 5% CO_2.

Phenotype and genetic analyses

Stocks prepared from the first passage of the plasmid-derived viruses BVDV-XR and BVDV-XR-INS- were used for phenotype and genetic analyses. Determination of plaque morphology of the recombinant viruses was carried out by plaque assay. Briefly, 80% confluent monolayers of BT cells grown in 6-well tissue culture plates were inoculated with BVDV-XR and BVDV-XR-INS-. After one hour adsorption the cell layers were washed with EMEM, overlaid with low-melting agarose (Sigma-Aldrich, St. Louis, MO, USA) containing EMEM and 2% FCS, and incubated at 37˚C in 5% CO_2 for 72 hours. The agarose overlay was removed, cells were washed with PBS, staining for visualisation of plaques was performed with a 1% (w/vol in 20% ethanol) crystal violet solution.

Antigenic analysis experiments were performed by IPX. Briefly: BT cells in 6-well tissue culture plates were inoculated with the respective virus strains. After one hour adsorption the cell layers were washed with EMEM, overlaid with low-melting agarose (Sigma-Aldrich, St. Louis, MO, USA) containing EMEM and 2% FCS, and incubated at 37˚C in 5% CO_2. The agarose overlay was removed 48 h post infection (p.i.), cells were washed three times with PBS, dried and fixed at 80ºC for three hours. BVDV polyclonal antiserum (VLA, UK) was added to the fixed cells, followed by incubation at 27ºC for 20 min. Serum was removed, the cells were washed and incubated with peroxidase-conjugated rabbit anti-cow immunoglobulins (Dako, Glostrup, Denmark) for 20 min at 27ºC. Following the washing steps, a developing solution (3-amino-9-ethylcarbozole and 3% hydrogen peroxide) was added to the cells and the plates were incubated until colour development (10-20 min).

For the analysis of growth kinetics of the recombinant viruses 80% confluent monolayers of BT cells grown in 6-well tissue culture plates were inoculated with BVDV-XR and BVDV-XR-INS-. Cells were harvested by freezing and thawing at 6, 24, 30, 48 and 72 h p.i. Titres were determined on new cells by plaque assay in the case of the BVDV-XR, and by IPX in the case of BVDV-XR-INS-.

For genetic analysis examinations total RNA was extracted from the supernatants of the first passage of BVDV-XR and BVDV-XR-INS-. RNA isolation, reverse transcription and PCR were carried out as described above. Two different RT-PCR assays were used to identify the recombinant viruses targeting the 5'UTR-E^{rns} with primers 13A and BVD7R and NS2 with primersV5F and V6R, respectively. To confirm the absence of the insertion in pBVDV-XR-INS-, PCR amplification was performed using primers V5F and 4815R targeting the flanking region of the insertion. The PCR products were separated on 1% agarose gel stained with ethidium bromide, and were visualised under UV light (UVP).

Results

Construction of a full-length cDNA clone of BVDV-X

The selected strategy for recovery of the recombinant BVDV-X virus based on the transfection of BT cells with the complete, genomic-like RNA of BVDV-X, that was generated *in vitro* by run-off transcription from full-length cDNA construct with T7 RNA polymerase described originally in [28]. A strict cloning and subcloning strategy was selected for the assembling of the full-length cDNA clone, since the genome of BVDV-X vaccine contains very few unique restriction sites. To avoid the instability problems of the large clones a low-copy-number plasmid pANCR1180 [27] and ElectroTenBlue recombinant *E. coli* competent cells (Stratagene, La Jolla, CA, USA) specially designed for propagation of large clones were used. Sequencing of the full-length clone showed eight nucleotide substitutions compared with the parent virus, BVDV-X. Four of them caused amino acid changes affecting the E2, NS4B, NS5A and NS5B regions, respectively (Table 2). A specific genetic tag by which the recombinant virus could be easily differentiated from the parent BVDV-X strain was found in the sequence of pBVDV-X: a silent mutation at nucleotide position 2448 generated an *ApaLI* site at nt 2447 to 2452 that is not present in the genome of BVDV-X as shown by sequencing of different passages of the parent virus. Restriction endonuclease digestion with *SmaI* was used to linearise the full-length cDNA clone prior to *in vitro* transcription to obtain the authentic 3' end of the BVDV-X genome.

Recovery of infectious virus from the transcribed RNA of pBVDV-XR

RNA was transcribed from the *SmaI*-linearised pBVDV-XR DNA using T7 RNA polymerase. The resulting RNA was transfected into BT cells by electroporation. Since BVDV-X is a cp BVDV

strain, transfection of infectious viral RNA should result in the formation of plaques. In cells transfected with pBVDV-XR derived RNA cytopathic effect (CPE) was first observed at about 48 h post-transfection (p.t.), and became advanced at 72 h p.t. (not shown). Plaque assay of the first passage of the recovered virus, BVDV-XR showed that the recovered virus has cp phenotype (Fig. 2A).

Identification of the virus-specific antigen

The IPX staining showed advanced cytoplasmic staining in BT cells infected with the first passage of BVDV-XR at 48 h p.i., showing that the recombinant virus is a BVDV strain. Cells infected with the original BVDV-X virus also showed the same feature, while non-transfected BT cells did not show staining (Fig. 2B).

Identification of the virus-specific nucleic acid sequences

The two different RT-PCR assays targeting the 5'UTR-E^{rns} and NS2 regions resulted in PCR products of 1318 and 854 bp, respectively, confirming the presence of BVDV-X-specific nucleic acid (Fig. 3). To exclude the possibility of carryover of plasmid DNA, the same PCR reactions were repeated without reverse transcription, and no PCR product was obtained. In addition, no RT-PCR products were obtained from the uninfected cell control (not shown).

Properties of the recovered virus from transcribed RNA

Analysis of the growth characteristics of the recovered virus and the parent BVDV-X virus by plaque assay revealed that the first signs of CPE occurred in both cases at 24 h p.i, and became advanced by 72 h. The recombinant virus reached a titre of $4x10^6$ PFU/ml, while the titre of the parent virus was $2x10^7$ PFU/ml at the same time point as shown in the growth curve in Fig. 4.

In vitro generation of noncytopathogenic BVDV

To verify that the 45-nt insertion is responsible for cytopathogenicity of BVDV-X, an insertion-negative mutant full-length infectious clone pBVDV-XR-INS- was constructed by PCR-directed mutagenesis. The only difference at genomic level between the original infectious clone and the insertion-negative mutant was the absence of the 45-nt insertion in the latter one. A PCR amplification reaction performed using primers flanking the insertion verified the absence of the 45-nt insertion in the virus recovered from pBVDV-XR-INS-. Using the original BVDV-X and BVDV-XR as template, a 549 bp product was obtained, whereas a 504 bp product was generated with the insertion-negative virus BVDV-XR-INS- (Fig. 3). Sequencing of the replaced part of the mutant full-length clone revealed two nucleotide substitutions compared with the pBVDV-XR clone. One of them caused amino acid change affecting the E^{rns} region (Table 2). After transfection of BT cells with RNA transcribed from the insertion-negative mutant CPE was not detected (Fig 2A). The mutant virus BVDV-XR-INS- kept its ncp characteristics after the third passage. However, IPX analysis with polyclonal antibodies clearly showed foci of positive cells (Fig. 2B). These data confirmed that the removal of the 45-nt insertion switched the originally cp BVDV-X to an ncp virus. Furthermore, BVDV-XR-INS- showed the same growth characteristics as its cp counterpart (Fig. 4).

Discussion

The majority of cp BVDV strains reveal alterations at two points of the viral genome, the so-called positions A and B [29]. The location of position A is not conserved, it appears approximately at

nucleotide position 4990 in the NS2 gene [30], whereas position B is located exactly at nucleotide position 5153 at the NS2/3 junction. In cp BVDV strains containing insertions at position A, the cleavage of NS2-3 occurs at the NS2-3 junction, indicating that the insertion generates the cleavage of the polyprotein *in trans*, while insertions at position B result in processing of NS2-3 by introducing a new cleavage site for cellular or viral protease at the carboxy terminus of the insertion, generating a *cis* proteolytic cleavage at the NS2/3 junction [11].

Recently, six cp BVDV field strains isolated between 1971 and 1978 in large cattle herds in Hungary were examined for the presence of cytopathogenicity markers in the viral genomes. The viruses originated from different forms of BVD, i.e., MD cases, which were observed after vaccination with a live attenuated vaccine, termed BVDV-X, and respiratory symptoms that were not connected to vaccination [22]. Interestingly, none of these strains contained genetic alterations at positions A or B. However, three different small insertions of viral and cellular origin were detected at nucleotide position 4355, upstream of position A.

These insertions represent so far seldom-observed cytopathogenicity markers. The common feature of these insertions is that they occurred at the same position where a 27-nt insertion was found in BVDV strain CP7 [23]. The insertion in BVDV CP7 originates from the NS2 gene. In contrast, the 45-nt insertion found in the BVDV-X live vaccine and in the four Hungarian cp BVDV strains isolated from early MD cases (which are likely identical with the vaccine strain) was in-frame, and contained bases originally located at nucleotide position 8402-8446 of the NS4B/5A junction region [22]. The two additional genomic alterations found in the respiratory isolates represent a small viral duplication originating from the NS5B gene and a so-far unidentified cellular insertion, respectively.

Following these observations, further studies were carried out focusing on the role of the first novel insertion at position 4355. To rule out that other genomic alterations may be involved in the cytopathogenicity of BVDV-X, the whole genome of this virus strain was sequenced. The thorough genome scanning revealed that further genetic patterns contributing to the cp phenotype of BVDV-X are not present in the vaccine virus [24].

As the first step to elucidate the role of the 45-nt insertion, expression studies were performed with the original and an artificially generated insertion-negative cloned NS2-3 of BVDV-X. The results clearly revealed that this small insertion is responsible for a partial cleavage of NS2-3, leading to expression of NS3, the marker protein of cp BVDV strains [22].

As the second step, reverse genetic methods were used in this study to obtain a final proof that the 45-nt insertion is not only responsible for NS2-3 cleavage, but also for the cytopathogenicity of BVDV-X. Reverse genetic studies clarifying the molecular basis of cytopathogenicity of different cp BVDV strains have been performed in several laboratories by using full-length infectious cDNA clones [20, 31, 32, 33]. By using such a cDNA clone of BVDV reference strain BVDV CP7, the crucial role of the 27-nucleotide insertion in cytopathogenicity of BVDV CP7 has been demonstrated [31]. The same strategy was applied in the present work to BVDV-X. In order to examine further the possible role of this insertion in cytopathogenicity, a full-length infectious cDNA clone of the BVDV-X vaccine was generated. The recovered virus, termed BVDV-XR, showed slight retardation in growth in comparison with the wild-type BVDV-X. The reason for this finding is probably the appearance of four amino acid substitutions in the polyprotein of BVDV-XR. This phenomenon is in accordance with the data of previous full-length cDNA clones, as the amplification and cloning steps may result in the occurrence of mutations [31, 32]. Since the ncp counterpart of the vaccine virus was not available, an ncp mutant full-length cDNA clone pBVDV-XR-INS- was generated by PCR-directed mutagenesis. This mutant clone differed from BVDV-X only by the absence of the 45-nt insertion. The recovered virus BVDV-XR-INS- also showed the same growth characteristics as its cp counterpart, but caused no CPE, clearly proving the role of the 45-nt insertion in BVDV-X cytopathogenicity.

So far, one cellular and four viral insertions were found in the genomes of different cp BVDV isolates at nucleotide position 4355. The development of these cytopathogenicity markers has not

yet been elucidated. One possible explanation is that position 4355 may represent a hot spot for recombination of viral and cellular sequences. On the other hand, a functional selection of the BVDV genomes containing insertions at this particular position as a result of “productive recombinations” cannot be excluded. The cytopathogenic variant of BVDV generated by recombination events at nucleotide position 4355 may have considerable advantage for replication in the animal, therefore may be selected from the pool of other recombinants.

At present, the exact role of insertions at position 4355 in the NS2 gene of cp BVDV strains is not known. A recently found cysteine protease generates efficient NS3-3 cleavage in both cp and ncp BVDV, but the autoproteolysis is downregulated at 9 h p.i. It is hypothesized that the insertion found in CP7 interferes with the downregulation process by making the autoproteolysis less sensitive to cofactors [34]. The newly found insertions probably support more data for the better understanding of this process.

In summary, together with the results of this study, by using full-length infectious cDNA clones, the crucial role of two different viral insertions at position 4355 in cytopathogenicity of BVDV strains CP7 and BVDV-X has been established, respectively. The pBVDV-XR plasmid backbone provides available tool to examine the possible role of the additional two insertions at position 4355 in the genomes of Hungarian cp BVDV isolates originating from respiratory disease cases, since it is genetically very close to these strains. All of the insertions found at position 4355, although vary in size and nucleotide composition, very likely act in a similar way. By the use of full-length cDNA clones, further experiments will determine their common mechanism that contributes to the maintaining of the cp phenotype of BVDV.

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Table 1. List of primers used in this study for generating overlapping fragments of the whole genome of BVDV-X to construct the full-length clones pBVDV-XR and pBVDV-XR-INS-. The sequences of restriction endonuclease sites are indicated by italic, the *SmaI* site used for linearisation of the full-length clones is in bold, and the T7 promoter site included in the 5EXTT7 primer is underlined.

Code of the primer	Nucleotide sequence (5'-3')	Nucleotide position (5'-3')*
5EXTT7 (49-mer)	ATGC*GTCGAC*TAATACGACTCACTATAGGTATACG AGAATTTGCCTAAC	1-21
13A† (22-mer)	GCTAGCCATGCCCTTAGTAGGA	101-122
BVD7R (21-mer)	ACCAACCATGCTTGTTCCACT	1419-1399
INSF (40-mer)	CTGATGTGGAAAAGAGTTGGCTAGGGGGGGTGGAC TACAA	4335-4374
V5F (20-mer)	TCTGATGATTGCCACCCTAT	4210-4229
INSR (40-mer)	TTGTAGTCCACCCCCCCTAGCCAACTCTTTTCCAC ATCAG	4374-4335
4815R (20-mer)	CTGGTTCCTAACCTTGTGCT	4741-4722
V6R (20-mer)	AATTGTCCCCTAGCGGCGTA	5045-5026
AF3V1F (20-mer)	TAGGACTGTCTACTGCTGAG	7193-7212
CL2R (20-mer)	TGATCTGACACCGATTCACC	7707-7688
3EXTSMA (31-mer)	AT*GAGCTC***CCCGGG**GCTGTTAGAGGTCTTGG	12308-12289

* Nucleotide position corresponding to BVDV SD-1 [35]

† [25]

Table 2. Sequence differences between the full-length clone pBVDV-XR and the BVDV-X parent virus. The additional nucleotide differences between the mutated full-length clone pBVDV-XR-INS- and the BVDV-XR are also indicated (†).

Nucleotide Position*	Nucleotide exchange	Amino acid substitution	Protein Coded for
393	T→C	Silent	N^{pro}
1448 †	A→G	Asn→Ser	E^{rns}
2448	A→T	Silent	E1
2685	A→G	Silent	E2
3073	T→C	Cys→Arg	E2
4392 †	T→C	Silent	NS2
7358	G→A	Silent	NS4A
7844	A→G	Tyr→Cys	NS4B
8531	G→A	Ser→Asn	NS5A
12047	T→C	Val→Gly	NS5B

* Nucleotide position corresponding to BVDV SD-1 [35]

Fig. 1. (a) Schematic representation of the genomic organization of the BVDV-X vaccine strain. The arrows indicate the 45-nt insertion (black boxes) in the NS2, and at its original position at the NS4B/5A junction region. The four PCR fragments overlapping the whole genome of BVDV-X are shown below. The primers applied to generate the PCR amplicons are indicated above the respective PCR products. The 5' and 3' nucleotide positions of each amplicon are indicated below the respective PCR products. (b) Schematic representation of the strategy used to construct the full-length pBVDV-XR. The four PCR fragments were cloned separately (pANCRI, pANCRII, pANCRIII and pANCRIV) in low-copy-number vector pANCR1180. The pANCRIII and pANCRIV clones were joined to obtain pANCRIII,IV. The pANCRIII,IV and pANCRI clones were joined to obtain pANCRI,III,IV. In the final step, pANCRI,III,IV and pANCRII were joined to obtain the final pBVDV-XR full-length cDNA clone. The restriction endonuclease sites used for subcloning and the *SmaI* site for linearisation of the final clone are indicated with vertical black lines. The nucleotide positions of the respective restriction endonuclease sites are indicated in brackets.

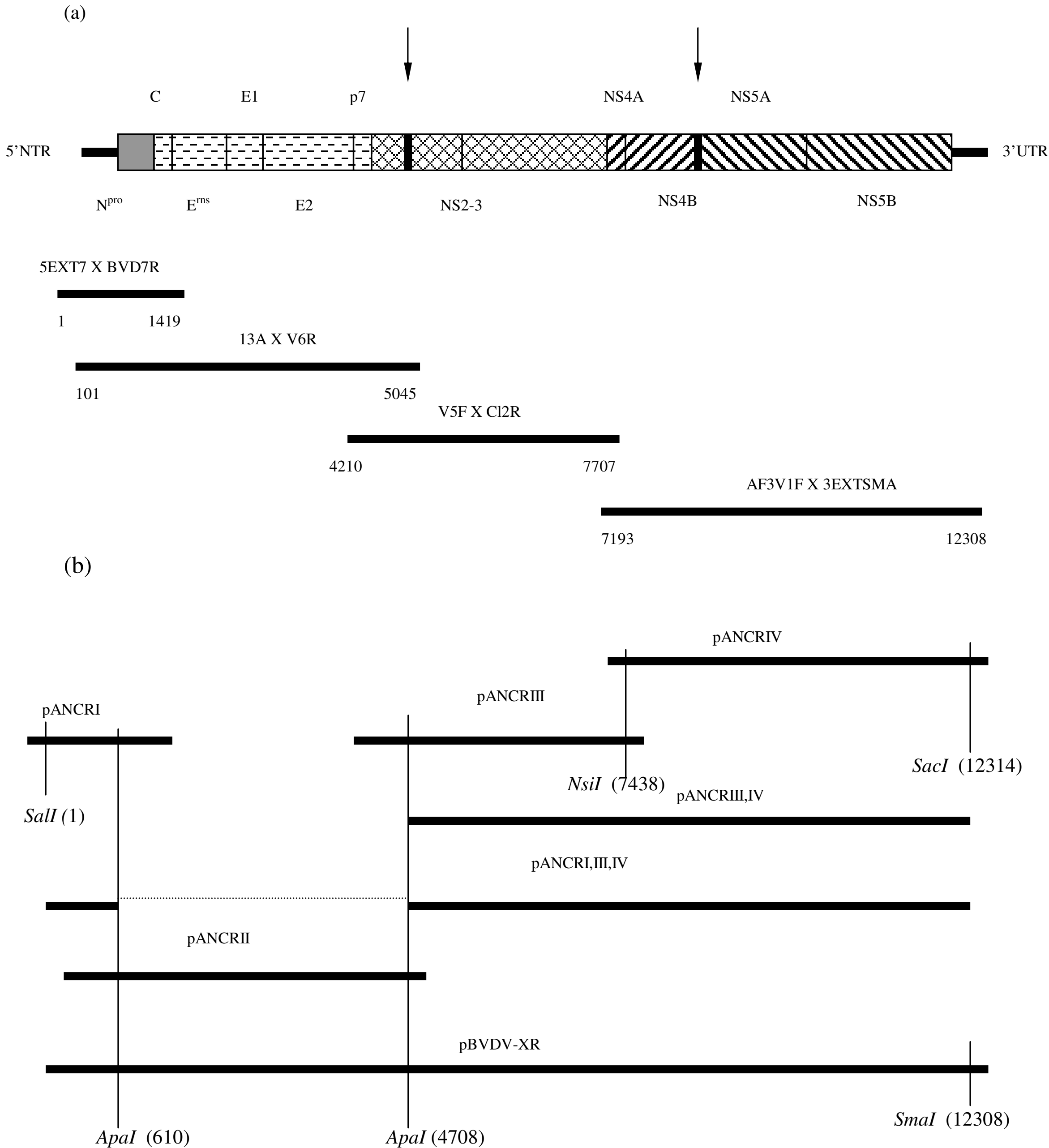
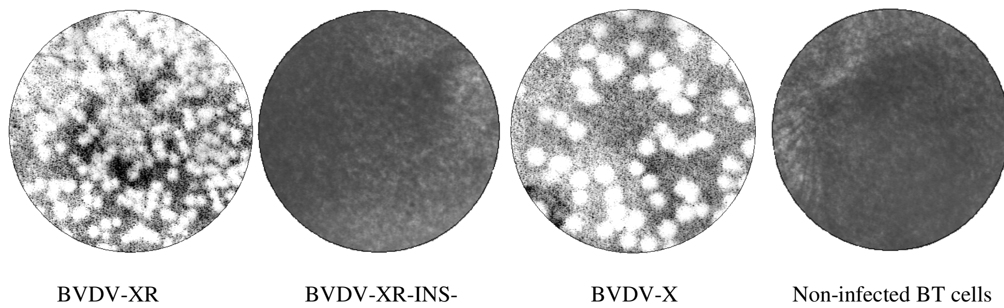


Fig. 2. (a) Crystal violet staining of BT cells 72h after infection with the first passage of viruses BVDV-XR and BVDV-XR-INS-, respectively. BT cells were inoculated with the respective viruses, overlaid with low-melting agarose containing EMEM and 2% FCS, and incubated at 37˚C in 5% CO_2 for 72 hours. Staining for visualisation of plaques was performed with a 1% (w/vol in 20% ethanol) crystal violet solution. The control panel shows BVDV-X and non-infected cells. (b) Immunoperoxidase assay of BT cells 48h after infection with the first passage of viruses BVDV-XR and BVDV-XR-INS-, respectively. BT cells were inoculated with the respective viruses, overlaid with low-melting agarose containing EMEM and 2% FCS, and incubated at 37˚C in 5% CO_2 for 48 hours. The IPX was carried out using BVDV polyclonal antiserum and peroxidase-conjugated rabbit anti-cow immunoglobulins. The control panel shows BVDV-X and non-infected BT cells.

(a)



(b)

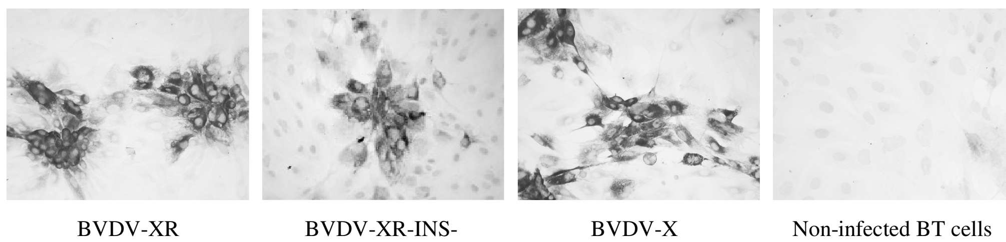


Fig. 3. RT-PCR assays for genetic identification of the first passages of the recombinant viruses BVDV-XR and BVDV-XR-INS-. Lane M: GeneRuler 1kb molecular weight marker (Fermentas); lanes a and b: PCR products of 1318 bp generated with primers targeting the 5'UTR-E^{rns} region using BVDV-X and BVDV-XR as templates, respectively; lanes c and d: PCR products of 854 bp generated with primers targeting the NS2 region using BVDV-X and BVDV-XR as templates, respectively; lanes e, f and g: PCR products of 549 and 504 bp generated with primers flanking the insertion using BVDV-X, BVDV-XR and BVDV-XR-INS- as templates, respectively.

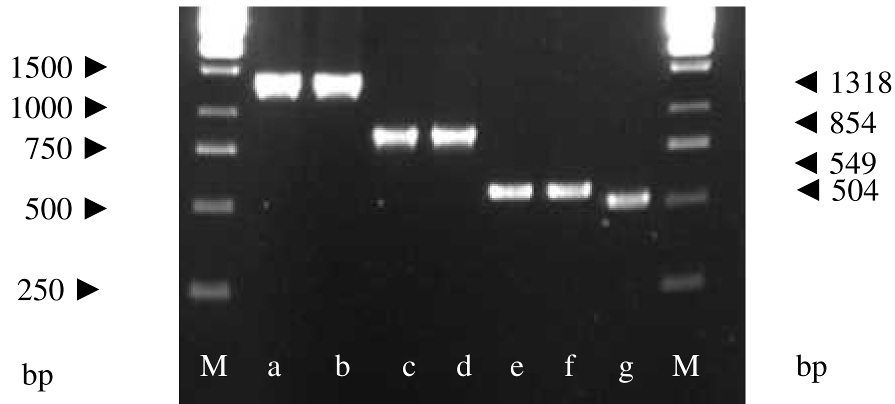


Fig. 4. Growth curve of BVDV-XR, BVDV-XR-INS- recombinant viruses and the BVDV-X parent virus. BT cells were infected with the respective viruses at an MOI of 0.1 and harvested at the indicated time points. Titres were determined on new BT cells by plaque assay in the case of BVDV-X and BVDV-XR, and by IPX in the case of BVDV-XR-INS-. The obtained titres are given in log_{10} PFU per millilitre (+1 standard deviation [error bars]). P.i. means post infection.