SZENT ISTVÁN UNIVERSITY
Doctoral Training Programme in Veterinary Sciences

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

Objectives of the PhD thesis

By

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Budapest
2005

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SCIENTIFIC BACKGROUND AND AIMS

Bovine viral diarrhoea virus (BVDV) is a major pathogen that causes significant economic losses worldwide in the cattle industry. Bovine viral diarrhoea (BVD) is one of the most important viral diseases of cattle all over the world. This revelation led in several European countries (Sweden, Finland, Denmark, Norway) to work out and start eradication programmes. In Hungary nearly 100% of cattle herds can be considered infected with BVDV and starting eradication will be necessary in the near future.

According to their ability to cause cytopathic effect (CPE) in infected cell cultures, BVDV strains can be distinguished into two biotypes: cytopathogenic (cp) or a non-cytopathogenic (ncp). Infection of pregnant cattle with ncp BVDV may result in birth of persistently infected (PI) calves. PI animals are immunotolerant against the respective ncp virus and are predisposed to develop fatal mucosal disease (MD). From animals succumbing to MD, both cp and the persisting ncp biotypes can be isolated. These viruses are called a “virus pair”.

Molecular analysis of different BVDV pairs elucidated that the cp biotypes are generated in the persistently infected (PI) cattle by genomic alterations of the ncp biotype. Insertions of cellular sequences, duplication of viral genomic regions with or without insertions, deletions and point mutations are present in the genomes of cp strains. These genetic alterations are termed cytopathogenicity markers (cp markers). In most cp BVDV strains the role of the cp markers is well defined, but in some cases further genetic and functional

analyses are needed to elucidate the basis of cytopathogenicity.

To protect cattle against BVDV, attenuated and inactivated vaccines are used in many countries. The main advantage of live attenuated vaccines is their low price and high efficacy. However, live BVDV vaccines cause several adverse effects. Besides causing immunosuppression and congenital defects, if PI animals are vaccinated, MD may occur. If the vaccine strain is antigenically closely related to the ncp BVDV strain in the PI animal, *early onset* of postvaccional MD can occur. If the antigenic relationship is distant, 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. In spite of the worldwide usage of the live vaccines, the exact molecular basis of attenuation of BVDV is not known. A better understanding of attenuation is needed to develop more efficacious and safe BVDV vaccines.

The main goal of our studies was to investigate the molecular biology of BVDV, with special regard to 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, termed here for ethical reasons BVDV-X, our further goal was to elucidate the scenario around the application of this vaccine. The question was studied by using methods of molecular biology in a retrospective study.

Specific aims of the studies

- To elucidate the molecular basis of cytopathogenicity of Hungarian cp BVDV strains isolated in the early 1970s from various forms of BVD (i.e., respiratory disease, enteritis and MD).
- To clarify the genetic factors contributing to the cytopathogenicity of recently isolated Hungarian cp BVDV strains.
- To clarify the observation that different BVDV strain-derived sequences are present in various batches of BVDV-X by determining the full-length genomic sequences of the marketed BVDV-X and of the BVDV-X pre-registration vaccine batches.
- To detect possible attenuation markers in the genome of BVDV-X.
- To confirm the role of the cytopathogenicity marker found in the genome of BVDV-X by using transient expression and reverse genetic studies.

MATERIALS AND METHODS

Cell cultures, infection and virus titration

The experiments were performed according to standard procedures. Secondary cultures of bovine turbinate (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.

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) was found to be the most suitable method, even in the case of generating long PCR products to assemble full-length cDNA.

Synthesis of cDNA

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

For generation of long cDNA copies, the Superscript II RT (Invitrogen) and an antisense primer VDAS1, complementary to the 3'UTR of the genome was used. Using double-stranded cDNA did not increase the signal from amplification of large fragments.

PCR

Single PCR assays using the Expand High Fidelity Kit and Expand Long Template Kit (Roche) 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) was used, since the efficacy and proof-reading activity was found better than those of other DNA polymerase enzymes.

Cloning and nucleotide sequencing

Cloning and subcloning procedures were performed according to standard procedures. For cloning the whole NS2-3 region of BVDV-X, the pCI mammalian expression vector (Promega) was used. To avoid the instability problems of the full-length cDNA clone of BVDV-X, a low-copy-number plasmid pANCR1180 and ElectroTen Blue recombinant *E. coli* competent cells (Stratagene) were selected. Sequencing work was carried out with the ABI Prism sequencer (Model 377), using the Big Dye Terminator V3.1 sequencing kit (Applied Biosystems). The obtained sequences were edited and analysed with the multiple programs of the DNAstar software package (Lasergene).

Western blot analysis

Transfected cells were scraped from the bottom of the wells of the tissue culture plates, centrifuged at 10 000xg 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 for SDS-PAGE and Western blot analyses.

Immunoperoxidase (IPX) staining

IPX is used for detection of BVDV specific antigens in serum samples of persistently infected animals and in preparations containing ncp virus. This technique was used to demonstrate the presence of viral antigens in cells transfected with RNA recovered from the full-length cDNA clones.

RESULTS AND DISCUSSION

In the first study the possible cytopathogenicity markers have been examined in the genomes of six “archive” cp BVDV strains, isolated in the 1970s. The viruses were selected as representatives of various forms of BVD: enteritis, and MD, as well as respiratory syndrome. The MD cases were presumably associated with the use of the live attenuated vaccine BVDV-X. The complete NS2-3 coding region of the six isolates and of the vaccine virus were amplified by RT-PCR and sequenced. The results showed that new cp markers were found in all the cp BVDV strains at nucleotide position 4355 in the NS2 gene. These cp markers resemble to a very rare cp marker that was observed for far only in the genome of reference strain BVDV CP7. The NS2-3 (and the 5’UTR and E2) regions of the isolates originated from the vaccination accidents and of the BVDV-X vaccine virus were identical, indicating that the vaccine caused *early onset* of MD. The cp markers 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. 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. 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 93 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.

In the second study two recently isolated cp BVDV strains were characterised. These viruses originated from MD cases. 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 and BVDV 2 strains. In the genome of cp BVDV strain H115/PCR, a large genome duplication was found. The duplication contained a complete ubiquitin monomer and the complete NS3 gene. 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 vaccine 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, preliminary sequencing data suggested the recombination between the vaccine and wild type strains of BVDV during vaccine production. Therefore, the genomes of pre-registration (BVDV-Xpre) and of marketed (BVDV-X) batches of the vaccine were 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 to BVDV reference strain Osloss. The identity to BVDV Oregon C24V was significantly lower (77.4%). 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. 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 last two studies, the role of the cytopathogenicity marker found in the genome of BVDV-X was examined. In the first step, the role of the 45-nucleotide insertion in the expression of NS3 was investigated. 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 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 on applied research in veterinary virology.

NEW SCIENTIFIC RESULTS

- 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.
- In the genomes of two cp BVDV strains, isolated recently from MD cases, two different, but similar to the earlier described cytopathogenicity markers were found, proving that recombinations leading to cytopathogenicity of BVDV frequently occur at positions A and B.
- 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.

- The whole genome of BVDV-X was sequenced, and the data revealed that the marketed vaccine did not contain Oregon C24V sequences, indicating a strain-switch during vaccine production. The marketed vaccine belongs to BVDV 1b subgroup and has the closest similarity to reference BVDV strain Osloss.
- 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.
- Construction and mutation of the full-length infectious clone of BVDV-X proved that a 45-nucleotide viral insertion is responsible for the cytopathogenicity of BVDV-X.
- The backbone of the cDNA clone will be a useful tool to examine the background of cytopathogenicity in the genomes of two newly detected BVDV variants.
- The constructed cDNA clone can serve as the basis of a genetically modified live vaccine.

PUBLICATIONS

Articles

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 diarrhoea virus as potential cytopathogenicity markers. *Virus Genes* **30:** 49-58.

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Abstracts

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Bálint, Á., Pálfi, V., Baule, C., Dencső, L., Hornyák, Á., Belák, S. Gene expression and reverse genetic studies to determine cytopathogenicity of bovine viral diarrhoea virus. *Hungarian Academy of Sciences*. Budapest, January 25, 2005. (lecture)

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