Veterinaria Italiana, **42** (3), 165-172

Characteristics of the 5´ untranslated region of wisent (*Bison bonasus*) and reindeer (*Rangifer tarandus*) *Pestivirus* isolates

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Summary

The 5´-untranslated region (5´-UTR) of the pestivirus strains isolated from wisent and reindeer suffering from severe diarrhoea in the Duisburg zoo in Germany were amplified by reverse transcription-polymerase chain reaction and sequenced for comparison with those of other pestiviruses from cattle, sheep, goats and swine. Phylogenetic trees constructed from the primary nucleotide sequences of these strains demonstrated that the 5´-UTR of both the wisent and reindeer isolates were identical and suggested that the isolates were allocated to a new cluster of Border disease virus (BDV). The BDV strains were further divided into at least three genotypes or subspecies by phylogenetic analysis and a newly proposed method based on palindromic nucleotide substitutions was used at the variable regions in the 5´-UTR.

Keywords

Border disease virus, *Pestivirus*, Phylogenic trees, Reindeer, Wisent.

Caratteristiche della regione non tradotta 5′ di isolati pestivirali di bisonte Europeo (*Bison bonasus*) e renna (*Rangifer tarandus*)

Riassunto

La regione non tradotta 5´ (5´-UTR) di ceppi di Pestivirus *isolati da un bisonte Europeo e una renna affetti da severa diarrea, provenienti dallo zoo di Duisburg, Germania, é stata amplificata con transcriptasi inversa - polymerase chain reaction e sequenziata al fine di compararla con altre ottenute da* Pestivirus *originati da bovini, pecore, capre e suini. Gli alberi filogenetici costruiti sulla base delle sequenze nucleotidiche primarie di questi ceppi suggerivano la classificazione degli isolati wisent e reindeer, la cui 5´-UTR era identica, in un nuovo gruppo di* Border disease *virus (BDV). I ceppi BDV sono stati ulteriormente divisi in almeno tre genotipi o sottospecie attraverso analisi filogenetica e un metodo proposto recentemente basato sulle sotituzioni nucleotidiche palindromiche nelle regioni variabili della 5´-UTR.*

Parole chiave

Border disease virus, *Pestivirus*, Bisonte europeo, Renna.

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Introduction

The genus *Pestivirus*, family *Flaviviridae*, is classified into four established species, Border disease virus (BDV), bovine viral diarrhoea virus 1 (BVDV-1) and 2 (BVDV-2), classical swine fever virus (CSFV) (hog cholera) and one tentative species, 'giraffe' pestivirus (24). These viral species are mostly identified according to the animal host species from which they were isolated. There is extensive antigenic cross-reactivity among them and they can cross the host-species barrier and infect different species of cloven-hoofed animals (7, 18, 22). Although CSFV has long been restricted to swine species, recently a CSFV strain was isolated from sheep in Spain (16). Serological data indicate that the host range of pestiviruses includes most even-toed ungulates (17). Pestiviruses have been isolated not only from domestic animals but also from wildlife, such as deer or giraffe (*Giraffa camelopardalis*). A taxonomic study of isolates from wildlife will provide useful information on the ecology and evolution of pestiviruses. Pestivirus strains isolated from deer in Germany, England and New Zealand have been assigned to BVDV-1 (3, 8) or to an unclassified group (19, 25), according to phylogenetic analyses of the N^{pro} (N-terminal autoprotease) and E2 (gp53) genes or the 5´-untranslated regions (5´-UTR). In November 1996, a wisent (*Bison bonasus*) and two reindeer (*Rangifer tarandus*), suffering from severe diarrhoea and anorexia, died in the Duisburg Zoo in Germany. These animals were about six months old at the time of death. The carcasses of the wisent and reindeer were autopsied. A pestivirus infection was diagnosed using the BVDV antigen capture enzyme-linked immunoassay (ELISA) and the causative viruses were isolated from leukocyte samples of these animals in Madin-Darby bovine kidney (MDBK) cells cultivated with medium supplemented with horse serum (MDBK-HS). Eleven animals from the reindeer herd in the Duisburg Zoo were examined serologically and all were found to be positive for BVDV antibody. This suggests that pestivirus infection was prevalent in the herd. The viral isolate from a reindeer was identified as a BDV in the phylogenetic trees based on the N^{pro} and E2 genes (5). In the present study, the 5´-UTR of the wisent and reindeer isolates were examined, in terms of palindromic nucleotide substitutions (PNS) which provide precise relationships among pestiviruses (14).

Materials and methods

Virus strain

The viral stains isolated from a wisent (European bison) and a reindeer, respectively, were subcultured four times in pestivirus-free MDBK cells. Their infective titres reached $10^{6.0\text{-}7.0}$/$TCID_{50}$/ml. The viral strains examined were held at the Institute of Immunology, Federal Research Centre for Virus Diseases of Animals in Tübingen, Germany. Both strains were used for reverse transcription-polymerase chain reaction (RT-PCR) of the 5´-UTR and sequence analyses. Nucleotide sequences from other pestiviruses were obtained from DNA databases or the literature. Primary and secondary structures of the wisent and reindeer strains were compared with those of other pestiviruses. Four palindromic sequences in the 5´-UTR of the two strains were subjected to PNS analysis.

Extraction of pestivirus RNA from virus cultures

Ribonucleic acids were isolated from pestivirus culture by the single-step guanidinium isothiocyanate-phenol-chloroform method (6) by using Trizol (Gibco-BRL, Gaithersburg, Maryland). Briefly, 200 µl of the virus culture fluids were mixed with 1 ml of Trizol solution containing guanidinium isothiocyanate-phenol in a 1.5-ml Eppendorf tube at room temperature for 5 min. To this, 200 µl of chloroform were added and the

tube was vigorously shaken by hand. The tube was incubated at room temperature for 3 min and spun in an Eppendorf centrifuge at 4°C for 15 min. The aqueous phase (500 µl) was transferred into a fresh Eppendorf tube and 500 µl of isopropanol were added. The RNA precipitate was collected by centrifugation at 8 000 x g at 4°C for 10 min. The pellet was washed three times with 1 ml of 75% ethanol. The pellet was air-dried, dissolved in 10 µl of sterile distilled water treated with 0.1% diethyl pyrocarbonate (DEPC). The RNA solution was subjected to RT reaction.

Oligonucleotides

Oligonucleotide primers used for the RT-PCR targeted at the 5´-UTR of pestiviruses were first described in 1993 (12). The first strand of pestivirus cDNA was synthesised by using an oligonucleotide primer R1 (5´-ACTCCATGTGCCATGTACAG-3´). Thereafter, primers F1 (5′-ATGCCC(A/T)(C/TAGTAGGACTAGC-3´) and R1 amplified an approximately 285 bp product from the pestivirus cDNA by the PCR. Positions of these primers relevant to the sequence of the NADL strain of BVDV-1 are F1=108-127 and R1=373-392. All oligonucleotides were custom-made by the Takara-Bio company in Kyoto, Japan.

Reverse transcriptase reaction

The cDNA synthesis and PCR were performed as described previously (10). To 8 µl of each RNA solution were added 8 µl of 5x buffer and 4 µl 0.1M dithiothreitol, 0.25 µl (25 units) of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL), 8 µl deoxynucleotide triphosphates (dNTPs) to a final concentration of 0.2 mM each, 0.2 µl (110 IU/ml) ribonuclease inhibitor (Takara-Bio), 0.25 µl R1 primer (40 pmol/µl), and DEPC-treated water to a final volume of 40 µl. The synthesis of cDNA was performed at 37°C for 90 min. A 5 µl portion of this reaction mixture was used directly for the PCR assay.

Polymerase chain reaction

The amplification of the 5´-UTR was performed according to the method described by Giangaspero *et al.* (10). Six microlitres of cDNA solution were added to 5 µl of 10x buffer (100 mM Tris-HCl, pH 8.9, 800 mM KCl, 15 mM $MgCl_2$, 5 mg/ml bovine serum albumin, 1% sodium chlorate, 1% Triton X-100), 1 µl (1.25 U) *Thermus thermophilus* (*Tth*) DNA polymerase (Chimerx, Madison, Wisconsin), 3 µl *Tth* buffer (10 mM Tris HCl, pH 7.5, 300 mM KCl, 1 mM DTT, 0.1 mM disodium ethylenediaminetetracetate [EDTA], 50% glycerol), dNTPs to a final concentration of 0.2 mM each, 0.25 µl of F1 and R1 primers (40 pmol/µl each) and water to a final volume of 50 µl. After the mixture was overlaid with mineral oil, the reaction cycle was conducted 30 times with denaturation at 94°C for 30 sec, annealing at 55°C for 120 sec and extension at 72°C for 120 sec in a thermal cycler.

Agarose gel electrophoresis

The PCR products were fractionated using agarose gel electrophoresis and visualised by staining with ethidium bromide. Ten-microlitre aliquots of the PCR products were mixed with 2 µl of 6x dye solution consisting of 0.25% xylene cyanol, 0.25% bromophenol blue and 40% sucrose in water, run on horizontal, submerged 2.0% NuSieve 3:1 agarose gels (FMC Bioproducts, Rockland, Maine) in TAE (40 mM Tris, pH 8.0, 5 mM sodium acetate, 1 mM disodium EDTA) buffer at 50 V for 50 min, and stained with ethidium bromide (0.4 µg/ml) for 15 min.

Analysis of the nucleotide sequences

Clearly visible bands of the expected size for the PCR primer pairs were extracted from the gels and were subjected to direct sequencing on each strand in an ABI Prism 310 genetic analyser (Perkin-Elmer Corporation, Norwalk, Connecticut). Secondary structures were predicted according to the algorithm of Zuker and Stiegler (27) and compared with those of BVDV and BDV. The

folding energies were calculated using the method of Freier *et al.* (9). Four variable regions in the 5´-UTR were subjected to PNS analysis (14). A phylogenetic tree based on the 5´-UTR was constructed from sequence alignment with Clustal X (23) by using the neighbour-joining method (21). The nucleotide sequences of relevant regions were obtained from the published data for reference pestivirus strains. Nucleotide sequences of the reference BVDV and CSFV strains, obtained from the DNA databases, are as follows [accession number is given in square brackets]: strains NADL [M31182], SD-1 [M96751] and Oregon [L32876] for BVDV-1a, strains Osloss [M96687], Draper [L32880], Sanders [L20928] and NY-1 [L32879] for BVDV-1b, strains SE5572 [Z79770] and Europa [AB000898] for BVDV-1c, strains 1373 [AF145967], 890 [L32886] and CD87 [L32887] for BVDV-2, and strains Brescia [AF091661], Alfort [J04358], and Alfort/187 [X87939] for CSFV. Nucleotide sequences of the BDV strains deposited to date in the DNA databases are as follows: strains 91/5809 [AF026768] and 90/8320/31 [AF026769] strain BD31 [U70263] (20), strain X818 [AF037405] (2), strain Ch1Es [D50816] (13), and strains Moredun cp [U65022], Moredun ncp [U65023], A841/1 [U65026], A1263/2 [U65027], A1870 [U65028] D1586/2 [U65034], G1305 [U65035], G2048 [U65036], JH2816 [U65037], K1729/3 [U65038], L991 [U65039], Q1488/1 [U66042], Q1488/6 [U65043],Q1673/2 [U65044], T1789/1 [U65045], T1802/1 [U65046], V1414 [U65047], V2377/12 [U65048], V2536/2 [U65049], V3196/1 [U65050] 137/4 [U65052],135 661 [U65054],170 337 [U65057], 8320-22NZ [U65063], and 8320-31NZ [U65064] (26), strains 2112/99 [AY159513], 79248/01 [AY159515], 80582/01 [AY159516] and 87877/01 [AY159513] (16). Accession numbers of the nucleotide sequences of the other deer strains 95-4845A and 95-4845B obtained from DNA databases are U80903 and U80904, respectively (3). The nucleotide sequence of strain SH9/11 isolated from roe deer (*Capreolus capreolus*) was obtained directly from the publication of Frolich and Hofmann (8). The accession number of the nucleotide sequence of strain V60 (or Reindeer-1) isolated from a reindeer is AF144618 (1).

Results

Phylogenetic trees based on sequence alignment

Nucleotide sequences of the 5´-UTR of the wisent and reindeer strains were identical, but were distinct from the reindeer strain V60 by a single nucleotide difference. Their taxonomic status at the species level was estimated by a phylogenetic tree constructed from the alignment with the representative strains from the four established species and 'giraffe' strain (Fig. 1). This phylogenetic tree at the species level indicated that the 23 pestivirus strains were divided into five clusters, BVDV-1, BVDV-2, BDV, CSFV and 'giraffe'. The roe deer strain SH9/11 was allocated to a group along with strains Europa and SE5572, which has previously been classified as BVDV-1c (15). The wisent and reindeer isolates were assigned to the BDV cluster in the tree, but were distinct from previously reported strains of BDV. The nucleotide sequences from the 5´-UTR of the strains were then aligned with other BDV strains for maximum matching (Fig. 2). A phylogenetic tree based on this alignment suggested that the BDV strains were divided into at least three genotypes or subspecies, BDV-1, BDV-2 and BDV-3 (Fig. 3). Although the nucleotide sequences at the 5´-UTR were well conserved among the strains, four palindromic nucleotide sequences designated as PNS-1, PNS-2, PNS-3 and PNS-4 were obvious in the 5´-UTR.

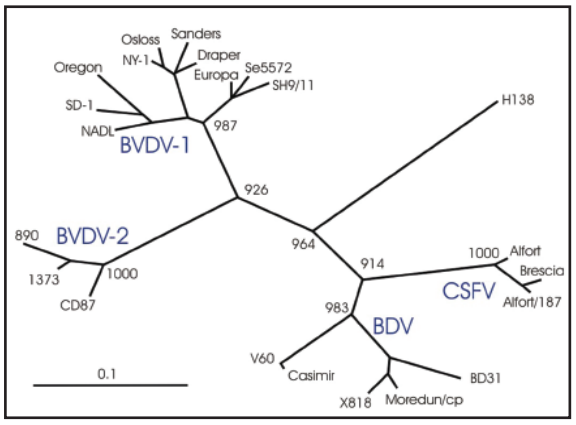

Figure 1
Phylogenetic tree based on the 5′-UTR comparison, suggesting a taxonomic position of the wisent and reindeer strains in the *Pestivirus* genus
Strains NADL, Oregon and SD-1 are references for the BVDV-1a genotype
Strains Osloss, NY-1, Draper and Sanders are references for the BVDV-1b genotype
Strains Europa and SE5572 are references for the BVDV-1c genotype
Strain SH9/11 is a deer isolate from Germany (8)
Strains 890, CD87 and 1373 are references for BVDV-2
Strains Moredun-CP, BD31 and X818 are references for BDV
Strains Alfort, Brescia and Alfort/187 are references for CSFV
Strain H138 is an isolate from giraffe (15)
Strain V60 is an isolate from reindeer (1)
Distances were computed with Clustal X using the neighbour-joining method
Numbers at the relevant branches refer to bootstrap values of 1 000 replications
Scale bar indicates 10 nucleotide substitutions per 100 nucleotides

Palindromic nucleotide substitutions analysis of the secondary structures at the 5′-UTR

Stable stem-loop structures with substantial negative free energies were predicted at the four palindromic sequences at the 5′-UTR of the strains. Three genotypes or subspecies in the BDV species were also evident by PNS analysis (Fig. 4). In the stem-loop structure at PNS-1, BDV-1 showed a characteristic U-A pairing at position 2 from the bottom of the stem, BDV-2 showed a characteristic C-G pairing at position 5, and BDV-3 showed a

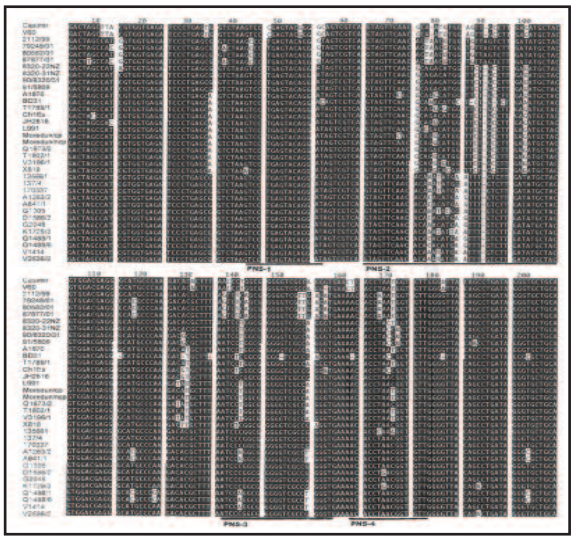

Figure 2
Nucleotide alignment of the 35 pestivirus cDNA sequences at the 5′-UTR
The wisent sequence was used in this alignment because the wisent and reindeer sequences were identical
Consensus nucleotides are shown as inverted characters
The nucleotide sequence numbers are given from a consensus alignment, which are relevant to positions 138-377 of the BVDV-1 strain NADL
Dashes represent spaces between adjacent nucleotides introduced for maximum alignment
Four palindromic sequences: PNS-1 (37 to 52) PNS-2 (61 to 100) , PNS-3 (131 to 154) and PNS-4 (158 to 173) are underlined

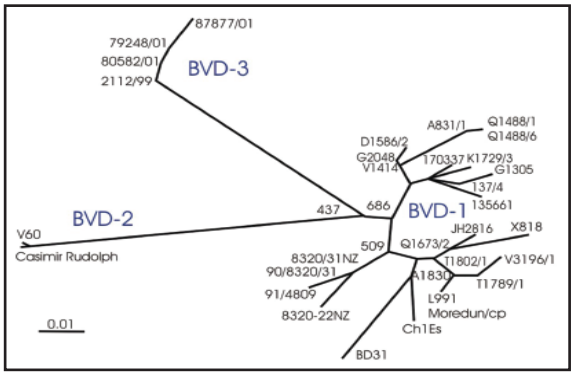

Figure 3
Phylogenetic tree based on the 5′-UTR comparison, suggesting a relationship among the wisent and reindeer strains and other Border disease virus strains
Distances were computed with Clustal X using the neighbour-joining method
Numbers at the relevant branches refer to bootstrap values of 1 000 replications
Scale bar indicates 10 nucleotide substitutions per 100 nucleotides

characteristic C-G pairing in position 2. In the stem-loop structure at PNS-2, BDV-3 showed characteristic base-pairings C-G and U-A at positions 17 and 14, respectively, from the bottom of the stem. The BDV-2 strains showed a remarkable PNS, U*G base-pairing at the 16th position at the PNS-2 region with minimum free energy of –10.71 Kcal/mol, and from U to C at the second position from the bottom of the stem-loop structure at the PNS-3 region with minimum free energy of –24 Kcal/mol. BDV-3 showed a remarkable PNS, A-U pairing, at position 6 in the PNS-3 structure. The number of base-pairings at the stem region for PNS-4 of the wisent and reindeer isolates in BDV-2 totalled four, which is smaller than that previously reported for BDV.

Nucleotide sequence accession numbers

The nucleotide sequence of the wisent and reindeer strains first presented in this paper have been deposited in the DDBJ, EMBL, GSDB and NCBI nucleotide sequence databases under accession numbers AB122085 and AB122086, respectively.

Discussion

Phylogenetic relationships of the pestivirus isolated from wild animals, including deer, have been reported previously (3, 4, 8, 25). Van Rijn *et al.* (25) reported that a United Kingdom deer isolate was distinct from other pestiviruses, but most similar to BVDV-1, based on a comparison of the E2 envelope glycoprotein gene. Although pestiviruses are traditionally designated by the host animal species, the strains isolated from deer or roe deer have been allocated to BVDV-1. Strains 95-4845A and 95-4845B isolated from deer in New Zealand, and strain 'Deer' isolated from deer in the United Kingdom have been assigned as BVDV-1 (3). Similarly, strain GB1 isolated from deer in the United Kingdom has been identified as BVDV-1 (4). Strain SH9/11 isolated from roe deer in Germany has also been classified as BVDV-1 based on the N^{pro} gene and 5´-UTR (8). These observations suggest that BVDV-1 strains can cross-infect deer as well as cattle, sheep, goats and swine. Our previous phylogenetic results based solely on the primary nucleotide sequences of the 5´-UTR also supported a notion that the SH9/11 strain was allocated to BVDV-1 (13). In the present study, the wisent and reindeer isolates were allocated to BDV by the phylogenetic analysis based on the 5´-UTR. Our results suggest that the BDV can cross-infect wisent and reindeer as well as goats and sheep, and that these wild ruminants may serve as a reservoir of BDV. Further examination of the genetic relationship among the BDV strains suggested that the BDV can be divided into at least three genotypes or subspecies based on the 5´-UTR.

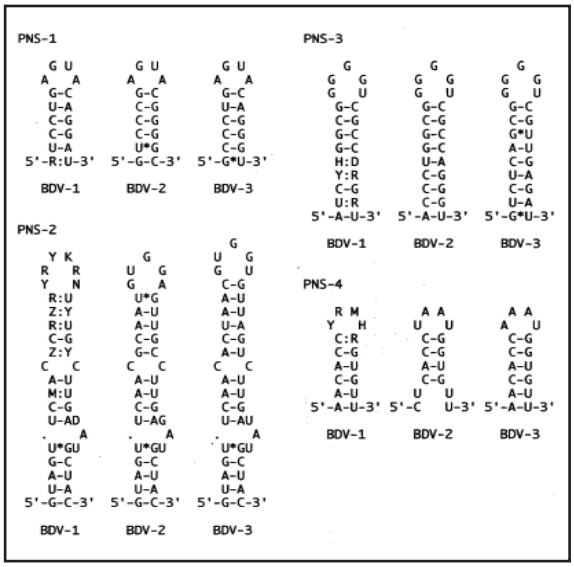

Figure 4
Secondary structures predicted for the four palindromic nucleotide sequences: PNS-1, PNS-2, PNS-3 and PNS-4 at the 5´-UTR of Border disease virus strains

– Watson-Crick base-pairing
* G and U pairing tolerated in secondary structures
: interchangeable base-pairings

Interchangeable nucleotides are shown by H for A, C, and U, K for G and U, M for A and C, R for A and G, W for A and U, and Y for C and U

Although the 5´-UTR is well conserved, incidence of some nucleotide substitutions observed in this region is biased by the selection of lethal mutations. Random mutations at the 5´-UTR have a high probability of incompatibility with viral survival since this region includes regulatory motifs which are indispensable to replication and translation of the virus RNA. We examined the secondary structures of the wisent and reindeer strains by PNS analysis, an independent and confirmative method, and found that they were distinct from the other BDV strains. This was compatible with the phylogenetic results based on the primary nucleotide sequences. The SH9/11 isolate from roe deer in Germany reported by Frolich and Hofmann (8) has been identified as BVDV-1c by the PNS method (15). The 95-4845A and 95-4845B strains described by Becher *et al.* (3) had characteristics of BVDV-1 at the PNS-3 and PNS-4 regions in the 5´-UTR, though their exact genotype has not been identified since their PNS-1 and PNS-2 regions were not available.

The analyses based on primary nucleotide sequence homology and on secondary palindromic sequence structures of the 5´-UTR of the wisent, reindeer and V60 strains suggested that they should be assigned to BDV-2, a new genotype or subspecies of BDV. Reindeer strain V60 has reportedly been allocated to genotype BDV-2 of the BDV species in the phylogenetic trees based on the N^{pro} and E2 genes (5). Our PNS as well as phylogenetic analyses on the 5´-UTR also suggested that the wisent and reindeer isolates were allocated to the BDV, but were distinct from previously reported BDV strains (11). We followed the nomenclature system proposed by Becher *et al.* (5), and demonstrated the PNS criteria to classify the BDV strain into at least three genotypes or subspecies, BDV-1, BDV-2 (representing the wisent, reindeer and V60 strains) and BDV-3 (representing 2112/99, 79248/01, 80582/01 and 87877/01 strains) in the present study.

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