Phylogenetic analysis of pestiviruses from domestic and wild ruminants

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Infections with pestiviruses occur in cattle, sheep, pigs and also in numerous other ungulate species. In the present study, pestiviruses from goat, buffalo, deer and giraffe were analysed at the molecular level; unusual strains from cattle and pigs were also included. A phylogenetic analysis of the respective pestiviruses was undertaken on the basis of a fragment from the 5′ non-coding region as well as the gene encoding autoprotease Npro. Statistical analyses of the respective phylogenetic trees based on the 5′ NCR revealed low confidence levels for most of the branches, while the structure of the tree based on the Npro gene was supported by high bootstrap values. Accordingly, the isolates from goat, buffalo and deer can be grouped together with bovine viral diarrhoea virus (pestivirus type 1); within this genotype three subgroups and one disparate virus have been identified. One isolate from pig and one from cattle belong to the group of ‘true’ border disease virus (pestivirus type 3), which can be further subdivided into two major subgroups. Interestingly, the giraffe isolate does not belong to one of the four established pestivirus genotypes. The phylogenetic analysis strongly suggests that genotype 1 pestiviruses occur world-wide in many ruminant species. Furthermore, phylogenetic trees based on the Npro gene nucleotide sequences show that the respective sequences do not segregate into discrete lineages based on host-species origin.

Introduction

The genus Pestivirus in the family Flaviviridae currently contains three accepted members: bovine viral diarrhoea virus (BVDV), classical swine fever virus (CSFV) and border disease virus (BDV) of sheep (Wengler et al., 1995). Traditionally pestiviruses are named after the affected species and the diseases they cause. Pestiviruses are, however, able to cross species barriers to infect a wide range of hosts within the Artiodactyla. This conclusion is mainly based on serological investigations, where pestivirus-specific antibodies have been detected in more than forty ruminant species (Hamblin & Hedger, 1979). In rare cases, pestiviruses have been isolated from some of these species, including goat (Fraser et al., 1981; Loken et al., 1982), buffalo (Plowright, 1969), deer (Doyle & Heuschele, 1983; Nettleton, 1990) and giraffe (Plowright, 1969). Little is known about the role of pestiviruses in causing disease in these species. For goats, spontaneous clinical border disease (BD) as well as isolation of pestiviruses from foetuses have been described (Fraser et al., 1981; Loken et al., 1982). For some other captive and free-living ruminants, outbreaks of disease resembling mucosal disease (MD) of cattle have been reported and pestiviruses have been isolated from such cases (Karstad, 1981).

Pestiviruses have single-stranded, positive-sense RNA genomes. For all three species, the viral RNA has been demonstrated to be generally approximately 12.3 kb in length and to contain one large open reading frame flanked by 5′ and 3′ non-coding regions (NCR) (Becher et al., 1994; Collett et al., 1988b; De Moerlooze et al., 1993; Deng & Brock, 1992; Meyers et al., 1989; Moormann et al., 1990). Viral gene expression is believed to occur via synthesis of a putative polyprotein and subsequent proteolytic processing mediated by cellular and viral proteases (Collett et al., 1988a). The first protein of the polyprotein is nonstructural, designated as

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The nucleotide sequences referred to in this paper have been submitted to GenBank and assigned the accession numbers U80897–U80907.
autoprotease N$^{pro}$ (Stark et al., 1993; Thiel et al., 1991; Wiskerchen et al., 1991). This protein is followed by the structural proteins C (Thiel et al., 1991), E$^{pro}$, E1 and E2 (Collett et al., 1988a; Thiel et al., 1991) and the nonstructural proteins p7 (Elbers et al., 1996), NS2-3, NS4A, NS4B, NS5A and NS5B (Collett et al., 1988a). Two biotypes of pestiviruses, cytopathogenic (cp) and noncytopathogenic (noncp) viruses, are distinguished by their effect on tissue culture cells (Becher et al., 1996; Gillespie et al., 1960; Meyers & Thiel, 1995, 1996; Vantiss et al., 1976).

Molecular analysis of pestivirus strains has concentrated on isolates from cattle, sheep and pigs. As a result of comparative sequence analyses, four different genotypes have been identified (Becher et al., 1995; Paton, 1995; Tiessen et al., 1996). One genotype consists exclusively of porcine isolates, while the other three comprise isolates mostly of bovine and ovine origin. Complete genomic sequences are available for all genotypes except for ‘true’ BDV strains. The identification of four different genotypes is in contrast to the currently used nomenclature that considers only the three mentioned species. In order to introduce the newly identified genotype and to reconsider the taxonomy of pestiviruses, it has been recently proposed to term BVDV strains pestivirus type 1, CSFV strains pestivirus type 2, ‘true’ BDV strains pestivirus type 3 and a new group including bovine and ovine isolates pestivirus type 4 (Becher et al., 1995). The separation of pestiviruses into four distinct groups is also supported by analyses with monoclonal antibodies (Becher et al., 1995; Paton, 1995). For pestivirus isolates derived from ruminants other than cattle and sheep, it is so far unclear whether they belong to one of the four established genotypes or whether they form additional groups.

The present study was initiated to characterize pestiviruses derived from an extended number of host species by nucleotide sequence determination and to investigate their phylogenetic relationships. So far, phylogenetic analyses of pestiviruses have been mostly based on small fragments derived from the 5′ NCR. We show that this region is not suited for a detailed analysis of pestivirus phylogeny, since confidence levels of most branches of the respective trees are low. In contrast, phylogenetic analysis using the entire N$^{pro}$-encoding gene is supported by high bootstrap values. For some pestivirus isolates, our grouping is different from the ones reported by others.

Methods

**Viruses and cells.** The pestivirus isolates analysed here were selected from different geographical regions and include strains from cattle, sheep, pig, goat, buffalo, deer and giraffe. Isolate CV 78/1274 was obtained from the pulmonary lung of a lamb, four-week-old goat kid in 1978 in Australia (Fraser et al., 1981). Isolate CV 94/4068 was isolated in 1994 from a buffalo foetal serum pool collected in the Northern Territory, Australia. Bovine isolate V-TOB was originally isolated from a case of acute MD in a Hereford steer in Australia (Snowdon, 1973). The two deer isolates 95-4845A and 95-4845B were obtained in New Zealand in 1980. Isolation of several pestiviruses from deer in 1979 and 1980 in New Zealand was associated with oral, ruminal and coronet erosions. The New Zealand goat pestivirus isolate 95-4845C was obtained from a case of abortion. Strain 695-361-96 was isolated in 1996 in Germany from a goat with acute diarrhoea and erosive oesophagitis. The giraffe pestivirus strain isolated in the 1960s in Kenya (Plowright, 1969) and the deer pestivirus strain Deer-GBI isolated in the UK in 1986 were kindly provided by Dr D. J. Paton (Central Veterinary Laboratory, New Haw, Weybridge, Surrey, UK). Porcine strain Frijters was isolated in 1994 from a congenitally infected piglet and obtained from the CDI-DLO, The Netherlands. By typing with monoclonal antibodies, strain Frijters was demonstrated to be a ‘non-CSFV’ pestivirus. Such a non-CSFV pestivirus was circulating in 1994 in a number of Dutch pig herds (Wensvoort et al., 1994). The British ovine field isolate R2727 (Brockman et al., 1988) was kindly provided by Dr S. Edwards (Central Veterinary Laboratory, New Haw, Weybridge, Surrey, UK). All pestivirus strains investigated in this study are listed in Table 1.

Pestiviruses were multiplied on Madin–Darby bovine kidney (MDBK) cells obtained from the American Type Culture Collection (Rockville, Md.). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS). Cells, medium and FCS were tested regularly for the absence of pestiviruses by RT–PCR and immunofluorescence. For FCS, the absence of anti-pestivirus antibodies was shown by lack of virus neutralization.

**Infection of cells.** Supernatants and lysates of infected cells were combined and used for infection of MDBK cells. Material for infection was prepared by freezing and thawing cultures 48 h post-infection and stored at $-70^\circ$C. An m.o.i. of about 0.1 was used for infections. All infections were controlled by indirect immunofluorescence with monoclonal antibody 8.12.7, kindly provided by Dr E. J. Dubovi (Cornell University, Ithaca, NY, USA).

**Oligonucleotides.** Oligonucleotides were purchased from Pharmacia Biotech. Sequences of oligonucleotides and their positions in the genome of BVDV NADL (Collett et al., 1988b) are as follows: 1400R, ACCAGTTGCTACCCATG (1448–1430); 17, GAGTCAGGAC-AGCTCGTCAG (179–198). Both primers were designed using a multiple sequence alignment including BVDV strains NADL (Collett et al., 1988b) and Osloss (De Moerloose et al., 1993), CSFV strains Alfort (Meyers et al., 1989) and Brescia (Moormann et al., 1990) as well as BDV strains X818, L83/84, 59386 and SCP (Becher et al., 1995, 1994).

**RT–PCR.** RNA from pestivirus-infected cells was prepared using RNeasy total RNA kit (Qiagen). Reverse transcription of approximately 200 ng heat-denatured RNA (2 min 92 °C, 2 min 4 °C in 11.5 µl water in the presence of 20 pmol reverse primer) was done after addition of 8 µl RT mix (125 mM Tris–HCl pH 8.3; 187 mM KCl; 7.5 mM MgCl$_2$; 25 mM dithiothreitol; 1.25 mM of each dATP, dCTP, dGTP, dTTP; 8 U of RNase inhibitor (RNasin, Promega). 50 U of reverse transcriptase (SuperScriptII, LifeTechnologies/BRL) and a piece of paraffin (Paraplast, melting point 55 °C) for 30 min at 37 °C. After reverse transcription, the reactions were heated to 80 °C for 2 min in order to melt the paraffin. After cooling, 30 µl of PCR mix [8.3 mM Tris–HCl pH 8.3; 33.3 mM KCl; 2.2 mM MgCl$_2$; 0.42 mM of each dATP, dCTP, dGTP, dTTP; 0.17% Triton X-100; 0.03% BSA; 20 pmol of the respective upstream primer; and 2.0 U Taq DNA polymerase (Appligene)] was added onto the solid paraffin surface. Amplification conditions were: 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 45 s.

**Nucleotide sequencing.** The DNA obtained after RT–PCR was separated by 10% agarose gel electrophoresis and purified using a Qiagen...
### Table 1. Pestivirus strains

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Abbreviation</th>
<th>Year of isolation</th>
<th>Species of origin</th>
<th>Region of isolation</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV 94/4068</td>
<td>Buffalo-A1</td>
<td>1994</td>
<td>Buffalo</td>
<td>Australia</td>
<td>U80901</td>
</tr>
<tr>
<td>95-4845A</td>
<td>Deer-NZ1</td>
<td>1980</td>
<td>Deer</td>
<td>New Zealand</td>
<td>U80904</td>
</tr>
<tr>
<td>95-4845B</td>
<td>Deer-NZ2</td>
<td>1980</td>
<td>Deer</td>
<td>New Zealand</td>
<td>U80904</td>
</tr>
<tr>
<td>'Deer'</td>
<td>Deer-GB1</td>
<td>1986</td>
<td>Deer</td>
<td>United Kingdom</td>
<td>U80902</td>
</tr>
<tr>
<td>'Giraffe'</td>
<td>Giraffe-1</td>
<td>1969*</td>
<td>Giraffe</td>
<td>Africa</td>
<td>U80907</td>
</tr>
<tr>
<td>695/361/96</td>
<td>Goat-G1</td>
<td>1986</td>
<td>Goat</td>
<td>Germany</td>
<td>U80898</td>
</tr>
<tr>
<td>CV 94/4068</td>
<td>Buffalo-A1</td>
<td>1994</td>
<td>Buffalo</td>
<td>Australia</td>
<td>U80901</td>
</tr>
<tr>
<td>R2727</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Year of isolation unknown. First referenced by Brockman et al. (1988).
‡ Year of isolation unknown. First referenced by Plowright (1969).

DNA purification kit (Qiagen). After denaturation with 0.2 M NaOH and neutralization with 0.2 M HCl about 50–100 ng DNA was incubated with the respective primer for 10 min at 37 °C. Dideoxy sequencing (Sanger et al., 1977) of double-stranded DNA templates was carried out using the T7 polymerase sequencing kit in the presence of [α-32P]dATP (Pharmacia). In the first round of nucleotide sequencing, primers 17 and 1400R were used. The obtained sequence data allowed derivation of additional oligonucleotides for determination of the nucleotide sequences of a 130 bp fragment from the 5' NCR and of the entire Npro gene. All sequences were confirmed by sequencing both strands. Sequences have been submitted to the GenBank nucleotide sequence database; accession numbers are listed in Table 1.

### Phylogenetic analysis

Sequence data were assembled and analysed using HUSAR (DKFZ, Heidelberg) which provides the GCG (Devereux et al., 1984) and PHYLIP (Felsenstein, 1985, 1993) software packages. Multiple sequence alignments of the DNA and deduced amino acid sequences were generated with GCG program PILEUP. Phylogenetic trees were constructed for the 130 bp fragments of the 5' NCR as well as for the entire Npro gene and deduced amino acid sequence data sets using the maximum likelihood, unweighted pair-group arithmetic averaging (UPGMA), neighbour-joining and Fitch–Margoliash methods (PHYLIP programs DNAML, NEIGHBOR and FITCH). In the case of the distance matrix programs, distances between sequences were estimated by using the six-parameter model (Felsenstein, 1993) and the Kimura 2-parameter method (Kimura, 1980) programs DNADIST and PROTDIST) with a transition/transversion ratio of 2:1. The robustness of the phylogenetic analysis and significance of the branch order were determined by bootstrap analysis carried out on 2000 replicates using PHYLIP programs SEQBOOT and CONSENSE (Felsenstein, 1985, 1993; Hedges, 1992).

### Results

#### Host species and geographical origin of the pestivirus isolates

In order to determine the genetic relatedness among pestiviruses derived from diverse host-species and geographical locations, three pestivirus isolates from goat, one from buffalo, three from deer and one from giraffe were characterized at the molecular level. Five of these are newly presented here: Goat-G1, Goat-NZ1, Buffalo-A1, Deer-NZ1 and Deer-NZ2. According to serological investigations, several porcine pestiviruses, for example strain Frijters (Wensvoort et al., 1994), have been demonstrated to be more closely related to ruminant pestiviruses than to CSFV. Such ruminant pestiviruses can cause significant problems in the diagnosis of classical swine fever. Also included in the present study are bovine isolate V-TOB (Snowdon, 1973) and the ovine genotype 1 strain R2727 (Brockman et al., 1988). The individual viruses characterized in this study are listed together with their corresponding host-species and region of isolation in Table 1. Isolates Giraffe-1, Deer-NZ2 and V-TOB exhibit cytopathogenicity, while all other viruses are noncytopathogenic.

#### Amplification and nucleotide sequencing of the 5' noncoding region and the gene encoding Npro

Genetic comparison of pestiviruses isolated from cattle, pigs and sheep has been the subject of several studies and different regions of the genome have been used for RT–PCR and comparative sequence analyses. Conserved primers in the 5' NCR are suited to detect pestiviruses. Respective fragments often comprising less than 1% of the entire pestiviral genome have been successfully used to differentiate virus genotypes (Becher et al., 1995; De Moerlooze et al., 1993; Hofmann et al., 1994; Paton, 1995; Pellerin et al., 1994; Ridpath et al., 1994). For a detailed phylogenetic analysis, however, these fragments might be too small. As an alternative to the 5' NCR, amplification and comparative sequence analysis of the genes encoding Npro and C of different pestivirus strains have been
reported, and the N\textsuperscript{pro} gene has proved to be well-suited for the study of genetic relationships within the genus \textit{Pestivirus} (Becher \textit{et al}., 1995). Both the 5' NCR and the entire N\textsuperscript{pro} gene are addressed in the present study.

For amplification of part of the 5' NCR and the genes encoding N\textsuperscript{pro} and C, oligonucleotide primers targeted at the 5' NCR and the C or E\textsuperscript{rns} gene were designed using a multiple sequence alignment of pestiviruses which included BVDV strains NADL and Osloss, CSFV strains Alfort and Brescia, and ovine pestiviruses X818 and L83/84. Of several primer combinations tested, two primers, 1400R and 17, were found to be reactive with all pestiviruses included in this study. Using this primer pair, a specific product of about 1200 bp was obtained for each virus strain (Fig. 1). Nucleotide sequences were determined by direct sequencing of the purified PCR products. Based upon data obtained in the first round of sequencing, specific primers were designed and used in additional rounds of sequencing to determine the nucleotide sequences comprising 130 nucleotides of the 5' NCR and the entire N\textsuperscript{pro} encoding region.

**Sequence comparison and phylogenetic analysis of pestivirus isolates using the entire N\textsuperscript{pro} gene**

The obtained nucleotide sequences of the N\textsuperscript{pro} gene and the respective deduced amino acid sequences were first subjected to pairwise comparisons and aligned with corresponding published pestivirus sequences using the PILEUP program (Deveraux \textit{et al}., 1984). N\textsuperscript{pro} of various pestivirus prototype strains has been reported to be encoded by the first 504 nucleotides of the pestivirus open reading frame (Becher \textit{et al}., 1995; Stark \textit{et al}., 1993). The alignment of the deduced amino acid sequences shows that the number of amino acids of N\textsuperscript{pro} is identical for all pestiviruses under study (Fig. 2). The C-terminal eight amino acids of N\textsuperscript{pro}, as well as the N-terminal two amino acids of C, are highly conserved among all these pestiviruses. N\textsuperscript{pro} has been demonstrated to represent a nonstructural autoprotease which generates its own C terminus (Stark \textit{et al}., 1993; Wiskerchen \textit{et al}., 1991). With respect to the type of proteinase, it has been suggested that N\textsuperscript{pro} belongs either to the serine-type proteinases or to the papain-like cysteine proteinases (Stark \textit{et al}., 1993; Wiskerchen \textit{et al}., 1991). For the latter, putative catalytic residues Cys\textsuperscript{69} and His\textsuperscript{130} are conserved among all pestiviruses including the eleven isolates described here (Fig. 2). Experimental studies concerning the nature of the protease are currently under way.

To define evolutionary relationships of the N\textsuperscript{pro} gene, a phylogenetic analysis of the viruses based on the nucleotide sequences of their N\textsuperscript{pro} gene was undertaken. In addition to the data obtained for the pestiviruses listed in Table 1, several published sequences derived from bovine, porcine and ovine pestiviruses were included. A phylogenetic tree was generated using the Kimura 2-parameter method (Kimura, 1980) for calculation of genetic distances, and the neighbour-joining method (Saitou \& Nei, 1987) for construction of the tree (Fig. 3). A bootstrap analysis inferred a robust phylogeny, with only three of the 24 interior branches of the tree receiving less than 83% support; 17 of the 24 branches were supported by more than 95% of bootstrap replicates. Phylogenetic trees using the same data sets and the maximum likelihood, UPGMA or Fitch–Margoliash methods had topologies identical to the tree generated by the neighbour-joining method (data not shown). It can be seen that the pestiviruses segregated into four major genotypes: type 1 containing BVDV strains with isolates from cattle, sheep, goat, deer and buffalo; type 2 containing CSFV strains; type 3 containing BDV strains from sheep together with bovine isolate V-TOB and two porcine viruses (Frijters, 87-6); and type 4 containing several sheep pestiviruses and BVDV-2 strain 890. Interestingly, the giraffe pestivirus isolate was found to be clearly distinct from these four genotypes (Fig. 3).

In pairwise comparisons, the nucleotide sequence homology for the N\textsuperscript{pro} gene ranged from 61.7% to 96.8% (data not shown). Between isolates of the same genotype, homology was at least 75.8%, while less than 71.4% identical nucleotides were found between isolates from different genotypes (data not shown). Nucleotide identities between isolate Giraffe-1 and any other pestivirus were below 69%.

It has been previously reported that genotype 1 (BVDV) isolates can be further divided into two subgroups, termed BVDV 1a and 1b (Pellerin \textit{et al}., 1994). According to the phylogenetic analysis presented here, these two subgroups can be confirmed. Subgroup 1a contains BVDV strains NADL and SD-1 as well as strains R2727, Goat-NZ1 and Deer-NZ2, while subgroup 1b includes BVDV strains Osloss and CP7 as well as strain Goat-G1. Interestingly, type 1 pestiviruses Buffalo-A1, Deer-NZ1 and Goat-A1 form a third cluster (1c) separate from
Phylogeny of ruminant pestiviruses

Fig. 2. Comparison of deduced amino acid sequences of nonstructural autoprotease Npro. The corresponding nucleotide sequences of the Npro gene of ovine strain R2727 (Brockman et al., 1988), porcine strain Frijters, bovine strain V-TOB (Snowdon, 1973), isolates Goat-G1, Goat-A1 (Fraser et al., 1981), Goat-NZ1, Deer-GB1, Deer-NZ1, Deer-NZ2, Buffalo-A1 and Giraffe-1 (Plowright, 1969) were determined by direct sequencing of RT–PCR products. The respective sequences of BVDV strains NADL (Collett et al., 1988b), Osloss (De Moerlooze et al., 1993), SD-1 (Deng & Brock, 1992) and CP7 (Meyers et al., 1996), CSFV strains Alfort (Meyers et al., 1989), Brescia (Moormann et al., 1990), Riems (accession no. U45477) and Glentorf (accession no. U45478), BVDV-2 strain 890 (Ridpath & Bolin, 1995b), ovine pestiviruses X818, L83-84 (Becher et al., 1995), 137-4 (Roehe et al., 1992), SCP (Becher et al., 1995), 59386 (Becher et al., 1995) and BD-78 (Sullivan et al., 1994), as well as porcine isolate 87-6 (Roehe et al., 1992) were extracted from the GenBank data library. The alignment was generated using PILEUP included in the GCG software package (Devereux et al., 1984). Only differences to the BVDV NADL sequence (top line) are indicated. It has been suggested that Npro belongs to the papain-like cysteine proteases (Stark et al., 1993). Suggested putative catalytic residues Cys69 and His130 are marked by asterisks.

subgroups 1a and 1b (Fig. 3). Isolate Deer-GB1 could not be assigned to any of the three subgroups, but was clearly placed within the group of genotype 1 viruses. With respect to genotype 3, two subgroups can be distinguished, one containing isolates X818, L83-84, V-TOB and Frijters, while the other comprises isolates 87-6 and 137-4. Segregation of genotype 1 and genotype 3 viruses into subgroups is supported by 100% of bootstrap replicates.

Genotypes 2 and 3 were found to be more closely related to each other than to the other genotypes. So far, type 2
pestiviruses (CSFV) have been isolated exclusively from pigs. In contrast, the other genotypes are not restricted to a single host-species. Type 1 and type 4 pestiviruses have been isolated from cattle and sheep, while type 3 pestiviruses were obtained mainly from sheep. In the present study, the first bovine type 3 strain, V-TOB, is described. This result strongly suggests that genotype 3 pestiviruses are capable of infecting both sheep and cattle. Furthermore, one genotype 3 strain (87-6) has been isolated from pigs (Roehe et al., 1992). The porcine pestivirus strain Frijters analysed here was also found to belong to genotype 3.

It is a noteworthy feature of the phylogenetic tree that the Npro sequences derived from ruminant pestiviruses do not segregate into discrete lineages based on host-species origin (Fig. 3). For example, the Npro sequence of the German isolate Goat-G1 is more closely related to the Npro sequence of cattle BVDV strains Osloss and CP7 than to those of other caprine pestivirus strains. With respect to genotype 4, ovine strain BD-78 is more closely related to bovine strain 890 than to the other sheep-derived pestivirus type 4 strains SCP and 59386. In summary, each of the ruminant pestivirus genotypes can be found in different ungulate species, while genotype 2 isolates are restricted to pigs.

**Sequence comparison and phylogenetic analysis of pestivirus isolates using a fragment of the 5′ NCR**

Partial sequences of the 5′ NCR have been previously shown to be useful for genotyping of pestiviruses (Becher et al., 1995; Hofmann et al., 1994; Pellerin et al., 1994; Ridpath et al., 1994). In the present study the genomic region comprising the last third of the 5′ NCR was also analysed. The respective sequences of the pestivirus isolates described here, and of published pestiviruses derived from cattle, sheep and pig, were aligned using the PILEUP program included in the GCG software package (Devereux et al., 1984) (Fig. 4). All sequences determined were unique. The lengths of the respective sequences varied between 127 and 131 nucleotides and the changes encompassed deletions and insertions as well as point mutations. The variation was most pronounced between virus genotypes, but could also be observed between strains within a genotype. The final 18 nucleotides upstream of the initiation codon were identical for all pestivirus strains (Fig. 4). In pairwise comparisons the identities of the respective sequences within each genotype were at least 87%, but less than 78% between strains of different genotypes (data not shown). When the sequence derived from the giraffe virus isolate was compared with any other pestivirus sequence, the variability was at least 28%. These results confirm that the giraffe pestivirus represents a member of a novel genotype within the genus *Pestivirus*.

In addition, a phylogenetic analysis of the fragments from the 5′ NCR was undertaken by using the neighbour-joining and UPGMA methods (Felsenstein, 1993) to construct phylogenetic trees (data not shown). The isolates from goat, deer, buffalo as well as ovine strain R2727 were grouped together with pestivirus type 1 strains, while bovine strain V-TOB and porcine strain Frijters were grouped together with pestivirus type 3 strains. In contrast, isolate Giraffe-1 could not be
Fig. 4. Alignment of the nucleotide sequences from the 5’ NCR encompassing nucleotides 256–385 with respect to the published sequence of BVDV NADL. The nucleotide sequences of ovine strain R2727, porcine strain Frijters, bovine strain V-TOB, isolates Goat-G1, Goat-A1, Goat-NZ1, Deer-GB1, Deer-NZ1, Deer-NZ2, Buffalo-A1 and Giraffe-1 were determined by RT–PCR driven direct sequencing. Other sequences were extracted from the GenBank/EMBL database [BVDV strains NADL (Collett et al., 1988b), SD-1 (Deng & Brock, 1992), Osloss (De Moerlooze et al., 1993), CP7 (Meyers et al., 1996); CSFV strains Alfort (Meyers et al., 1989), Brescia (Moormann et al., 1990), Riems (accession no. U45477), Glentorf (accession no. U45478); ovine pestiviruses X818, L83-84, Moredun, SCP, 59386 (Becher et al., 1995), BD-78 (Sullivan et al., 1994) and BVDV-2 strain 890 (Ridpath & Bolin, 1995b)]. Sequences were aligned with PILEUP included in the GCG software package (Devereux et al., 1984). Only differences from the BVDV NADL sequence on the top line are indicated. Base deletions are shown by dots.

assigned to one of the established pestivirus genotypes (data not shown). A bootstrap analysis (Felsenstein, 1985; Hedges, 1992) carried out on 2000 pseudoreplicate data sets using either the neighbour-joining or UPGMA method revealed that 16 of the 23 interior branches of the respective consensus trees received less than 80% bootstrap support; only four branches were supported by $\geq 95\%$ of bootstrap pseudoreplicates (data not shown). Further subdivision of pestivirus genotypes into subgroups was not supported by statistically significant ($P \geq 95\%$) bootstrap values. The low confidence levels obtained for the phylogenetic analysis based upon part of the 5’ NCR demonstrate that this region is not suited for a detailed analysis of phylogenetic relationships between pestiviruses.

**Discussion**

Differentiation of pestiviruses has been the subject of several studies and there is general agreement about the presence of four genotypes comprising isolates from cattle, sheep and pigs (Becher et al., 1995; Paton, 1995; Tijssen et al., 1996). Based on the sequences of an entire pestiviral gene, we present here the first phylogenetic analysis of pestiviruses where isolates from goats and nondomestic ruminant species
are included. The structure of the phylogenetic tree was supported by high bootstrap values, which allowed a detailed analysis of the genetic relatedness of pestiviruses. An interesting feature of the phylogenetic tree is that diversity of the $N^{pro}$ gene does not correlate with host species, region of isolation or age of virus isolates. Furthermore, three isolates from goat, one from buffalo, and three from deer belong to the genotype 1 (BVDV-1) pestiviruses, while a giraffe isolate is clearly different from the described genotypes and has to be considered as the first member of a novel pestivirus genotype (Fig. 3). The analysis of additional isolates, especially obtained from wild ruminants, will show whether more than five pestivirus genotypes exist.

So far, studies on the genetic relatedness of pestiviruses have been mostly based on short fragments from the 5' NCR (Harasawa, 1996; Hofmann et al., 1994; Paton, 1995; Pellerin et al., 1994; Ridpath et al., 1994). One major disadvantage connected with analyses of the 5' NCR is the lack of collinearity, especially when different genotypes are considered; consequently, several gaps are obligatory in multiple sequence alignments of this region. When the respective alignments from individual investigations are considered, these gaps vary significantly in number, position and size (Becher et al., 1995; De Moerlooze et al., 1993; Harasawa, 1996; Hofmann et al., 1994; Paton, 1995; Pellerin et al., 1994; Ridpath et al., 1994). Furthermore, the nucleotide sequences analysed in many of these cases comprise less than 120 bp, which corresponds to less than 1% of the genome. While it is generally accepted that studies of phylogenetic relationships should include a statistical analysis, the trees reported so far have not been statistically evaluated and the confidence levels of the respective groupings are therefore not known. When we analysed the 5' NCR sequences, multiple datasets (2000 replicates) were used. The resulting consensus trees showed that the individual isolates could be segregated into four major groups and one disparate virus; clustering within the genotypes, however, was not supported by significantly high bootstrap values. It is therefore not surprising that our grouping of pestiviruses based on the $N^{pro}$ gene is different from the ones reported by others on the basis of 5' NCR sequences. These differences are not restricted to clustering within the genotypes but also concern the segregation of some pestiviruses into genotypes, i.e. the giraffe isolate was reported to cluster with genotype 4 viruses (Paton, 1995), while in our analysis it represents a disparate virus clearly different from members of genotype 4 as well as of the other three genotypes. In addition, ovine pestivirus 59386 was grouped together with genotype 3 viruses (Paton, 1995), but our analysis shows that it represents a genotype 4 virus. The differences between our grouping and the ones reported by others demonstrate that phylogenetic analyses of pestiviruses without an appropriate statistical test can result in questionable conclusions. Furthermore, Harasawa (1996) has suggested using the assumed secondary structure of this region as an additional parameter for differentiation of pestivirus genotypes. In summary, nucleotide sequences from the 5' NCR can be used for segregation of pestiviruses into genotypes when multiple datasets are used. The 5' NCR, however, is not suited to the analysis of genetic relationships of pestiviruses within a genotype.

In addition to comparative sequence analyses, several other methods have been used to differentiate pestiviruses. Segregation of pestiviruses into four major groups is also supported by serological investigations using binding assays with MAbs (Becher et al., 1995; Paton, 1995) and cross-neutralization assays with polyclonal sera (Dekker et al., 1995; Paton, 1995). The study by Dekker et al. (1995) led to the identification of two additional antigenic clusters, one represented bystrain Deer-GB1 and the other represented by strain Giraffe-I. The separation of strain Giraffe-I from all other pestiviruses is in agreement with our grouping (Fig. 3). With respect to strain Deer-GB1, however, our data show that this strain represents a genotype 1 virus; this conclusion is also confirmed by binding assays with MAbs (unpublished results).

Pestiviruses isolated from goats have been usually termed BDV (Loken et al., 1982; Nettleton, 1990). However, all three caprine isolates investigated in this study represent genotype 1 pestiviruses (BVDV-1). Accordingly, the term BDV for pestiviruses of goats is, at least for these three isolates, not appropriate. Analyses of additional caprine viruses will show whether pestivirus type 3 (BDV) isolates occur in goats under natural conditions. Isolation of pestiviruses from aborted goat foetuses as well as a disease similar to border disease of sheep have been described (Fraser et al., 1981; Loken et al., 1982). Interestingly, a goat with severe enteric disease was the source for isolate Goat-G1. Further studies are necessary to investigate the role of pestiviruses as pathogens of goats.

It is not known whether wild ruminants harbour their ‘own’ pestiviruses, but the results obtained with the giraffe virus isolate suggest that this can be the case. In addition, cross-species transmissions of pestiviruses between domestic and wild ruminants might occur and could play an important role in the spread of pestiviruses. This assumption is supported by the presented analysis, which does not allow a distinction of pestiviruses from deer and buffalo from BVDV isolates of cattle, sheep and goats. For most wild ruminant species, seroprevalence rates are significantly lower when compared to those of cattle (Nettleton, 1990). Transmission of pestiviruses from domestic to wild ruminants seems therefore to be more probable than vice versa. Little is known with respect to the significance of pestiviruses for causing disease in nondomestic ruminants. Several pestiviruses have been isolated from wild ruminants with clinical signs and/or pathological changes resembling MD (Karstad, 1981; Nettleton, 1990). Interestingly, several pestiviruses isolated from such affected wild ruminants are cytopathogenic, including strains Deer-NZ2 and Giraffe-I. With respect to MD of cattle, it is generally accepted that a cp BVDV mutant generated by RNA
recombination from the persisting noncp virus causes the outbreak of fatal disease (Thiel et al., 1996). Assuming a similar situation for wild ruminants, isolation of cp pestiviruses from animals with MD-like disease would indicate the existence of persistent infections in deer and other free-living ruminant species. Persistently infected animals have a central role in the maintenance of BVDV in cattle and sheep; the existence of persistently infected animals within wild ruminant populations, however, has not been established.

Pestiviruses are presumably derived from a common ancestor. The present genotypes can thus be considered as results of divergent evolution, and possibly recombination. The extent of recombination events between pestiviruses and their significance in the generation of pestivirus diversity is unknown. Recombination between BVDV strains has been shown to occur in persistently infected cattle after vaccination with cp BVDV strain NADL (Ridpath & Bolin, 1995). With regard to evolution of pestiviruses, it is useful to consider acute and persistent infections separately. For other RNA viruses, it is known that persistent infections contribute to virus variability (Gebauer et al., 1988). However, the comparison of partial sequences of viruses obtained from persistently infected cattle over a time period of at least one year failed to detect any base substitutions (Hertig et al., 1995; Paton et al., 1994). On the basis of these data it has been suggested that the acquired immunotolerance prevents survival of virus variants which arise during persistent infection. In contrast, mutations occur during acute infections. According to the present analysis, Npro sequences cannot be segregated by the age of the respective virus isolates.

Classical swine fever has been successfully eradicated in several countries, while ruminant pestiviruses have a worldwide distribution with seroprevalence rates up to 90% in cattle. Recently, Denmark, Norway, Sweden and Finland have started programmes with the goal to eradicate BVDV infection in cattle. According to this study, a comparison of Npro sequences does not allow a distinction of pestiviruses from goat, deer and buffalo from bovine and ovine genotype 1 (BVDV-1) viruses. Accordingly, goats and wild ruminants might serve as a reservoir for pestiviruses. The putative presence of such pestivirus reservoirs in wild ruminants might seriously endanger the success of eradication programmes.

This study was supported by Intervet International BV (project 75/73.1808.720).

References


Received 7 November 1996; Accepted 6 March 1997