

Short  
CommunicationA novel pestivirus associated with deaths in  
Pyrenean chamois (*Rupicapra pyrenaica  
pyrenaica*)

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During investigations into recent population decreases in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) 21 animals found dead or dying were necropsied. Immunohistochemistry revealed the presence of a pestivirus in organs from two of the 21 chamois. From one of these animals a pestivirus was isolated from the spleen, skin and serum. The virus had better growth in ovine than in bovine cells and was neutralized most effectively by an anti-border disease virus (BDV) reference antiserum. Using panpestivirus and genotype-specific primers selected from 5'-untranslated region (UTR) of the pestivirus genome, BDV RNA was demonstrated by RT-PCR. Comparison of the chamois sequences from 5'-UTR, entire N<sup>pro</sup> and E2 gene coding regions with those of other pestivirus genotypes revealed that this virus did not fall into any of the pestivirus genotypes identified so far. Results of phylogenetic analysis suggested that the chamois pestivirus was closely related to BDV and it was typed as BDV-4 genotype.

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Pestiviruses (family *Flaviviridae*) affect ruminants and suids. There are four accepted pestivirus species: *Border disease virus* (BDV), *Bovine viral diarrhoea virus-1* (BVDV-1), BVDV-2 and *Classical swine fever virus* (CSFV); and an isolate tentatively classified as a pestivirus from a giraffe (Heinz *et al.*, 2000). Genetic and antigenic characterization of new pestiviruses isolated from sheep has led to the proposal that BDV strains can be allocated into one of three genotypes, BDV-1 to -3 (Becher *et al.*, 2003).

The knowledge of pestivirus infections in wild animals is limited. Pestiviruses have been isolated from giraffe (Plowright, 1969), deer, buffalo, bison, bongo, alpaca and reindeer. The deer, buffalo, alpaca and bongo isolates had BVDV-1 genotypes. The bison and reindeer isolates were closer to BD virus (Becher *et al.*, 1997, 1999) and the reindeer isolate was classified into the BDV-2 genotype (Becher *et al.*, 2003). Serological surveys have shown that many species of free-living ruminants have varying prevalence of antibody to pestiviruses (Nettleton, 1990).

The Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*)

known locally as sarrio and isard, is a free-living ruminant grazing with domesticated cattle and sheep in the Pyrenean mountains, with a population of about 25 000 animals (Pérez *et al.*, 2002). Recently, a population decrease has been observed in both the French and Spanish Central Pyrenees, and the possible involvement of pestiviruses has been reported (Guffond *et al.*, 2003; Marco *et al.*, 2003; Schelcher & Alzieu, 2003). The study reported here was undertaken in the Principality of Andorra and four hunting reserves in Aragon (Spain): Benasque, Los Circos, Viñamala and Los Valles. The area in which chamois deaths were excessive lies between Andorra to the east and Benasque reserve to the west.

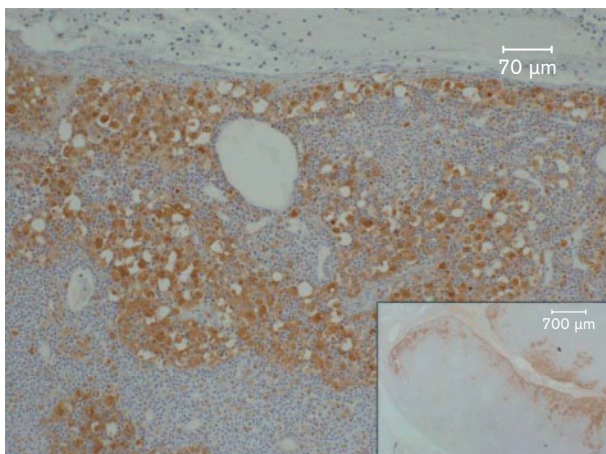
A serological survey was conducted to investigate the prevalence of pestivirus antibody in Pyrenean chamois. An ELISA was used to detect anti-pestivirus antibodies in 200 sera using a standard method employing the Oregon C24V strain of virus (Brockman *et al.*, 1988; Nettleton *et al.*, 1998; OIE, 2000). Thirty-three sera were positive (OD values from 0.16 to 0.87) and 167 were negative (OD values < 0.1). Chamois from Andorra and Benasque were significantly more likely to have anti-pestivirus antibody (24/88) than those sampled in the three regions further west (9/120) ( $\chi^2 = 17.64$ , d.f. = 1,  $P = < 0.001$ ).

The GenBank/EMBL/DDBJ accession numbers of the four sequences reported in this paper are AY738080–AY738083.

Pathological and virological examinations conducted on 21 Pyrenean chamois in 2002–2003 failed to show any consistent post-mortem findings. Tissues from two animals were positive for the presence of pestivirus by immunohistochemistry using mAbs 15C5 and WB 103/105 (Corapi *et al.*, 1990; Thür *et al.*, 1997). No pestivirus was detected by immunohistochemistry in the spleens of 145 hunted animals.

One pestivirus-positive chamois (H2121) was an adult female estimated to be 9 years old, found in August 2002 in Andorra. It had cachexia, alopecia, skin hyperpigmentation and winter coat retention on the face and legs. There was hyperaemia and irregular thickening of the colon and caecum with excess mucus in the large intestine and rectum. Histopathology revealed inflammatory lymphoproliferative lesions in the skin, central nervous system (CNS) and gastrointestinal tract. Necrosis of cortical lymphoid cells and an abundant presence of macrophages was observed in the subcutaneous and mesenteric lymph nodes. Immunohistochemistry revealed widespread pestivirus antigen principally in macrophages of subcutaneous (Fig. 1) mesenteric and mammary lymph nodes and spleen, epithelial cells of the skin, kidney and rumen, and CNS and bone marrow cells. The pathological changes and widespread pestivirus antigen suggest that this chamois died of a mucosal disease-like syndrome similar to that described in persistently infected sheep (Barlow *et al.*, 1983; Gardiner *et al.*, 1983).

A second pestivirus-positive Pyrenean chamois was a 5-year-old male from Benasque (Aragon, Spain). Pestivirus antigen was detected in cells of bone marrow and spleen using both mAbs. However, no virus was isolated from tissues and RT-PCR tests using 5'-UTR primers were inconclusive.



**Fig. 1.** Immunohistochemistry showing brown pestivirus antigen predominantly in macrophages in a formalin fixed section of a subcutaneous lymph node from chamois H2121 stained using mAb 15C5. Inset: low power view of the same lymph node.

Virus isolation was attempted on 15 specimens from seven dead chamois. Virus was isolated only from all three specimens (spleen, skin and serum) from chamois H2121, with significantly better growth in fetal lamb kidney (FLK) than in bovine embryonic kidney cells. Stocks were prepared from the spleen virus after two and three passages in FLK cells. A cytopathic effect (CPE) was observed during the second and third passages. The FLK-2 stock has a titre of 500 TCID<sub>50</sub> ml<sup>-1</sup> as judged by CPE and 6 × 10<sup>4</sup> TCID<sub>50</sub> ml<sup>-1</sup> non-cytopathic (ncp) virus following immunoperoxidase staining of FLK cells grown in 96-well microtitre plates (OIE, 2000). A biologically cloned stock of ncp virus has been established following three terminal dilutions in FLK cells from the FLK-2 stock. Cloning of the cytopathic (cp) virus away from the predominant ncp virus has not yet been achieved.

The H2121 chamois pestivirus (Chamois-1) was compared with reference strains of BDV (Moredun CP strain) and BVDV-1 (NADL strain) in cross-neutralization tests employing four chamois sera and two pestivirus reference antisera. Co-efficients of antigenic similarity (Becher *et al.*, 2003; Nettleton *et al.*, 1998) showed the chamois virus to be related to BDV and serologically distinguishable from BVDV-1. Specific reference antisera against the four pestivirus species (VLA Weybridge, UK) confirmed Chamois-1 to be most closely related to BDV.

To detect pestivirus RNA in clinical specimens, total RNA was extracted using the RNeasy mini kit (Qiagen) from 25 mg spleen, bone marrow, skin or brain from the same seven Pyrenean chamois selected for virus isolation. Preparation of cDNA used random hexamers (MWG-Biotech) and Moloney Murine Leukaemia Virus reverse transcriptase (Promega). A pestivirus-specific RT-PCR product (Vilcek *et al.*, 1994) was only detected in the spleen, skin and serum of Chamois H2121. A differential nested RT-PCR performed using primers 324 and 326 (Vilcek *et al.*, 1994) for the first round amplification followed by second round reactions specific for BVDV-1a, BVDV-1b, BVDV-2 or BDV (Ridpath & Bolin, 1998; Letellier *et al.*, 1999; Vilcek & Paton, 2000) provided a positive result only with BDV primers.

The N<sup>PRO</sup> region was amplified with BD1 and BD2 primers (Vilcek *et al.*, 1997). The majority of the E2 gene was amplified using primers P2256 (5'-ACTGGTGGCCNTATGARAC-3'; position 2256–2274 relative to SD1 strain) and P3422R (5'-TGAGCATGTATTGYTGGGAARTA-3'; position 3422–3401). The E2 gene was also amplified using the primers P2256 and P5175R (5'-ATCTTCTTGACACCGGCNGG-3'; position 5175–5156) or P5337R [5'-CC-TTGGTG(T/G)GTGTANGCCCA-3'; position 5337–5318] by the Expand Long Template system (Roche). PCR amplicons were purified using the QIAquick PCR purification kit (Qiagen). Sequencing primers (CH1–CH5) were obtained by primer walking. Mixed nucleotide peaks were found in direct sequencing of an E2 PCR-product so it was cloned using the TOPO TA cloning kit (Invitrogen) to facilitate sequencing. Sequencing was performed using Big

Dye terminator v1 and 1.1 (Applied Biosystems) on a Perkin Elmer Biosystems 377 DNA sequencer.

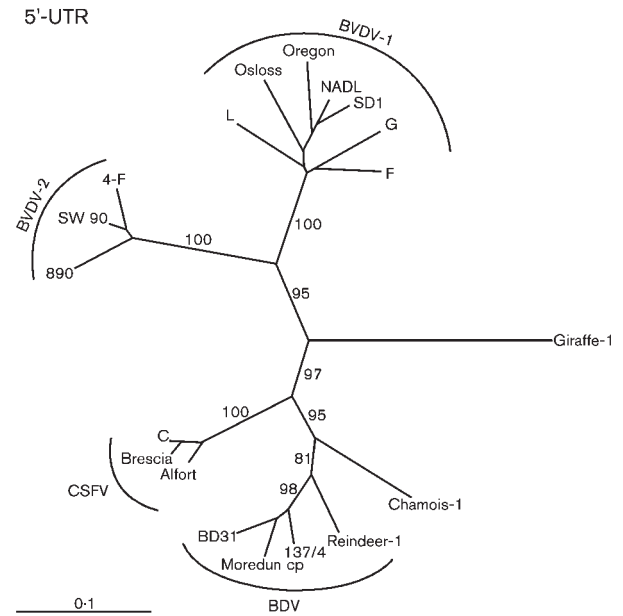
Nucleotide sequences were proof-read using the SeqMan II program from the DNASTAR multiple program package and compared using the CLUSTAL W program (Thompson *et al.*, 1994). Evolutionary distances were calculated using the program DNADIST, employing the Kimura two-parameter method (Kimura, 1980). Phylogenetic analysis was performed using the NEIGHBOR program based on the Neighbour-joining method (Saitou & Nei, 1987) from PHYLIP inference package programs (Felsenstein, 1985, 1993). The nucleotide and amino acid percentage similarities were calculated using the MegAlign program of DNASTAR.

The GenBank accession numbers of the four sequences reported in this paper are AY738080–AY738083.

Phylogenetic analysis of 5'-UTR sequences showed that the strain Chamois-1 formed a separate branch, which was closer to the BDV genotype but did not fall into any phylogenetic group identified so far (Fig. 2).

Phylogenetic analysis of the entire 504 nt N<sup>pro</sup> region clearly confirmed genetic typing of seven recently recognized pestivirus genotypes: BVDV-1, BVDV-2, CSFV, BDV-1, BDV-2, BDV-3 and Giraffe. However, the Chamois-1 strain was not located into any of these genotypes. The nearest location of this strain in the phylogenetic tree was to BDV-1, BDV-2 and BDV-3 genotypes but it topologically formed an additional clearly separated branch, which could be labelled as BDV-4 (Fig. 3). The percentage of nucleotide similarity between the Chamois-1 strain and BDV-1, BDV-2 and Gifhorn (BDV-3) strains is 68.1–73.2, 70.2–73.0 and 69.2%, respectively. Similar comparison between the Chamois-1 strain and Alfort (CSFV), NADL (BVDV-1), 890 (BVDV-2) as well as Giraffe-1 strains provided slightly lower values – 65.7, 62.3, 60.9 and 63.3%, respectively. The same tendency was observed at the amino acid level.

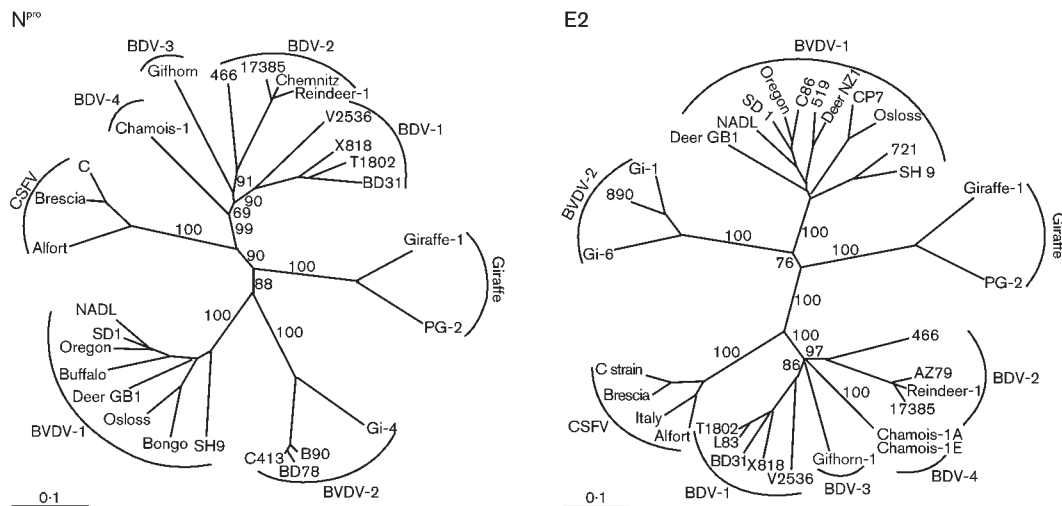
The presence of mixed nucleotide peaks in the PCR product of the E2 region suggested a mixture of viral populations. The PCR product was cloned and nine individual clones sequenced. The alignment of nucleotide sequences revealed that there were two main populations, labelled Chamois-1A and Chamois-1E. E2 for Chamois-1A was 1116 nt long, and E2 for Chamois-1E was 1119 nt long. The discrepancy was due to a three nucleotide (GCT) deletion in E2 of Chamois-1A and consequently one amino acid deletion (alanine). The position of this alanine residue in E2 of Chamois-1E is 89. Overall nucleotide similarity between E2 of Chamois-1A and 1E is 98.8%. RT-PCR and sequencing of cell culture grown master stock and biologically cloned ncp chamois virus revealed only Chamois-1E-type virus. The Chamois-1A virus demonstrated in the spleen may represent cp virus but this is not verifiable until the cp virus has been cloned away from the predominant ncp population.



**Fig. 2.** Phylogenetic tree showing the genetic relationship between pestivirus strains in 5'-UTR. The unrooted tree was based on analysis of partial 245 nt long sequences (position in NADL 130–374). It was prepared using the Neighbour-joining method (Kimura two-parameter method, transition/transversion 2). Sequences for other strains were taken from GenBank with the following accession numbers: BD31, U70263; Moredun Cp, U65022; 137/4, U65052; Reindeer-1, AF144618; Giraffe-1, AF144617; Alfort, J04358; Brescia, M31768; C strain, Z46258; NADL, M31182; SD1, M96751; Oregon, AF041040; Osloss, M96687; 890, U18059; SW 90, AB003622. Sequences for F, G, L and 4-F strains were taken from Vilcek *et al.* (2001). Numbers indicate the percentage of 1000 bootstrap replicates that support each phylogenetic branch.

The phylogenetic analyses performed over the entire E2 region confirmed the results from the N<sup>pro</sup> region (Fig. 3). The BVDV-1, BVDV-2, CSFV and Giraffe genotypes were clearly separated into phylogenetic groups supported by 100% bootstrap values. In the BDV group, four significant phylogenetic branches were observed, namely BDV-1, BDV-2, BDV-3 and BDV-4 represented by Chamois-1A and Chamois-1E. The BDV-4 branch was supported by 100% bootstrap value as was observed for the whole BDV cluster.

The percentage of similarity at the nucleotide and amino acid levels revealed the same tendency as observed for N<sup>pro</sup> region. The Chamois-1 strain is slightly more similar to BDV strains than to other strains belonging to different pestivirus genotypes. Namely, the following values were observed: nucleotide similarity between Chamois-1 and BDV-1, BDV-2, Gifhorn (BDV-3), Alfort (CSFV), NADL (BVDV-1), 890 (BVDV-2) and Giraffe-1 strains was 61.8–68.9, 67.2–69.3, 69.3, 59.4, 54.2, 48.9 and 49.6%, respectively. The corresponding amino acid similarity varied in



**Fig. 3.** Phylogenetic tree showing the genetic relationship between pestivirus strains in the N<sup>pro</sup> and E2 regions. The unrooted trees visualized by TREEVIEW program (Page, 1996) were based on analysis of the entire 504 nt of N<sup>pro</sup> or 1116–1119 nt of E2. They were prepared using the Neighbour-joining method (Kimura two-parameter method, transition/transversion 2). The N<sup>pro</sup> sequences for other strains were taken from GenBank with following accession numbers: PG-2, AY163647; V2536, AY163648; T1802, AY163649; 466, AY163650; 17385, AY163651; AZ79, AY163652; Gifhorn, AY163653; BD31, U70263; X818, AF037405; Reindeer-1, AF144618; Giraffe-1, AF144617; Alfort, J04358; Brescia, M31768; C strain, Z46258; NADL, M31182; SD1, M96751; Oregon, AF041040; Osloss, M96687; Deer GB1, U80902; Buffalo, U80901; Bongo, AF144474; SH9, AF144473; 890, U18059; Gi-4, AF144468; C413, AF002227; BD78, U18330. The nucleotide sequences for E2 region were acquired from GenBank with the same accession numbers except: PG-2, AY163654; V2536, AY163655; T1802, AY163656; 466, AY163657; 17385, AY163658; AZ79, AY163659; Gifhorn, AY163660; L83, U00890; Italy, AY027672; 721, AF144609; 519, AF144610; C86, AF144611; Deer NZ1, AF144614; Deer GB1, AF144615; SH9, AF144616; CP7, AF220247; Gi-1, AF104030; Gi-6, AF144612. Numbers, which were calculated by SEQBOOT and CONSENCE program from PHYLIP, indicate the percentage of 1000 bootstrap replicates that support each phylogenetic branch.

the range of 68.8–76.4, 76.1–79.4, 78.0, 67.1, 61.7, 56.2 and 57.5%, respectively. Again, the similarity values between Chamois-1 and BDV strains were closer than to other pestivirus genotypes.

No unambiguous reason for the deaths among Pyrenean chamois has been established during this investigation. The only known pathogen to be demonstrated was a pestivirus but its role in the deaths of chamois remains uncertain.

The typing by RT-PCR using pestivirus-specific primers suggested that the new pestivirus could be a BD virus, as did the limited antigenic comparison. Genetic typing using computer-assisted phylogenetic analysis of the Chamois-1 strain in the 5'-UTR (244 nt) and entire N<sup>pro</sup> (504 nt) as well as E2 (1116–1119 bp) coding regions revealed that this virus did not fall into any known pestivirus genotype identified so far. It has been located into a separate branch near or inside the BDV cluster. BD viruses were typed recently into three BDV genotypes (Becher *et al.*, 2003). Accepting this taxonomic classification the Chamois-1 strain represents the first member of the BDV-4 genotype. Our findings extend evidence that BDV strains are more diverse than viruses of other pestivirus genotypes (Becher *et al.*, 2003).

Previous results on genetic typing of pestiviruses (Becher *et al.*, 2003; Harasawa *et al.*, 2000; van Rijn *et al.*, 1997) and the results presented here, describe eight pestivirus genotypes, namely BVDV-1, BVDV-2, CSFV, BDV-1, BDV-2, BDV-3, BDV-4 and Giraffe. Six pestivirus genotypes have been identified in farm animals and three (giraffe, reindeer – BDV-2; chamois – BDV-4) in wild animals. Although not all pestiviruses infecting wild animals represent new pestiviruses (Becher *et al.*, 1997, 1999; Fisher *et al.*, 1998; Van Campen *et al.*, 2001; Vilcek *et al.*, 2000), it seems that wild animals may be a significant source of new pestivirus genotypes.

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