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Detection of a Newly Described Pestivirus of Pyrenean Chamois (*Rupicapra pyrenaica pyrenaica*) in France

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ABSTRACT: A pestivirus was detected and characterized in chamois (*Rupicapra pyrenaica pyrenaica*) originating from the French part of the Pyrenees. Phylogenetic analysis of the pestivirus was done on the basis of a fragment from the 5′ noncoding region including 22 published nucleotide sequences of different pestivirus strains. Our strain was grouped within the clade of border disease viruses (BDV). However, it had an intermediate position between clade BDV and classical swine fever viruses representing a basal position to BDV strains of domestic sheep. Our strain was grouped as a sister unit to a novel pestivirus (Chamois-1) recently described from chamois in Spain. Therefore, we postulate that this virus occurs in the entire population of Pyrenean chamois. On the basis of the phylogenetic grouping of this isolate, a postulated cross-species transmission of pestivirus from domestic sheep to chamois via shared pastures seems to be unlikely.

Key words: Border disease virus, chamois, cross-species transmission, French Pyrenean, *Pestivirus*, phylogenetic analysis, *Rupicapra pyrenaica pyrenaica*.

The genus *Pestivirus* within the family *Flaviviridae* is divided into bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus (BDV) (Becher et al., 1997). Traditionally, these viruses were classified primarily according to the host from which they were isolated. It is now known that cross-infection among species occurs readily. Consequently, these viruses are now grouped more according to their reactivity with monoclonal antibodies and to their nucleotide sequences. Analysis of antigenic similarities indicated the presence of seven major antigenic groups corresponding to BVDV-1, BVDV-2, CSFV, BDV-1, BDV-2, BDV-3 (Becher et al., 2003), and "giraffe," represented by a single strain (H138) isolated from a giraffe in Kenya (Avalos-Ramirez et al., 2001). However, a novel pestivirus isolated from pronghorn (*Antilocapra americana*) seems to be distinct from the established groups (Cornish et al., 2003). Moreover, a novel pestivirus was recently detected in Spanish chamois and did not fall into any of the previously identified pestivirus genotypes. Phylogenetic analysis suggested that this virus was closely related to BDV and it was typed at BDV-4 genotype.

The endangered Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) belongs to the family Bovidae and the subfamily Caprinae. Nearly 25,000 Pyrenean chamois currently inhabit the Pyrenean range in southwest France. A regular health monitoring scheme for ungulates in the Orlu Reserve has been in existence for more than 15 yr by the Office National de la Chasse et de la Faune Sauvage. Their studies compare the health status of wild ungulates and domestic livestock to clarify the risk of interspecific transmission; cattle and domestic sheep share pastures with chamois. Although no unusual mortality in chamois was observed in the Orlu Reserve, several sick and dead chamois from other parts of the French Pyrenees and the Spanish Pyrenees (Catalonia) were observed. These animals developed emaciation within 2 to 3 wk, general alopecia, and skin hyperpigmentation. At clinical examination, the chamois were cachectic and anemic. Nonspecific lesions were observed in the central nervous system. However, in a few animals a nonsuppurative encephalitis was detected (Marco and Lavin, 2003). Pestiviruses were detected by poly-

merase chain reaction (PCR) from two free-ranging Pyrenean chamois, in France. The objective of this study was to characterize these two viruses to determine if they represent recognized domestic animal pestiviruses or a novel pestivirus recently described from chamois in Spain.

The Reserve Nationale de Chasse et de Faune Sauvage Orlu is located in the east of the Pyrenean Mountain Range in the Département of Ariège (42°26' to 42°41'N, 1°45' to 2°0'E). This reserve has an area of 4,200 ha with an altitude between 900 and 2,700 m. Approximately 1,000 chamois inhabit the study area. Fifty spleen samples were taken from chamois hunted in the Orlu Reserve between 2001 and 2002.

Reverse transcription (RT) followed by PCR (Wirz et al., 1993) was performed to detect pestiviral RNA in spleen samples. Total RNA from tissue samples (50–100 mg) was isolated using the RNeasy® Mini Kit (Qiagen, Venlo, The Netherlands). Reverse transcription was performed in 20 μl, containing 10–100 ng of RNA, 2.5 μM random hexamer primers, 5× M-MLV buffer, 20 U of M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA), 2 U of RNase inhibitor (Applied Biosystems, Foster City, California, USA), and 1 mM of each dNTP (Boehringer, Mannheim, Germany). First, the mixture was incubated for 15 min at 37 C followed by 30 min at 42 C. Reverse transcriptase was inactivated (5 min, 99 C) and cDNA samples were stored at 4 C. Following RT, a PCR (50 μl) was performed with 10 μl of the cDNA reaction under the following conditions: 1 U of Taq-polymerase (Qiagen), 10× PCR buffer, 0.5 mM $MgCl_2$ (both Applied Biosystems), 0.5 mM of each dNTP (Boehringer), and 10 pM of each primer. After an initial denaturation period (5 min at 95 C), DNA was amplified throughout 35 cycles (94 C, 30 sec; 55 C, 45 sec; 72 C, 45 sec) followed by a terminal elongation phase (10 min, 72 C). The following primers were used: BVD3: 5′-GTGGACGAGGGCATGCCCA-3′ (nucleotides 237–255) and Pest2: 5′-TCAA CTCCATGTGCCATGTAC-3′ (nucleotides 395-375) (Collett et al. 1988). As negative controls, RT-PCR experiments were performed without the RT step to avoid misleading false-positive results due to contaminating DNA in RNA preparation. Positive controls were performed using BVDV-strain SH9/11.

Polymerase chain reaction products were purified using the QIAquick PCR purification kit (Qiagen). Direct sequencing was done using the same primers and a BigDye Cycle Sequencing Kit on a 3100 Genetic Analyzer (Applied Biosystems). The following nucleotide sequences of other pestiviruses were obtained from DNA databases for phylogenetic reconstructions [accession numbers in 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 EBTr [D50817], 890 [L32886] and CD87 [L32887] for BVDV-2, strains BD31 [U70263], Moredun cp [U65022], Ch1Es [D50816] and X818 [U17142] for BDV-1, strain Reindeer-1 V60 [AF144618] for BDV-2 and strains Brescia [AF091661], Alfort [J04358], and GPE- [AB019152] for CSFV. Additionally, we included the roe deer strain SH9/11 (Fischer et al., 1998), the H138 Giraffe strain [AB040131], and the very recently described novel pestivirus Chamois-1 [AY738080]. The length of the diagnostic fragment used for phylogenetic analyses was 114 base pairs. Alignments were done using CLUSTAL W (Thompson et al., 1994) and proofed visually by eye. We used the computer package MEGA v. 2.1 (Kumar et al., 1994) to calculate Kimura 2-parameter distances and neighbor-joining trees. The significance of the branches was examined by bootstrap analysis (1,000 replications) as implemented in MEGA.

Two of 50 spleen samples were identified as pestivirus positive in RT-PCR. All other spleen samples were negative. Se-

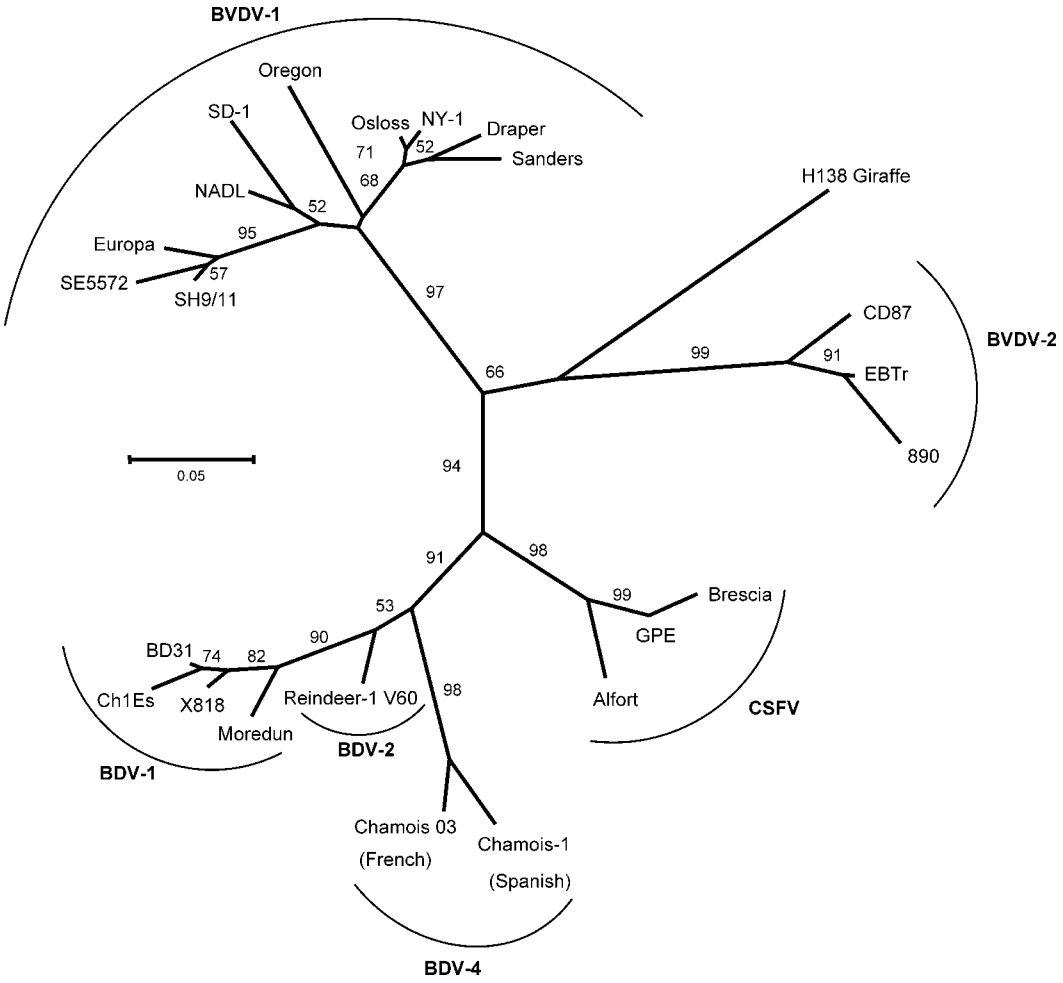

FIGURE 1. Neighbor-joining tree based on Kimura 2-parameter distance values. Numbers indicate percentage bootstrap support. CSFV, classical swine fever viruses; BDV, border disease viruses; BVDV, bovine viral diarrhea viruses. Sequence information of the BDV-3 group 5'UTR is not available in public databases. The Spanish isolate (Chamois-1) was described by Arnal et al. (2004).

quence analysis showed that the two positive samples had no differences. Comparative alignment of 24 sequences included 123 nucleotides. We observed 67 variable sites; 58 of these were phylogenetically informative. The new fragment sequenced from French Pyrenean chamois contained three diagnostic sites according to all other strains with the exception of the Spanish chamois-1 sequence (Arnal et al., 2004). This sequence shows the same diagnostic differences. The French and the Spanish chamois virus (Chamois-1, Arnal et al., 2004) sequences differ by seven substitutions with a corresponding pairwise nucleotide distance of 0.061 [standard error (S.E.) 0.022]. Computed overall average pairwise nucleotide distances was 0.276 [S.E. 0.036]. The observed sequence was phylogenetically grouped as sister unit to Chamois-1 (bootstrap support 98%) with a basal position of this group relative to the BDV sequences Ch1Es, BD31, Moredun, Reindeer-1, and X818 (Fig. 1). This grouping was similar to that of Arnal et al. (2004). Regarding the strong phylogenetic relationship of Chamois-1 and our sequence and following the arguments of

Becher et al. (2003) and Arnal et al. (2004), the pestivirus sequence found in this study represents a strain within the BDV-4 group. This strain was named Chamois 03.

Differentiation of pestiviruses has been the subject of several studies (Becher et al., 1997; Fischer et al., 1998; Harasawa et al., 2000; Avalos-Ramirez et al., 2001; Becher et al., 2003). Suspected sources of the virus for wild animals include direct contact with infected livestock, shared feed and watering areas, or the presence of pestivirus-infected individuals within wild populations (Van Campen and Williams, 1996).

For the first time we described a pestivirus in French Pyrenean chamois showing a basal position to the BDV strains of sheep. However, recently a similar BDV strain was detected in chamois originating from the Spanish part of the Pyrenees (Arnal et al., 2004).

Because of the fact that both isolates from French chamois are identical as shown by their 5' UTR sequences and both animals were from the same location, these chamois were probably involved in the same BDV epidemic.

Border disease is a congenital infection of sheep and goats first reported from the border region between England and Wales in 1959; the distribution of the virus is currently worldwide. Prevalence varies in sheep populations from 5% to 50%. Clinical signs in sheep include barren ewes, abortions, stillbirths, and birth of small weak lambs. Affected lambs may show tremor, abnormal body conformation, and hairy fleeces (Nettleton et al., 1998).

In conclusion, our phylogenetic analysis indicates that French Pyrenean chamois harbor a pestivirus distinct from the established pestivirus genotypes showing a basal position to the BDV strains of sheep. Therefore, cross-species transmission of pestivirus from domestic sheep to chamois via shared pastures is unlikely for this virus strain. Several investigators have also speculated that an independent cycle of pestivirus infection occurs among wild ruminants (Elazhary et al., 1981; Weber et al., 1982; Liebermann et al., 1989; Frölich, 1995). In addition, we postulate that this BDV strain occurs in the entire population of Pyrenean chamois throughout this ecosystem, because an identical sequence (Hurtado et al., 2004) and a similar strain (Arnal et al., 2004) to our isolate were recently detected in Spanish chamois. Our results seem to be valuable for management of the nature reserve, because it does not appear to be necessary to separate domestic sheep and Pyrenean chamois because of concern over transmission of this pestivirus strain.

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