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Genetic characterization of ovine pestiviruses isolated in France, between 1985 and 2006

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Abstract

In order to investigate the genetic heterogeneity of ovine pestivirus isolates from France, 32-clinical samples collected between 1985 and 2006, previously detected positive by indirect immuno-fluorescence for the presence of border disease virus (BDV), were tested by reverse transcription-polymerase chain reaction (RT-PCR). Parts of the 5'-non coding region (5'-NCR) and the Npro-coding region were successfully amplified from 23 samples. An internal control was designed and used to assess the validity of negative RT-PCR results, which could be explained by RNA degradation during the storage of samples at –20°C for at least 16 years. A phylogenetic study was performed by using sequences obtained from the two loci. French isolates clustered into four distinct groups. Six isolates were assigned to the previously described BDV-3 group. However, some isolates could not be assigned to any existing phylogenetic BDV groups, and therefore, tentative new

phylogenetic groups are suggested. The AV strain, isolated in 1984 from sheep showing a severe hemorrhagic syndrome in the rearing region of Aveyron in France and sequenced during this study, should be considered as the strain model of the BDV-5 group. Nine viral sequences clustered in a set distinct from all other groups were assigned to the BDV-6 group. Two viral sequences were distinct from the BDV phylum and composed the last set assigned to the group of unclassified pestivirus that had been previously isolated in Tunisia. The marked diversity of pestiviruses might reflect the sheep trade in France and with foreign countries.

Keywords: Border disease virus; sheep; RT-PCR; autoprotease coding sequence; genotyping

1. Introduction

Pestiviruses are single-stranded positive RNA viruses belonging to the family *Flaviviridae,* the genus *Pestivirus*. These viruses infect a wide range of ungulate species like swine, cattle and sheep. They can cause prenatal and postnatal infections and heavy economical losses. A virus infection in pregnant ewes during early or mid-gestation frequently results (up to 67%) in foetal death with resorption, mummification, abortion, stillbirth or birth of unviable lambs (Scherer et al., 2001). Persistently infected (PI) animals are the main source of infection in a flock. The foetus infected between days 50 and 60 of gestation, before the onset of immunological competence, may remain healthy to a mature age but constantly excretes large amounts of infectious virus (Nettleton et al., 1992). The clinical signs exhibited by PI lambs are variable but usually described

as those of the hairy shaker syndrome. Affected lambs are small and weak, with abnormal developed hairy fleeces, and they have tonic-clonic tremors. With careful nursing PI lambs can be reared. The nervous signs decline but the infected lambs often grow slowly and under normal field conditions many will die after birth or around weaning time. Less frequently, PI sheep succumb to mucosal disease-like syndrome resulting from a genetic rearrangement of the virus showing a cytopathogenic biotype (Monies et al., 2004; Becher et al., 1996).

The pestiviruses are classified into different viral species according to their host origin: *Classical swine fever virus* (CSFV) in pig, *Bovine viral diarrhea virus 1 and* 2 (BVDV-1 and BVDV-2) in cattle, and *Border disease virus* (BDV) in sheep. Today, isolates are classified according to their genetic relatedness to viral strains that are used to define the type species. The genome of pestiviruses has a length of approximately 12.3 kb. The viral genome contains one large open reading frame (ORF), which encodes a polyprotein, and is flanked by 5' and 3' non-coding region (NCR). The virus-encoded polyprotein is cleaved into structural (C, Erns, E1 and E2) and non-structural proteins (Npro, P7, NS2-3, NS4A, NS4B, NS5A, NS5B). Previous reported phylogenetic analysis used different genomic regions, namely 5'-NCR, Npro or E2 genes, to distinguish genotypes within each viral species. Npro refers to a N-terminal autoprotease that has no counterpart in other flaviviruses, whereas the E2 protein plays a major role in virus attachment and entry. In addition, E2 is also important for the induction of neutralizing antibodies. So far, genotyping by using 5'-NCR, Npro or E2 sequences has given consistent results i.e grouping of isolates was identical. As the 5'-NCR is relatively more conserved, this genomic region was used to define pan-pestivirus reactive primers (Vilček et al., 1994), and was also more frequently utilised in genotyping

studies. However, despite the lack of consensual sequences into the Npro or E2 genes to select efficient primers, these genes appear more relevant to distinguish different genotypes (Becher et al., 2003). Currently, 3 genotypes are distinguished inside CSFV, 11 genotypes inside BVDV-1 (Vilček et al., 2001), 2 genotypes inside BVDV-2 (Vilček et al., 2005), and 4 genotypes inside BDV (Valdazo-González et al., 2007). Considering BDV-1, BDV-2, and BDV-3 as separate genotypes, the range of sequence divergence of Npro region were 4.1-13.8% between virus isolates of one subgroup, 16.4-27.7% between sub-groups, and 28.9-37.6% between genotypes (Becher et al., 2003).

Recently, pestiviruses havebeen also isolated from other domestic and wild ruminants. Although some pestivirus strains were classified as new species, in particularthe viruses from exotic animals (Becher et al., 2003; Vilček and Nettleton, 2006), most fell into established genotypes described for swine, cattle or ovine pestiviruses. On the other hand, initial characterisation of the BDV-4 genotype, was made after the discovery of a pestivirus infecting Pyrenean chamois - *Rupicapra pyrenaica* - (Arnal et al., 2004), but more recently viruses of this genotype have been also isolated from ovine (Valdazo-González et al., 2006, 2007).

Generally, sheep seem naturally sensitive to the infection by pestiviruses from different viral species. In sheep, BDV, but also BVDV-1 strains, were isolated from animals reared in different countries such as Germany, Sweden, United Kingdom, and United States (Becher at al., 1994; Vilček et al., 1997; Willoughby et al., 2006). In Germany, United Kingdom, and United States, sheep were also found to be infected by BVDV-2, which had been frequently isolated from cattle since 1994, (Becher et al., 1995; Sullivan et al., 1997; Vilček et al.,

1997). The transmission of pestiviruses between different animal species was associated to common pasture or to close contact between animals (Valdazo-González et al., 2006). In addition, pestiviruses belonging to an intermediate group, which is genetically close to CSFV but antigenically related to BDV, were isolated in Tunisia from sheep (Thabti et al., 2005). These strains were associated to clinical cases due to the use of a vaccine produced on contaminated cell lines derived from ovine, but natural infections also occurred. Because no other pestiviruses were assigned to this group, it could be hypothesised that they might be representative of local strains. So far, the genetic diversity of ruminant pestiviruses from France was only reported in cattle on a limited number of BVDV-1 and BVDV-2 isolates (Vilček et al., 2001), but no characterization of pestiviruses from sheep was published.

The aim of this study was to genetically characterize the pestiviruses isolated in France by our laboratory since 1985 and to establish their phylogenetic relationship with other pestiviruses previously isolated in various animal species throughout the world.

2. Materiel and methods

Virus isolates

Thirty two samples, obtained from clinical cases of abortion or hemorrhagic syndrom in 26 sheep flocks, between 1985 and 2006, and previously tested BDV-positive by indirect immuno-fluorescence, were kept at –20°C since

126 their date of reception (Table 1). Most of them (22/32) came from large flocks
127 located in the South-East of France. Seven clinical samples came from Southwest
128 regions (Aquitaine and Midi-Pyrénées) close to the Pyrenean, one sample from the
129 Centre of France (85-F-488) and two samples from the East region (90-F-6159 and
130 90-F-6233).

131 The Moredun cythopatic strain of BDV-1 kindly provided by Pr. P.
132 Nettleton (Moredun Institute, United Kingdom) was produced and titrated on the
133 ETM52 cell line (Thabti et al., 2002), and kept at –80°C until used. The strain AV,
134 a generous gift of Dr. G. Chappuis (Merial, France), was conserved in liquid
135 nitrogen until used.

136

137 **Viral RNA purification**

138

139 Total RNA was extracted from 100 µl of serum or from 10%-homogenate
140 (w/v) of tissue in Minimum Essential Medium (MEM-Gibco) using the RNeasy®
141 mini kit (Qiagen) according to the manufacturer's instructions. The RNA was
142 recovered in 30 µl of RNAse free water and kept at –80°C until used.

143

144 **Amplification of 5'-NCR by RT-PCR**

145

146 The genomic region encoding the highly conserved 5'-non coding region
147 (5'-NCR) of the genome was amplified using the primers 324 and 326 flanking a
148 249 bases fragment (Vilček et al., 1994). Briefly, RNA extract (2 µl) was analysed
149 by RT-PCR in a single tube. The 10-µl RT mixture was made of 50 mM Tris-HCl,
150 pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 500 µM of the four deoxynucleoside

triphosphates, 1 µM of downstream primer (326), 20 U of RNase inhibitor (RNaseOUT ribonuclease, Invitrogen), and 100 U of M-MLV reverse transcriptase (SuperCript III reverse transcriptase, Invitrogen). The mixture was incubated for 1 h at 55°C. PCR was carried out in a 50 µl reaction mixture containing the 10 µl of RT-mixture supplemented with 40 µl of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 250 µM deoxynucleoside triphosphates, 0.25 µM of downstream primer (324), and 2.5 U of *Taq* DNA polymerase (Platinium *Taq* DNA polymerase, Invitrogen). The cycling conditions were as follows: 94°C for 2 min; followed by 45 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 15 min. Amplified products were detected by electrophoresis on a 2% agarose gel stained with ethidium bromide. In order to avoid carry-over contamination, separate rooms and dedicated material were used for master-mix preparation, sample processing, RNA transcription, DNA amplification, and amplicon detection. A negative amplification control (water) was included in each amplification series.

Competitive RT-PCR 5'-NCR

A competitive internal control (CIC) was included in every RT-PCR reaction in order to assess the presence of eventual inhibitors. This CIC was produced by amplifying the multi-cloning site of pBlueScript II KS (+-) plasmid with chimeric primers harbouring homologies with the plasmid and with the pestivirus primer 324 (5' ATG CCC TTA GTA GGA CTA GCA GCG GCC GCT CTA GAA CTA GTG GA 3') or 326 (5' TCA ACT CCA TGT GCC ATG TAC CAC TTT ATG CTT CCG GCT CGT ATG TT 3'). A second amplification

reaction was performed using the primers 326 and 324 flanking at its 5' termini with the sequence of the T7 promoter (5' GCG TAA TAC GAC TCA CTA TAG GGA TGC CCT TAG TAG GAC TAG CA 3'). Amplified DNAs were purified with the QiaQuick® PCR Purification kit (Qiagen) and 1 µl of DNA was used as template for *in vitro* transcription using the RiboMax Large Scale production Systems T7 Kit (Promega), according to the manufacturer's instructions. After 4 h of incubation at 37°C, the DNA template was cleaved with 10 U of RNase free DNase I, during 15 min at 37°C. A subsequent digestion of residual DNA was performed using 5 µl of transcript supplemented with 1X Buffer and 5 U of Dnase I Rnase free in a final volume of 500 µl. The neo-synthesised RNA was quantified by agarose gel electrophoresis. Aliquots containing 25 ng of CIC were kept at –80°C. For competitive RT-PCR, 1.5 µl of a 10^{-7} fold dilution in water was mixed with 13.5 µl of viral RNA. Co-amplification of the CIC and the viral 5'-NCR were performed by RT-PCR by using 2 µl of this mixture and primers 324 and 326 as described in the previous section. The amplified CIC had a length of 260 pb compared to about 288 pb for the 5'-NCR amplicon. A negative amplification control (water) was included in each amplification series.

Amplification of Npro regions by RT-nested PCR

The 5'-NCR/Npro/Erns genomic region was amplified by using the primers P324 and 1400RC, recently published by Vilček et al (2005). RNA extract (2 µl) was analysed by RT-PCR in a single tube essentially as described for 5'-NTC region except that the quantity of M-MLV (200 U) in the RT mixture was increased to 200 U, and that 35 cycles were performed using an annealing

temperature of 52°C. This first RT-PCR amplification step yielded an amplicon with a size of 1,349 bp. A nested PCR was performed using the primers BD1 and BD2 (Vilček et al., 1997) and the same reaction conditions as the first amplification. The amplified products were about 738 pb in length.

Sequence analysis

RT-PCR products characterized by a single band and an intense fluorescent signal on the agarose gel were purified by using the QiaQuick® PCR Purification kit (Qiagen) and direct sequencing of both strands was performed by Millegen (France). Each sample was treated in duplicate. GenBank accession numbers of the obtained sequences are indicated in Table 1. Because segments of genome were the most frequently analysed and deposited in the GenBank database, consensus of the sequences were checked in shorter genomic regions of above 212 bases in the 5'-NCR and above 489 bases in the Npro coding region using the Lasergene software (DNAStar, Madison, Wisconsin). Sequence alignments and phylogenetic trees were calculated with the CLUSTAL X (version 1.81) analysis program (Thompson et al., 1997). Confidence values were determined by the bootstrapping method, as implemented in CLUSTAL X. Visualization of the phylogenetic trees was performed using TREEVIEW, version 1.6.6 (Page, 1996).

3. Results

Detection of viral RNA by competitive RT-PCR

In order to check if the use of the CIC could affect the sensitivity of the 5'-NCR RT-PCR, step-fold dilutions of RNA extracted from a titrated culture of strain PBII were analysed with and without CIC (data not shown). The limit of detection of the competitive RT-PCR was estimated to 0.7 $TCID_{50,}$ whereas without CIC, a 10-fold increase of sensitivity was noted. By agarose gel electrophoresis, bands of equal intensity were observed for a dilution of viral RNA equivalent to about 7 $TCID_{50}$/reaction. However, the band corresponding to the CIC could not be observed when viral RNA template equivalent to 700 $TCID_{50}$ was amplified. Among the 32 BDV positive samples, as determined by IIF, 23 were found positive by competitive RT-PCR (Fig. 1). Most of the positive samples contained high quantities of viral RNA since CIC specific amplicons were not detected (Fig. 2, lanes 2 and 4). The viral RNA and the CIC were detected simultaneously in few samples only (Fig. 2, lanes 5 and 6). Negative RT-PCR samples were found (Fig. 2, lanes 1, 7 and 8). For these negative samples, the intensity of the CIC-specific band was equivalent to that observed for the negative control (Fig. 2, lane CIC). No viral RNA was detected from the negative samples by RT-PCR performed without CIC (data not shown).

Genetic typing in the 5'-NCR

The nucleotide sequence obtained from the 23 RT-PCR positive samples was used to infer the phylogenetic relationship with pestivirus sequences already deposited in the GenBank database (Table 1). Ovine pestiviruses from France segregated into four genotypes (Fig. 3). Three groups were clearly related to the BDV strains. One of these groups included the first strain of pestivirus isolated in

France, in 1984 (strain AV, GenBank accession number: EF693984) and four viruses infecting ovine between 1985 and 1996, in the Pyrenean mountains (89-F-5415, and 96-F-7624), in the Centre of France (85-F-488) and in the Alpine mountains (93-F-7289). Notably, one virus (85-F-488) showed 100% identity with the AV strain. This group of viruses was the most closely related to the Spanish strains of BDV and clustered with the viruses from chamois, all previously classified as group BDV-4. A second group of viruses obtained from samples collected between 1990 and 2006, in the Southeast region of France, was bounded with a low bootstrap value to a phylogenetic node that bifurcate to form the lineage of the viruses classified in the BDV-4 group. The third group of French pestiviruses was the most distant from the others but still related to the phylogenetic branch of the BDV group. This set of viruses included isolates collected between 1985 and 2006, also in the Southeast region of France.

Two pestivirus sequences (91-F-6731 and 91-F-6732) obtained from ovine isolates collected in 1991, in Vaucluse (one of the six counties of the Provence-Alpes-Côte d'Azur region), were clearly related to the Tunisian isolates that, until now, were the only viruses related to this group. This group of ovine pestiviruses segregate on a branch located close to viruses classified in the CSFV type. However, this branch is not supported by a strong bootstrap value (53%).

Genetic typing in the Npro region

All the samples previously found positive by RT-PCR were also amplified in the starting coding region of the pestivirus genome. However, few of them provided quantities of cDNA enabling a direct sequencing (data not shown). Re-

amplification was performed by nested PCR, which finally made possible to sequence the Npro/C region (about 780 bp between primers BD1 and BD2) from all positive samples. Twenty-one different viral sequences were found. The sequences obtained from the three sub-samples 06-F-0083 showed a complete identity in the Npro/C region as well as in 5'-NCR region. A phylogenetic tree was constructed (Fig. 4). As previously, four genetic groups of French viruses were found.

The cluster of French pestiviruses, previously related to the group DBV-4 by analysing the 5'-NCR, was also more closely related to the pestivirus strains isolated from ovine in Spain and from chamois. However, the phylogenetic analysis did not find statistically available ancestral taxa between the two sets of viral sequences. Inside this new set of viruses, the sequence 85-F-488 had complete identity with the sequence of the strain AV (GenBank accession number: EF693962). The range of sequence divergence reached 3.5% between virus isolates of this set of pestiviruses and was about 25.1-33.9% between these viruses and the BDV-4 viruses. Consequently, we proposed to designate this group as BDV-5. The second group of viruses (range of divergence: 0.0 to 5.3% between isolates) was not clearly related to other pestivirus sequences and formed a distinct genotype inside the BDV group. The lower sequence divergence (26.0 to 32.4%) was found between this set of isolates and the BDV-1 strains. We proposed to designate this second new group of viruses as BDV-6. The third group of French viruses, previously found distant from the other pestiviruses, was closely related to the viruses of the BDV-3 group (range of divergence: 8.8 to 12.4%). The two pestiviruses sequences (91-F-6731 and 91-F-6732), obtained from ovine samples

300 performed in 1991 from the Vaucluse county, also clustered in the Tunisian group
301 of unclassified viruses (range of divergence: 8.6 to 14.5%).
302
303 **4. Discussion**
304
305 In this study, the diversity of ovine pestiviruses isolated in France was
306 investigated by viral RNA amplification in the 5'-NCR and the Npro region, direct
307 sequencing, and phylogenetic analysis. Only 26 sheep flocks were taken into
308 account in this study among about 40,000 professional flocks, most of them
309 located in the south half of France. Nevertheless, the results provide the first
310 sequences of 21 different French pestiviruses obtained over more than 20 years
311 from ovine with symptoms of border disease. The sampling began in 1985, less
312 than two years after the first strain of BDV (the AV strain) was isolated in France,
313 (in Aveyron, one of the height counties of the Midi-Pyrénées region), from sheep
314 having hemorrhagic syndrome. The last sample was collected in 2006, when we
315 started the present study.
316 Thirty-two samples, which had been found positive for BDV by viral
317 isolation and identification by indirect immuno-fluorescence, were tested. Only 23
318 of them were successfully amplified by RT-PCR in two distinct parts of the
319 pestivirus genome. No inhibition of the RT-PCR reactions was detected, since an
320 internal control RNA (ICC), specifically designed during the time course of this
321 study, was successfully amplified in all samples by using the 5'-NCR RT-PCR.
322 Therefore, it is likely that RNA degradation had occurred in RT-PCR negative
323 samples during prolonged storage at –20°C, or that the quantity of RNA was
324 below the limit of detection. The recent procedure of storage of samples using

guanidinium compounds that overcome the action of nuclease and/or the storage of samples at –80°C are now currently available to reduce such RNA degradation process.

The phylogenetic analysis of the viral sequences made possible the classification of the French viruses in four genetic groups. Some of the ovine pestiviruses were closely related to the AV strain and were obtained from samples collected between 1985 and 1996 in several rearing regions from the Alpine to the Pyrenean mountains of France. The similarity between the viruses isolated from distant rearing regions might be associated with the trade in animals, particularly of the Lacaune species of dairy sheep. By analysing the 5'-NCR of these AV-like viruses, we found similarities with the Spanish strains of ovine pestiviruses (Hurtado et al., 2003; Valdazo-González et al., 2006a, 2006b) and with viruses previously isolated from Pyrenean chamois (Arnal et al., 2004). However, no common node was found by the phylogenetic analysis of the Npro region. If the phylogenetic analysis was performed without the Chamois-1 sequence (as proposed by Valdazo-González et al., 2007), the bootstrap value at this node was still non-significant (under 70%; data not shown). Considering the range of sequence divergence found by Becher et al. (2003) between different genotypes of BDV, we suggest that the AV strain should be the reference strain of a new genetic group named BDV-5.

Most of the French pestiviruses analysed in this study were classified into two genetic groups. The first group was composed of pestiviruses (found over a period of time of 16 year) with no clear phylogenetic similarity to the previous known pestiviruses. Therefore, it is proposed to classify these viruses also in a new genetic group (BDV-6). The viruses of the second group were found during the

longest period of time: 21 years. These viruses were related to the BDV-3, a group previously reported in Germany and Switzerland (Becher et al., 2003; Stalder et al., 2005). It is remarkable that viruses belonging to different genetic groups (BDV-3 and BDV-6) could be detected in the same rearing area (Alpes de Haute-Provence) during the same period of time (i.e. 2006). It is likely that pestiviruses belonging to these groups were endemic to the Southeast region of France. However, we did not exclude the possibility that strains related to the AV strain (BDV-5) were still circulating because only few clinical samples from different sheep flocks were conserved over the sampling period and because no samples were available in our laboratory for the period of time between 1997 and 2005.

Another striking result is that French pestiviruses collected in 1991 clustered with Tunisian isolates. Although these viruses were genetically related to the group of CSFVs, Thabti et al. (2005) assigned Tunisian isolates as BDV according to their antigenic properties. We excluded a possible contamination of our samples by the Tunisian samples analysed by Thabti et al. (2005), which were all analysed in our laboratory, because of the long delay of time that separates the studies. Besides, in the present study original non-cultivated clinical samples were used to avoid possible contamination during cell cultivation particularly by pestiviruses from the foetal calf serum or the cell culture itself. Although it is not possible to find any epidemiological link between Tunisian and French strains, this finding might reflect the trade in sheep between France and North African countries, and emphasizes once more the importance of sanitary controls of the imported and exported animals. However, French viruses sharing similarities with Tunisian pestiviruses were only observed from samples collected in 1991 in two

neighbouring flocks. Consequently, we could not conclude that this genetic type is still circulating in France.

During this study, pestiviruses belonging to the BDV-1 group were not detected, despite the fact that they were reported to be widely distributed (Vilček et al., 1997; Hurtado et al., 2003). Besides, no viruses close to BVDV were found during our study despite that the bovine viruses might infect the sheep (Scherer et al., 2001).

In conclusion, the French ovine pestiviruses analysed in this study clustered in four genetic groups. Two of them were closely related to previously described genetic groups of pestiviruses (BDV-3 isolated until now in Germany and Switzerland, and unclassified Tunisian isolates). Two sets of BDV sequences could not be clustered with sequences of viruses obtained from GenBank database and consequently we suggest BDV-5 and BDV-6 as additional genotypes. However, further genetic studies should be carried out to make possible a more definitive classification of the BDV.

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References

398 Arnal, M., Fernández-de-Luco, D., Riba, L., Maley, M., Gilray, J., Willoughby, K.,
399 Vilcek, S., Nettleton, P. F., 2004. A novel pestivirus associated with deaths in
400 Pyrenean chamois (Rupicapra pyrenaica pyrenaica). J.Gen.Virol. 85, 3653-3657.
401 Becher, P., Avalos, R. R., Orlich, M., Cedillo, R. S., König, M., Schweizer, M.,
402 Stalder, H., Schirrmeier, H., Thiel, H. J., 2003. Genetic and antigenic
403 characterization of novel pestivirus genotypes: implications for classification.
404 Virology 311, 96-104.
405 Becher, P., Meyers, G., Shannon, A. D., Thiel, H. J., 1996. Cytopathogenicity of
406 border disease virus is correlated with integration of cellular sequences into the
407 viral genome. J.Virol. 70, 2992-2998.
408 Becher, P., König, M., Paton, D. J., Thiel, H. J., 1995. Further characterization of
409 border disease virus isolates: evidence for the presence of more than three species
410 within the genus pestivirus. Virology 209, 2000-2006.
411 Becher, P., Shannon, A. D., Tautz, N., Thiel, H. J., 1994. Molecular characterization
412 of border disease virus, a pestivirus from sheep. Virology 198, 542-551.
413 Hurtado, A., Aduriz, G., Gomez, N., Oporto, B., Juste, R. A., Lavin, S., Lopez-Olvera,
414 J. R., Marco, I., 2004. Molecular identification of a new pestivirus associated with
415 increased mortality in the Pyrenean Chamois (Rupicapra pyrenaica pyrenaica) in
416 Spain. J.Wildl.Dis. 40, 796-800.
417 Hurtado, A., García-Pérez, A. L., Aduriz, G., Juste, R. A., 2003. Genetic diversity of
418 ruminant pestiviruses from Spain. Virus Res. 92, 67-73.
419 Monies, R. J., Paton, D. J., Vilček, Š., 2004. Mucosal disease-like lesions in sheep
420 infected with Border disease virus. Vet.Rec. 155, 765-769.

421 Nettleton, P. F., Gilmour, J. S., Herring, J. A., Sinclair, J. A., 1992. The production
422 and survival of lambs persistently infected with border disease virus. Comp
423 Immunol.Microbiol.Infect.Dis. 15, 179-188.
424 Page, R. D., 1996. TreeView: an application to display phylogenetic trees on personal
425 computers. Comput.Appl.Biosci. 12, 357-358.
426 Scherer, C. F., Flores, E. F., Weiblen, R., Caron, L., Irigoyen, L. F., Neves, J. P.,
427 Maciel, M. N., 2001. Experimental infection of pregnant ewes with bovine viral
428 diarrhea virus type-2 (BVDV-2): effects on the pregnancy and fetus.
429 Vet.Microbiol. 79, 285-299.
430 Stalder, H. P., Meier, P., Pfaffen, G., Wageck-Canal, C., Rufenacht, J., Schaller, P.,
431 Bachofen, C., Marti, S., Vogt, H. R., Peterhans, E., 2005. Genetic heterogeneity of
432 pestiviruses of ruminants in Switzerland. Prev.Vet.Med. 72, 37-41.
433 Sullivan, D. G., Chang, G. J., Akkina, R. K., 1997. Genetic characterization of
434 ruminant pestiviruses: sequence analysis of viral genotypes isolated from sheep.
435 Virus Res. 47, 19-29.
436 Thabti, F., Letellier, C., Hammami, S., Pépin, M., Ribière, M., Mesplede, A.,
437 Kerkhofs, P., Russo, P., 2005. Detection of a novel border disease virus subgroup
438 in Tunisian sheep. Arch.Virol. 150, 215-229.
439 Thabti, F., Fronzaroli, L., Dlissi, E., Guibert, J.M., Hammami, S., Pépin, M., Russo,
440 P., 2002. Experimental model of border disease virus infections in lambs:
441 comparative pathogenicity of pestiviruses isolated in France and Tunisia. Vet. Res.
442 33, 35-45.
443 Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., Higgins, D. G., 1997.
444 The CLUSTAL_X windows interface: flexible strategies for multiple sequence
445 alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.

Valdazo-González, B., varez-Martínez, M., Greiser-Wilke, I., 2006a. Genetic typing and prevalence of Border disease virus (BDV) in small ruminant flocks in Spain. Vet.Microbiol. 117, 141-153.

Valdazo-González, B., varez-Martínez, M., Sandvik, T., 2006b. Genetic and antigenic typing of border disease virus isolates in sheep from the Iberian Peninsula. Vet.J.

Vilček, Š., Nettleton, P. F., 2006. Pestiviruses in wild animals. Vet.Microbiol. 116, 1-12.

Vilček, Š., Durkovic, B., Kolesarova, M., Paton, D. J., 2005a. Genetic diversity of BVDV: consequences for classification and molecular epidemiology. Prev.Vet.Med. 72, 31-35.

Vilček, Š., Ridpath, J. F., Van, C. H., Cavender, J. L., Warg, J., 2005b. Characterization of a novel pestivirus originating from a pronghorn antelope. Virus Res. 108, 187-193.

Vilček, Š., Paton, D. J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S., Scicluna, M. T., Paifi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch.Virol. 146, 99-115.

Vilček, Š., Nettleton, P. F., Paton, D. J., Belák, S., 1997. Molecular characterization of ovine pestiviruses. J.Gen.Virol. 78 (Pt 4), 725-735.

Vilček, Š., Herring, A. J., Herring, J. A., Nettleton, P. F., Lowings, J. P., Paton, D. J., 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Arch.Virol. 136, 309-323.

469 Willoughby, K., Valdazo-González, B., Maley, M., Gilray, J., Nettleton, P. F., 2006.
470 Development of a real time RT-PCR to detect and type ovine pestiviruses.
471 J.Virol.Methods 132, 187-194.

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Table 1
Clinical samples from ovine flocks analyzed in this study

Flock no. /Sample ref.	Sub-ref.	Date of reception	Geographical origin	Nature of sample	GenBank accession no.[a]	
					5'-NCR	Npro
1/85-F-488		May 1985	Centre	Spleen	EF693985	EF693963
2/85-F-517		May 1985	Aquitaine	Spleen	-	-
3/85-F-588		Jun 1985	PACA[b]	Spleen	EF693986	EF693966
4/85-F-864		August 1985	Midi-Pyrénées	Spleen	-	-
5/89-F-5374		April 1989	PACA	Spleen	EF693987	EF693964
6/89-F-5415		May 1989	Aquitaine	Lung	EF693988	EF693965
7/89-F-5867		November 1989	Midi-Pyrénées	Spleen	-	-
8/89-F-5906		November 1989	Aquitaine	Spleen	-	-
9/90-F-6146		February 1990	Aquitaine	Serum	-	-
10/90-F-6159		March 1990	Bourgogne	Lung	-	-
11/90-F-6172		March 1990	PACA	Serum	-	-
12/90-F-6173		March 1990	PACA	Serum	-	-
13/90-F-6227		April 1990	PACA	Serum	EF693989	EF693967
14/90-F-6233		April 1990	Bourgogne	Spleen	-	-
15/90-F-6335		May 1990	PACA	Serum	EF693990	EF693968
16/90-F-6338		May 1990	PACA	Serum	EF693991	EF693969
17/90-F-6339		May 1990	PACA	Serum	EF693992	EF693970
18/91-F-6731		January 1991	PACA	Spleen	EF988632	EF693982
19/91-F-6732		January 1991	PACA	Liver	EF988633	EF693983
20/91-F-7014		December 1991	PACA	Serum	EF693993	EF693971
21/92-F-7119		April 1992	PACA	Serum	EF693994	EF693972
22/93-F-7289		February 1993	PACA	Intestine	EF693995	EF693973
23/94-F-7446	1	April 1994	PACA	Spleen	EF693996	EF693974
	2				EF693997	EF693975
24/96-F-7624		February 1996	Midi-Pyrénées	Spleen	EF693998	EF693976
25/06-F-0083	834	February 2006	PACA	Serum	EF693999	EF693977
	835					
	836					
26/06-F-0299	60357	April 2006	PACA	Serum	EF694000	EF693978
	60369				EF694001	EF693979
	60420				EF694002	EF693980
	60477				EF694003	EF693981

[a] -: no viral sequence detected by RT-PCR
[b] PACA: Provence-Alpes-Côte d'Azur

Fig. 1. Map of France with the region borders, indicating the geographical location (black circles) of the clinical samples found positive for pestivirus by RT-PCR.

Fig. 2. Competitive RT-PCR with RNA extracted from clinical samples. The amplification reactions were performed using 1 µl of the dilution 10^{-7} of the CIC mixed with water (lane CIC) or RNA extract from negative samples (lanes 1, 7 and 8) and positive samples (lanes 2 to 6). Lanes 0: negative control (water). Lane +: viral RNA of BPII virus. Lanes MW: molecular length marker (SmartLadder, Eurogentec).

Fig 3. Neighbour-joining phylogenetic tree constructed using 212 nt from the 5'-NCR of the pestivirus sequences found during this study and from the database. Sequences taken from GenBank database with the following accession numbers: Giraffe, NC003678; 890, U18059; C413, AF002227; NADL, NC001461; Osloss, M96687; 35, AF462001; 33S, AF462002; BM01, AY453630; 544099, AY159514; Alfort, X87939; Brescia, AF091661; Reindeer, NC003677; BD31, U70263; X818, AF037405; Moredunncp, U65023; 832031NZ, U65064; 1374, U65052; D15862, U65034; BU1CRA22, DQ275622; C27, DQ275623; Chamois1, AY738080; OrluR36, DQ898294; M3, DQ275626; C121, DQ275625; LE31C2, DQ361072. The tree was outgrouped to the sequence of the Giraffe pestivirus. The numbers close to the major nodes indicate the bootstrap values (in percent; 1000 replicates). Bar, number of substitutions per site.

Fig 4. Neighbour-joining phylogenetic tree constructed using 489 nt from the Npro region of the pestivirus sequences found during this study and from the database. Sequences taken from GenBank database with the following accession numbers: Antelope, AY781152; 890, U18059; C413, AF002227; NADL, NC001461; Osloss,; Giraffe, NC003678; HoBi,

AY735486; 35, AF462014; BM01, AY452482; 33S, AF462015; Alfort, X87939; Brescia, AF091661; 71202, AJ829444; CHBD1, AY895008; Gifhorn, AY163653; CHBD2, AY895009; 466, AY163650; 1738500, AY163651; Chemnitz, AY163652; Reindeer1, NC003677; Bison1, AF144476; 1374, L05402; X818, AF037405; BD31, U70263; CB5, AF145358; Chamois1, AY738083; M3, DQ273163; C121, DQ273159; LE31C2, DQ273161; BU1CRA22, DQ273155; C27, DQ273156. The tree was outgrouped to the sequence of the Antelope pestivirus. The numbers close to the major nodes indicate the bootstrap values (in percent; 1000 replicates). Bar, number of substitutions per site. Nomenclature of species, genotype and subtype were described according to Becher et al. (2003), and Valdazo-González et al. (2007).