



**HAL**  
open science

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

Eric Dubois, Pierre Russo, Myriam Prigent, Richard Thiéry

### ► To cite this version:

Eric Dubois, Pierre Russo, Myriam Prigent, Richard Thiéry. Genetic characterization of ovine pestiviruses isolated in France, between 1985 and 2006. *Veterinary Microbiology*, 2008, 130 (1-2), pp.69. 10.1016/j.vetmic.2008.01.002 . hal-00532384

**HAL Id: hal-00532384**

**<https://hal.science/hal-00532384>**

Submitted on 4 Nov 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

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

Authors: Eric Dubois, Pierre Russo, Myriam Prigent, Richard Thiéry



PII: S0378-1135(08)00007-2  
DOI: doi:10.1016/j.vetmic.2008.01.002  
Reference: VETMIC 3938

To appear in: *VETMIC*

Received date: 5-7-2007  
Revised date: 16-11-2007  
Accepted date: 15-1-2008

Please cite this article as: Dubois, E., Russo, P., Prigent, M., Thiéry, R., Genetic characterization of ovine pestiviruses isolated in France, between 1985 and 2006, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.01.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



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

34

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

37

## 38 **1. Introduction**

39

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

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

58 The pestiviruses are classified into different viral species according to their  
59 host origin: *Classical swine fever virus* (CSFV) in pig, *Bovine viral diarrhea virus*  
60 *1 and 2* (BVDV-1 and BVDV-2) in cattle, and *Border disease virus* (BDV) in  
61 sheep. Today, isolates are classified according to their genetic relatedness to viral  
62 strains that are used to define the type species. The genome of pestiviruses has a  
63 length of approximately 12.3 kb. The viral genome contains one large open  
64 reading frame (ORF), which encodes a polyprotein, and is flanked by 5' and 3'  
65 non-coding region (NCR). The virus-encoded polyprotein is cleaved into structural  
66 (C, Erns, E1 and E2) and non-structural proteins (Npro, P7, NS2-3, NS4A, NS4B,  
67 NS5A, NS5B). Previous reported phylogenetic analysis used different genomic  
68 regions, namely 5'-NCR, Npro or E2 genes, to distinguish genotypes within each  
69 viral species. Npro refers to a N-terminal autoprotease that has no counterpart in  
70 other flaviviruses, whereas the E2 protein plays a major role in virus attachment  
71 and entry. In addition, E2 is also important for the induction of neutralizing  
72 antibodies. So far, genotyping by using 5'-NCR, Npro or E2 sequences has given  
73 consistent results i.e grouping of isolates was identical. As the 5'-NCR is relatively  
74 more conserved, this genomic region was used to define pan-pestivirus reactive  
75 primers (Vilček et al., 1994), and was also more frequently utilised in genotyping

76 studies. However, despite the lack of consensual sequences into the Npro or E2  
77 genes to select efficient primers, these genes appear more relevant to distinguish  
78 different genotypes (Becher et al., 2003). Currently, 3 genotypes are distinguished  
79 inside CSFV, 11 genotypes inside BVDV-1 (Vilček et al., 2001), 2 genotypes  
80 inside BVDV-2 (Vilček et al., 2005), and 4 genotypes inside BDV (Valdazo-  
81 González et al., 2007). Considering BDV-1, BDV-2, and BDV-3 as separate  
82 genotypes, the range of sequence divergence of Npro region were 4.1-13.8%  
83 between virus isolates of one subgroup, 16.4-27.7% between sub-groups, and  
84 28.9-37.6% between genotypes (Becher et al., 2003).

85 Recently, pestiviruses have been also isolated from other domestic and wild  
86 ruminants. Although some pestivirus strains were classified as new species, in  
87 particular the viruses from exotic animals (Becher et al., 2003; Vilček and  
88 Nettleton, 2006), most fell into established genotypes described for swine, cattle or  
89 ovine pestiviruses. On the other hand, initial characterisation of the BDV-4  
90 genotype, was made after the discovery of a pestivirus infecting Pyrenean chamois  
91 - *Rupicapra pyrenaica* - (Arnal et al., 2004), but more recently viruses of this  
92 genotype have been also isolated from ovine (Valdazo-González et al., 2006,  
93 2007).

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

101 1997). The transmission of pestiviruses between different animal species was  
102 associated to common pasture or to close contact between animals (Valdazo-  
103 González et al., 2006). In addition, pestiviruses belonging to an intermediate  
104 group, which is genetically close to CSFV but antigenically related to BDV, were  
105 isolated in Tunisia from sheep (Thabti et al., 2005). These strains were associated  
106 to clinical cases due to the use of a vaccine produced on contaminated cell lines  
107 derived from ovine, but natural infections also occurred. Because no other  
108 pestiviruses were assigned to this group, it could be hypothesised that they might  
109 be representative of local strains. So far, the genetic diversity of ruminant  
110 pestiviruses from France was only reported in cattle on a limited number of  
111 BVDV-1 and BVDV-2 isolates (Vilček et al., 2001), but no characterization of  
112 pestiviruses from sheep was published.

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

117

118

## 119 **2. Materiel and methods**

120

### 121 **Virus isolates**

122

123 Thirty two samples, obtained from clinical cases of abortion or  
124 hemorrhagic syndrom in 26 sheep flocks, between 1985 and 2006, and previously  
125 tested BDV-positive by indirect immuno-fluorescence, were kept at  $-20^{\circ}\text{C}$  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 cythopathic 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  $-80^{\circ}\text{C}$  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  $\mu\text{l}$  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  $\mu\text{l}$  of RNase free water and kept at  $-80^{\circ}\text{C}$  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  $\mu\text{l}$ ) was analysed  
149 by RT-PCR in a single tube. The 10- $\mu\text{l}$  RT mixture was made of 50 mM Tris-HCl,  
150 pH 8.3, 75 mM KCl, 3 mM  $\text{MgCl}_2$ , 500  $\mu\text{M}$  of the four deoxynucleoside



151 triphosphates, 1  $\mu$ M of downstream primer (326), 20 U of RNase inhibitor  
152 (RNaseOUT ribonuclease, Invitrogen), and 100 U of M-MLV reverse transcriptase  
153 (SuperCript III reverse transcriptase, Invitrogen). The mixture was incubated for 1  
154 h at 55°C. PCR was carried out in a 50  $\mu$ l reaction mixture containing the 10  $\mu$ l of  
155 RT-mixture supplemented with 40  $\mu$ l of 20 mM Tris-HCl, pH 8.4, 50 mM KCl,  
156 2.5 mM MgCl<sub>2</sub>, 250  $\mu$ M deoxynucleoside triphosphates, 0.25  $\mu$ M of downstream  
157 primer (324), and 2.5 U of *Taq* DNA polymerase (Platinum *Taq* DNA  
158 polymerase, Invitrogen). The cycling conditions were as follows: 94°C for 2 min;  
159 followed by 45 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a  
160 final extension step at 72°C for 15 min. Amplified products were detected by  
161 electrophoresis on a 2% agarose gel stained with ethidium bromide. In order to  
162 avoid carry-over contamination, separate rooms and dedicated material were used  
163 for master-mix preparation, sample processing, RNA transcription, DNA  
164 amplification, and amplicon detection. A negative amplification control (water)  
165 was included in each amplification series.

166

### 167 **Competitive RT-PCR 5'-NCR**

168

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

176 reaction was performed using the primers 326 and 324 flanking at its 5' termini  
177 with the sequence of the T7 promoter (5' GCG TAA TAC GAC TCA CTA TAG  
178 GGA TGC CCT TAG TAG GAC TAG CA 3'). Amplified DNAs were purified  
179 with the QiaQuick® PCR Purification kit (Qiagen) and 1 µl of DNA was used as  
180 template for *in vitro* transcription using the RiboMax Large Scale production  
181 Systems T7 Kit (Promega), according to the manufacturer's instructions. After 4 h  
182 of incubation at 37°C, the DNA template was cleaved with 10 U of RNase free  
183 DNase I, during 15 min at 37°C. A subsequent digestion of residual DNA was  
184 performed using 5 µl of transcript supplemented with 1X Buffer and 5 U of Dnase  
185 I Rnase free in a final volume of 500 µl. The neo-synthesised RNA was quantified  
186 by agarose gel electrophoresis. Aliquots containing 25 ng of CIC were kept at –  
187 80°C. For competitive RT-PCR, 1.5 µl of a 10<sup>-7</sup> fold dilution in water was mixed  
188 with 13.5 µl of viral RNA. Co-amplification of the CIC and the viral 5'-NCR  
189 were performed by RT-PCR by using 2 µl of this mixture and primers 324 and 326  
190 as described in the previous section. The amplified CIC had a length of 260 pb  
191 compared to about 288 pb for the 5'-NCR amplicon. A negative amplification  
192 control (water) was included in each amplification series.

193

#### 194 **Amplification of Npro regions by RT-nested PCR**

195

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

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

205

### 206 **Sequence analysis**

207

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

221

## 222 **3. Results**

223

### 224 **Detection of viral RNA by competitive RT-PCR**

225

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

243

#### 244 **Genetic typing in the 5'-NCR**

245

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

251 France, in 1984 (strain AV, GenBank accession number: EF693984) and four  
252 viruses infecting ovine between 1985 and 1996, in the Pyrenean mountains (89-F-  
253 5415, and 96-F-7624), in the Centre of France (85-F-488) and in the Alpine  
254 mountains (93-F-7289). Notably, one virus (85-F-488) showed 100% identity with  
255 the AV strain. This group of viruses was the most closely related to the Spanish  
256 strains of BDV and clustered with the viruses from chamois, all previously  
257 classified as group BDV-4. A second group of viruses obtained from samples  
258 collected between 1990 and 2006, in the Southeast region of France, was bounded  
259 with a low bootstrap value to a phylogenetic node that bifurcate to form the  
260 lineage of the viruses classified in the BDV-4 group. The third group of French  
261 pestiviruses was the most distant from the others but still related to the  
262 phylogenetic branch of the BDV group. This set of viruses included isolates  
263 collected between 1985 and 2006, also in the Southeast region of France.

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

270

### 271 **Genetic typing in the Npro region**

272

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

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

283 The cluster of French pestiviruses, previously related to the group BDV-4  
284 by analysing the 5'-NCR, was also more closely related to the pestivirus strains  
285 isolated from ovine in Spain and from chamois. However, the phylogenetic  
286 analysis did not find statistically available ancestral taxa between the two sets of  
287 viral sequences. Inside this new set of viruses, the sequence 85-F-488 had  
288 complete identity with the sequence of the strain AV (GenBank accession number:  
289 EF693962). The range of sequence divergence reached 3.5% between virus  
290 isolates of this set of pestiviruses and was about 25.1-33.9% between these viruses  
291 and the BDV-4 viruses. Consequently, we proposed to designate this group as  
292 BDV-5. The second group of viruses (range of divergence: 0.0 to 5.3% between  
293 isolates) was not clearly related to other pestivirus sequences and formed a distinct  
294 genotype inside the BDV group. The lower sequence divergence (26.0 to 32.4%)  
295 was found between this set of isolates and the BDV-1 strains. We proposed to  
296 designate this second new group of viruses as BDV-6. The third group of French  
297 viruses, previously found distant from the other pestiviruses, was closely related to  
298 the viruses of the BDV-3 group (range of divergence: 8.8 to 12.4%). The two  
299 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  $-20^{\circ}\text{C}$ , or that the quantity of RNA was  
324 below the limit of detection. The recent procedure of storage of samples using

325 guanidinium compounds that overcome the action of nuclease and/or the storage  
326 of samples at  $-80^{\circ}\text{C}$  are now currently available to reduce such RNA degradation  
327 process.

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

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



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

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

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

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

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

389

#### 390 **Acknowledgements**

391

392 We are most grateful to Jean-Luc Champion (FDGDS 04, Digne, France) and  
393 veterinary practitioners for providing clinical samples. Ms Cristina Gastaldi is also  
394 acknowledged for her help to improve the English form of the manuscript.

395

#### 396 **References**

397

- 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., Schirrmeyer, 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.

- 446 Valdazo-González, B., varez-Martínez, M., Greiser-Wilke, I., 2006a. Genetic typing  
447 and prevalence of Border disease virus (BDV) in small ruminant flocks in Spain.  
448 Vet.Microbiol. 117, 141-153.
- 449 Valdazo-González, B., varez-Martínez, M., Sandvik, T., 2006b. Genetic and antigenic  
450 typing of border disease virus isolates in sheep from the Iberian Peninsula. Vet.J.  
451 Vilček, Š., Nettleton, P. F., 2006. Pestiviruses in wild animals. Vet.Microbiol. 116, 1-  
452 12.
- 453 Vilček, Š., Durkovic, B., Kolesarova, M., Paton, D. J., 2005a. Genetic diversity of  
454 BVDV: consequences for classification and molecular epidemiology.  
455 Prev.Vet.Med. 72, 31-35.
- 456 Vilček, Š., Ridpath, J. F., Van, C. H., Cavender, J. L., Warg, J., 2005b.  
457 Characterization of a novel pestivirus originating from a pronghorn antelope.  
458 Virus Res. 108, 187-193.
- 459 Vilček, Š., Paton, D. J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A.,  
460 Rossmannith, W., Vega, S., Scicluna, M. T., Paifi, V., 2001. Bovine viral diarrhoea  
461 virus genotype 1 can be separated into at least eleven genetic groups. Arch.Virol.  
462 146, 99-115.
- 463 Vilček, Š., Nettleton, P. F., Paton, D. J., Belák, S., 1997. Molecular characterization of  
464 ovine pestiviruses. J.Gen.Virol. 78 ( Pt 4), 725-735.
- 465 Vilček, Š., Herring, A. J., Herring, J. A., Nettleton, P. F., Lowings, J. P., Paton, D. J.,  
466 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least  
467 three genogroups using polymerase chain reaction and restriction endonuclease  
468 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.

472

473

474

475

Accepted Manuscript

1 Table 1  
2 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. <sup>a</sup>	
					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 <sup>b</sup>	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

3 <sup>a</sup> -: no viral sequence detected by RT-PCR

4 <sup>b</sup> PACA: Provence-Alpes-Côte d'Azur

5

6

7

8

9

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

3

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

9

10 Fig 3. Neighbour-joining phylogenetic tree constructed using 212 nt from the 5'-NCR of the  
11 pestivirus sequences found during this study and from the database. Sequences taken from  
12 GenBank database with the following accession numbers: Giraffe, NC003678; 890, U18059;  
13 C413, AF002227; NADL, NC001461; Osloss, M96687; 35, AF462001; 33S, AF462002;  
14 BM01, AY453630; 544099, AY159514; Alfort, X87939; Brescia, AF091661; Reindeer,  
15 NC003677; BD31, U70263; X818, AF037405; Moredunncp, U65023; 832031NZ, U65064;  
16 1374, U65052; D15862, U65034; BU1CRA22, DQ275622; C27, DQ275623; Chamois1,  
17 AY738080; OrluR36, DQ898294; M3, DQ275626; C121, DQ275625; LE31C2, DQ361072.  
18 The tree was outgrouped to the sequence of the Giraffe pestivirus. The numbers close to the  
19 major nodes indicate the bootstrap values (in percent; 1000 replicates). Bar, number of  
20 substitutions per site.

21

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



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







