Epidemiology of pestivirus infection in wild ungulates of the French South Alps

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Abstract

Interspecies transmission is often incriminated in epidemiology of *Pestivirus* diseases. The purpose of this study was to investigate the prevalence of *Pestivirus* in some mountain wild ungulates and to determine their role in *Pestivirus* transmission, as mountain pastures are a place where cohabitations between wild and domestic ungulates are particularly high. Between 2003 and 2007, a longitudinal epidemiological study was carried out on hunted ungulates in the French Hautes-Alpes department. Pestivirus-specific antibodies against p80 protein (named also NS3) common to all Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV) were found in 45.9% (95% confidence interval [CI95%]: 40.5-51.3%) of the 343 tested chamois (*Rupicapra rupicapra*). In addition, mouflons (*Ovis gmelinii musimon*) were shown for the first time to be strongly infected (61.1%; CI95%: 38.6-83.6) by a *Pestivirus*. These serological ELISA results were confirmed by comparative virus neutralization tests, performed on 7 *Pestivirus* strains by using 15 seropositive samples. The highest antibody titers were directed against 2 BDV strains (Av and 33s strains), rather than BDV-4, a strain responsible for Pyrenean-chamois epizooties. Virus neutralization tests confirm a BDV circulation in wild ungulates in the French South Alps. However, no *Pestivirus* RNA was detected by reverse-transcriptase polymerase chain reaction in serum and spleen samples from seronegative animals and no virus was isolated from those samples either. Efforts should be made to improve the protocol in order to be able to isolate and characterize the local strain. Finally, the oldness (age) and femaleness (gender) increase the risk of seroconversion in chamois.

Keywords

Wild ruminants, *Pestivirus*, Epidemiology, Alps (France), Inter-species transmission, Risk factors

1. Introduction

Pestiviruses, together with the genera *Flavivirus* and *Hepacivirus*, constitute the *Flaviridae* family. Four species of *Pestiviruses* are officially accepted by the International Committee on Taxonomy of

Viruses (ICTV): *Bovine Viral Diarrhea Virus 1* (BVDV-1); *Bovine Viral Diarrhea Virus 2* (BVDV-2); *Border Disease Virus* (BDV) and *Classical Swine Fever Virus* (CSFV). A strain isolated from a giraffe (*Giraffa cameleopardis*) is tentatively classified as a species within the genus (Becher et al., 1999). The ICTV defines nucleotide sequence relatedness, serological relatedness and host of origin as the 3 main criteria for species discrimination. The subdivision between all species is also antigenically supported by poor serological cross-reactivity (Avalos-Ramirez et al., 2001).

Pestivirus are enveloped spherical viruses, 40 to 60 nm in diameter approximately. The genome is a positive single-stranded and nonpolyadenylated RNA molecule, 12.3 kb in length (Meyers and Thiel, 1996). Classification into genotypes and into species mostly relies on phylogenetic analysis, usually performed after alignment of 5'UTR, Npro or E2 sequences (Becher et al., 1999). Currently, BVDV-1 is divided into 11 genetic subgroups (Vilček et al., 2001), BVDV-2 into 2 subgroups (Vilček et al., 2005), CSFV into 3 subgroups (Paton et al., 2000) and BDV into 7 subgroups (Valdazo-Gonzalez et al., 2007).

Ruminant *Pestivirus* are world-wide distributed and have economically important consequences (Houe, 1999). The associated clinical signs mainly include reproductive failure such as abortion, stillbirth or decrease of fertility. The immunosuppressive effects of *Pestivirus* infection increase the severity of other opportunistic infections. In small ruminants, especially sheep, neurological signs, abnormal body conformation or small lambs with poor growth rate and viability are often associated with the infection (Nettleton, 2000). The presence of persistently infected animals (PI) is a very critical point to be checked before considering control measures against *Pestivirus* infections (Letellier and Kerkhofs, 2003).

In wildlife, *Pestivirus* infections have been widely described. Strains have been isolated from many artiodactyls such as camelids (Evermann, 2006); cervids (Frolich and Hofmann, 1995), and in a great number of *Bovidae* (Vilček and Nettleton, 2006). Hamblin and Hedger (1979) described an important seroconversion in several African bovids. In Pyrenean chamois, a recent outbreak of BDV was described, associated with a BDV-4 strain (Marco et al., 2008; Pioz et al., 2007).

In mountain areas, common pasturing in summer seasons represents an important risk of contamination of both wild and domestic animals, either through direct contacts, or sharing same places, such as salt deposits (Richomme et al., 2006).

The goal of this study was first to investigate the seroprevalence of *Pestiviruses* in chamois (*Rupicapra rupicapra*), mouflon (*Ovis gmelinii musimon*) and roe deer (*Capreolus capreolus*) in the French South Alps and to identify the most relevant exploratory variables associated (risk factors). Then, we wanted to isolate the circulating strain(s) in order to sequence it (them) and to compare them with circulating strains already known. However, isolation of viral strains was not possible and comparative virus neutralization test (VNT) was performed in order to determine the most probable *Pestivirus* species circulating in the studied area.

2. Materials and methods

2.1. Samples and data

A total of 381 blood samples were collected during 2003-2007 hunting seasons from 343 chamois, 18 mouflons and 20 roe deer. Samples (blood and spleens) were collected whenever possible directly after shot by hunters themselves, or within 8 hours after shooting. Blood samples were centrifugated and sera were frozen at -20°C within 12 hours after shooting. A total of 53 spleens originating from chamois only were frozen at -20°C within 12 hours after their sampling. Species, sex, age, location of shot and assessment of health status of each hunted animal were given as complementary data.

2.2. Study area

The study was carried out in two areas located in the French South Alps, in the Hautes-Alpes department (respectively 44°46’ N, 6°57’ E and 44°58’ N, 6°30’ E) (Figure 1). Altitudes range between 1300m to 3000m. The first area is the Game and Wildlife National Reserve of Ristolas, located in the Queyras district and bordered in the west by the Monte Viso area, the Italian border separating these 2 areas. The second area is the Briançon district. It is composed by the Clarée Valley (Val de la Clarée), the Stretta Valley (Vallée étroite), and the left-bank of Guisane Valley. They are bordered by the High Valley of Susa (Italia) in the east. Investigations were initiated in these two areas consequently of demographic troubles reported in chamois in previous years. The vegetation is a

111 mixture of pine, foliage trees, larch forests and alpine pasture. Contacts may occur between chamois,
112 mouflon, roe deer, alpine ibex (*Capra ibex ibex*), red deer (*Cervus elaphus*), and wild boar (*Sus*
113 *scrofa*). Domestic livestock (small and large ruminants) share the pastures with wild animals during
114 the grazing season from June to September (Figure 2).

115 *2.3. Serological tests*

116 Serological samples were screened for *Pestivirus*-specific antibodies against p80 protein (also named
117 NS3), common to all BVDV and BDV strains, using a blocking enzyme linked immunosorbent assay
118 (ELISA) (Synbiotics, Lyon, France) according to the manufacturers' recommendations.

119 In order to confirm positive ELISA results, a total of 53 randomly selected ELISA positive sera (49
120 chamois and 4 mouflons) were tested with a VNT against the BDV strain Av (Chappuis et al., 1986).
121 Titers obtained in the 2 ELISA positive roe deer were also investigated. Besides, comparative VNT
122 were then performed on 15 positive chamois sera (randomly chosen between all positive sera) against
123 7 strains: BVDV-1 strain NADL (Collett et al., 1988 ; Gen Bank accession number M31182), BVDV-
124 2 strain 3534 (Letellier and Kerkhofs, Gen Bank accession number AM181232), BDV-1 strain 137/4
125 (Vilček et al., 1997 ; Gen Bank accession number U65052), BDV-1 strain Frijters (Becher et al., 1997
126 ; Gen Bank accession number U80905), Tunisian-BDV strain 33s (Thabti et al., 2005 ; Gen Bank
127 accession number AF462002), BDV-5 strain Av (Dubois et al., 2008 ; Gen Bank accession number
128 EF693984), and BDV-4 strain named 02/1517. This latter strain does not have a Gen Bank accession
129 number but was isolated in France in 2002 in Pyrenean chamois (Alzieu et al., 2004) and shares more
130 than 99% of genetic identity with the BDV-4 chamois-01 strain (Arnal et al., 2004, Gen Bank
131 accession number AY738080) both in the 5' UTR and the Npro regions.

132 A fixed virus dose (fixed amount between 30 and 200 CCID50) was incubated for 2 hours at 37°C
133 with twofold serum serial dilutions in an antibiotics enriched growth medium (i.e. penicillin,
134 gentamicin and amphotericin B). MDBK cells (ATCC Number CCL-22) were added and the cultures
135 were grown for 72 hours at 37°C in a CO2 incubator. All sera were tested in duplicate, using a 1:2 or a
136 1:10 as starting dilution. Viruses were titrated in all assays. Titers were expressed as the reciprocal of
137 the highest serum dilution yielding virus growth neutralization.

138

139 *2.4. Virus detection*

140 2.4.1. ELISA

141 Antibody negative serum samples collected between 2003 and 2006 were screened for *Pestivirus*
142 specific antigens (p80) using a sandwich ELISA antigen test (Synbiotics, Lyon, France). Sera
143 collected in 2007 were analyzed by an Erns-capture ELISA (Idexx, Liebefeld-Bern, Switzerland).

144 2.4.2. RT-PCR (reverse transcription-polymerase chain reaction)

145 Conventional and real-time reverse transcription-polymerase chain reactions (respectively RT-PCR
146 and real-time RT-PCR) were performed on each serum and on each spleen, using previously described
147 assays (Letellier et al., 1999, Letellier and Kerkhofs, 2003).

148 The RNA was extracted using QIAamp RNeasy® Mini kit (QIAGEN) and was resuspended in 40 µL
149 DEPC-treated water. The reverse transcription was carried out in a volume of 20 µl containing 50 mM
150 Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM dNTP, 150 pmol of the reverse
151 primer B2, 200 U MMLV reverse transcriptase (GIBCO BRL) and 10 µl RNA. The cDNA was
152 synthesized at 37°C for 15 min and the enzyme was inactivated for 5 min at 95°C. For conventional
153 PCR, the 5'UTR region was amplified using primers BE 5' CATGCCCTTAGTAGGACTAGC 3'and
154 B2 5'TCAACTCCATGTGCCATGTAC 3' to amplify a 287 base pair fragment. In vitro
155 Amplification was realised in a thermocycler in a 50 µL- solution containing 20 mM Tris-HCl (pH
156 8.4), 3mM MgCl2, 0,5 mM dNTP, 75 pmol of each primer, 2.5 u. Taq DNA polymerase (Invitrogen®)
157 and 2 µL cDNA. Conditions of amplification were a first enzymatic activation for 5 min at 95°C
158 followed by 35 cycles of amplification (each cycle 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C).
159 Amplified products were separated by electrophoresis in 1.5% agarose gel in Tris-borate EDTA buffer
160 (Letellier *et al.*, 1999).

161 For real-time RT-PCR, the primer pair F2: 5'CTCGAGATGCCATGTGGAC 3' (position 224–242 of
162 the NADL sequence) and PESTR: 5'CTCCATGTGCCATGTACAGCA3' (position 391–371 of the
163 NADL sequence) and the 5'FAM CAGCCTGATAGGGTGCTGCAGAGGC TAMRA 3' and the
164 5'VIC CACAGCCTGATAGGGTGTAGCAGAGACCTG TAMRA 3' probes were used. The Applied
165 Biosystems ABI-PRISM 7900HT sequence detection system was used. The PCR conditions were as
166 followed: 10' 95°C and 45 cycles 15" 95°C and 1'60°C. Fluorescent measurements were carried out

during the elongation step. Each PCR reaction was run in 25 µl containing 2X Universal Master Mix (Applied Biosystems), 300nM of both primers and 200nM of both fluorescent probes. Clinical samples were also run in the presence of TaqMan Exogenous Internal Positive Control Reagents (IPC, Applied Biosystems) in order to avoid false negative results (Letellier and Kerkhofs, 2003).

2.4.3. Virus isolation

All ELISA positive samples but RT-PCR negative were tested for virus isolation. Briefly, 200µl of each serum were inoculated onto duplicate wells on 24-well microtitre plates containing MDBK cells, After 1 hour of incubation at 37°C in 5% CO_2, the wells were drained and an antibiotics enriched growth medium (i.e. penicillin, gentamicin and amphotericin B) was added. Cultures were incubated for 5 days at 37°C in 5% CO_2. After cell fixation at -20°C, an Immuno Peroxydase Monolayer Assay was used to control presence of virus plaques.

2.5. Statistical analysis

The relationship between 2 qualitative variables was studied using Pearson chi squared test as a relationship statistical test (Toma et al., 2001). The apparent prevalence was standardized on age distribution in the studied population. Animals were separated in 6 groups: animals under 2 years, from 2 to 3 years, 4 to 5 years, 6 to 7 years and animals over 8 years. This allows a comparison of the epidemiological situation between different populations, as it takes into account their demographical differences (Toma et al., 2001). Wilcoxon rank sum test (Dagnelie, 1998) was used to compare optical density (OD) values. WinEpiscope ® software (Thrusfield et al., 2001) was used to calculate odds ratio (OR) in order to identify the main exploratory variables (risk factors). Adjusted odds ratio was used when zero values were observed (Grenier, 1990). Finally a logistic regression analysis was used to check the relation between the serological status of animals, their location, gender and age (Statacorp, 2007). The limit of statistical significance of the conducted tests was defined as $P \leq 0.05$. Comparison between combinations of VNT titers against each viral strain was performed using Wilcoxon signed rank test (Dagnelie, 1998), given that titers values are quantitative, paired, not normally distributed and, furthermore, that the variances were unequal. Because of the multiple comparisons of VNT titers against each viral strain, a Bonferroni correction was applied to reduce the

risk of type I error (conservative approach); it involves dividing the *P*-value obtained with any of the test by the number of multiple comparisons performed (i.e. 21 comparisons in this study).

3. Results

3.1. Descriptive epidemiology

3.1.1 Demographic data

Demographic data are available for the Game and Wildlife National Reserve of Ristolas. Size of the chamois population is reported in Table I: a decrease was noticed nearly by 2004. The reproduction rate (calculating by the number of kids divided by the number of females) is deteriorated in 2003-2004, and is increasing after (Table II).

3.1.2. Serological results

Table III presents seroprevalence according to species and years: 41 % among 381 wild ungulates has antibodies against *Pestiviruses*.

For mouflon, 11 animals among 12 were p80 antibodies positives in 2006, whereas no other was found positive, neither in 2003 (n = 3) nor in 2007 (n = 3). OD are significantly higher for mouflon than for chamois and roe deer (Wilcoxon signed rank test, $P<0.0001$ in both case; results not shown). These ELISA positive results were confirmed by VNT, according to a survey on four ELISA positive sera randomly chosen. Neutralizing antibodies titers against the BDV Av strain ranged from 1:128 to 1:512. For roe deer, only 2 were antibodies positive among 20 tested, in 2003 (Table III). Titers obtained in VNT were for the first one superior to 1:256 and, for the second one, inconclusive results (toxicity until 1:4 and negative results from 1:8). Nevertheless, considering the small effective of roe deer and mouflon, all following results are given for the chamois species only.

For chamois, the high size of the samples allows to standardize apparent prevalence on age distribution and to study related risk factors. Among 338 samples (age is unknown in 5 animals) collected between 2003 and 2007, 45.9% (95%CI: 40.5-51.4 %) were tested positive with the p80 *Pestivirus* antibodies ELISA test. Forty nine of these positive sera were randomly chosen and tested by

VNT (using BDV-Av strain): all were confirmed positive. Values of apparent prevalence ranged from 60.6% in 2003 (CI95%: 42.1-77.1%) to 36.5% in 2007 (95% CI: 25.6-48.5%). Apparent prevalence evolution in function of the chamois birth year (determined by horn ring-counting) is represented on figure 3. The apparent prevalence is steadily going down. Interestingly, the prevalence drop every 4 years (1997, 2001 and 2005).

In the VNT directed against Av-strain, mean titers obtained in 2007 amounted to 7857 (Standard Error, S.E.: 3038) and were higher than those obtained either in 2003 (Mean= 267; S.E=152), in 2004 (Mean=238; S.E=109) or in 2006 (Mean=269; S.E=50).

3.1.3. Virological results

Antigen ELISA was only performed on seronegative samples. In 2004 all tested samples were positive for *Pestivirus* antigen (apparent prevalence: 100%; 95% CI: 85-100%) whereas no antigen was detected in 2005 (95%CI: 0-8%). In 2006 and 2007, respectively 2.6% (95% CI: 0.3-9%) and 7.8 % (95% CI: 3.51-11.87%) of the tested animals were positive. Nevertheless, no *Pestivirus* was detected using RT-PCR either in seropositive and seronegative sera or in the spleens tested (no spleens were available in 2004). No virus was isolated in cell culture.

3.2. Analytical epidemiology

3.2.1. Apparent seroprevalence related factors

Seroprevalence was significantly lower in younger chamois (OR≤2years= 0.40; 95% CI: 0.25-0.63) than in older animals. The risk to be seropositive significantly increased in older animals: OR>8years= 2.90 (95% CI: 1.74-4.82). In addition, the p80 antibodies prevalence is significantly higher in females than in males (OR♀=2.39; 95% CI: 1.50-3.80). There is a significant difference between the 2 studied areas: the apparent prevalence was higher in the Briançon district than in the Queyras district (OR Briançon = 1.62 (95% CI: 1.05-2.51). However, the seroconversion status of animals was assessed in function of the location, the gender and the age of animals using a logistic regression. In fact, only the oldness (age) and femaleness (gender) increase the risk of seroconversion in chamois.

3.2.2. Comparative VNT

VNT titers were obtained against 7 *Pestivirus* strains and are reported in Table IV.

The Wilcoxon signed rank test was performed on the data sets (21 different combinations were tested) and the results showed that Av and 33s titers are higher than those obtained for each of the remaining strain (Table V). However, the difference between Av and 33s is not significant (Wilcoxon signed rank test, P=0.28). Titers against the BVDV-2 3534 strain were significantly lower than those against all other strains (Wilcoxon signed rank test with Bonferroni correction, P<0.001 in each case) demonstrating that the circulating virus could probably not be classified as BVDV-2 genotype. Nevertheless, there is no significant difference among the NADL, Frijers, 137/4 and 02/11517 strains (Figure 4).

4. Discussion

The presence of pestivirus infection in chamois and mouflons, investigated by serological ELISA tests, and confirmed in VNT, is obvious. For mouflon, it's the first published description of pestivirus positive seroprevalence confirmed by VNT.

At methodological level, p80 *Pestivirus* antibodies ELISA test appears to be an excellent sensitive screening tool for these wild ungulates, as 100 % of positive sera were confirmed by VNT. Infection seems to be of major impact in 2003 and 2004, both with population downfall and with detection antigen positive animals in Ristolas in 2004. Then, it is important to know if the circulating strain is indigenous among wild ungulates like in the Pyrenean chamois, or if the strain is shared between wild and domestic ungulates. Indeed, in the Pyrenean Mountains, several *Pestivirus* strains all clustered in the BDV-4 genotype are circulating (Marco et al., 2008; Pioz et al., 2007).

For direct viral detection, positive antigen ELISA results suggested that a pestivirus strain was circulating especially in 2004; unfortunately, these results could not be confirmed by the RT-PCR test. Three hypotheses could explain these diverging results.

The first hypothesis is the degradation of samples quality during freezing storage process. Indeed, RNA labile nature has already been pointed (Blacksell et al., 2004). Some RNA storage methods such as the use of guanidinium compounds (Dubois et al., 2008) or RNA*later*TM have to be evaluated. The higher titers obtained in 2007 with VNT using Av strain, compared to others years, may be another index. Then, in 2004, no spleens were available while the p80 antigen ELISA (prevalence antigen)

was the highest. It was therefore impossible to use this organ of choice to confirm these results. Another hypothesis is the lack of specificity of the antigen ELISA test when performed on wild ruminants serum samples. Indeed, for two chamois shot in 2007, the virus status of the animals (determined with an antigen ELISA test performed on serum samples) were not confirmed by the RT-PCR carried out on the corresponding spleens, even if samples were adequately stored. This underlines the problem of commercial kits use in conditions different from those recommended by the manufacturers. This is in accordance with a previous study conducted on Pyrenean chamois by Marco et al., (2008); 4 samples on 18 were false positive with antigen ELISA test manufactured by Synbiotics firm. Sensitivity and specificity of the commercial tests used are therefore known for domestic animals only. Moreover, in this study, sera samples were collected in dead animals, within 12 hours after shooting. Specificity and sensitivity values are therefore lower than in live animals (Olde Riekerink et al., 2005).

The RT-PCR method, considered as reference in this study, has been previously performed on various *Pestivirus* strains. All the results provided by real time RT-PCR were in concordance with conventional PCR. At least 60 strains can be detected by this test, classified in the 4 *Pestivirus* species (BVDV-1, BVDV-2, BDV including a BDV-4 virus isolated from Pyrenean chamois or CSFV). This test was thus suitable to detect unknown *Pestivirus* strains, like in wild animals (Letellier and Kerkhofs, 2003).

Since we were not able to isolate the local strains, comparative VNT against different Pestivirus strains were performed in order to characterize the local strain antigenically. There could be a bias in the comparative VNT conclusions as the 15 selected sera were randomly chosen among all samples with titers against Av superior to 1/128. Av strain was chosen as reference strain for the initial VNT for several reasons. First of all, a BDV strain was preferred to a BVDV strain as chamois are phylogenetically closer to sheep and goats than to bovines. Then, a recent study performed in Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) showed that a BDV-4 was enzootic (Pioz et al., 2007). Among all BDV strains available in this study, we chose the Av strain, mainly for geographical reasons. 137/4 strain was isolated from sheep in the United Kindom (Vilček et al., 1997), Frijters from pigs in Germany (Vilček and Belak, 1996), and 33s in Tunisians vaccines (Thabti et al., 2005). Strains

Av and 02/1517 were isolated in France and Av strain comes from an acute Border Disease outbreak of Aveyon department in 1985 (Dubois et al., 2008). The 02/1517 strain was isolated in a Pyrenean chamois (Letellier, personal communication). Vilček Dubois et al. (2008) showed that an Alpine mountain strain isolated in 1993 from sheep was closely related to the Av strain. Assuming that a contamination could come from domestic ungulates in a same pasture, we chose Av strain as reference. However, construction of a dendrogram based on antigenic coefficient similarities (Archetti and Horsfall, 1950) was not possible, due to the unavailability of isolated circulating strain. Our comparative VNT results tend to show that this strain may be classified into the BDV genotype. Indeed, significantly lower titers were obtained against the 3534 strain, indicating that the circulating virus was not clustered in the BVDV-2 species. Then, Av and 33s strains, 2 BDV strains, were more neutralized than the other ones. Although these results are not significant with Bonferroni correction, they are of great importance. In France, ovine *Pestivirus* strains distribution has only been published by Dubois and collaborators (2008). They sequenced 23 of 32 strains isolated between 1985 and 2006 in 4 French districts. Thirteen ovine strains circulating in PACA (Provence Alpes Côte d'Azur) regions were classified among BDV-3, BDV-5, BDV-6 and Tunisian genotypes. Among these 13 strains, 3 are especially relevant. The first was collected in the Alpine mountains in 1993 (named 93-F-7289), and clustered with the Av strain. The 2 other ovine isolate, collected in 1996 in Vaucluse (one of the 6 PACA departments), clustered with the Tunisians isolates (Dubois et al., 2008). Thus, our results feel with this description. The circulating strain seems to be quite different from the *Pestivirus* strain circulating among the Pyrenean chamois population, as titers directed against 02/1517 are lower than those obtained against Av and 33s. Interactions are frequently observed between wild and domestic ungulates, mainly on salt points during grazing season (Richomme et al., 2006). In this region, small ruminants herds are in contact with wild populations analyzed (e.g., in Ristolas, around 7500 sheep and 50 goat were present in all pastures) and may have played an important role in the transmission of *Pestiviruses*. This can be confirmed by the assumption made by Olde Riekerink and collaborators, in 2005. Unfortunately, analysis of seroprevalence and circulating strains among domestic ruminants could not be included in this study.

The prevalence of *Pestivirus* antibodies based on the animals year of birth has been decreasing since

1991. It is interesting to note the presence of a regular, acute decrease every 4 years (1997, 2001 and 2005). This could be explained by the circulation of a *Pestivirus* strain, which could be enzootic, with periodical active circulation periods, or by more appropriated samples conservation since 2007. One fifth of the youngest animals have antibodies. On average, juveniles are 6 months old at the time of sampling which is the limit of colostral antibodies duration in cattle. It is thus difficult to assume whether they are still under colostral protection or have their own antibodies. However, 29.5 % (95% CI: 16.8-45.2%) of the chamois yearlings are seropositive. This confirms the hypothesis of a recent circulation of a *Pestivirus.* This is also supported by 2 epidemiological studies conducted in the Pyrenean Mountains, in which a *Pestivirus* seems to be enzootic in the Pyrenean chamois populations, either in France or in Spain (Marco et al., 2008; Pioz et al., 2007). In Lecco province, in the Italian Alpine mountains, Citterio and collaborators (2003) have shown the absence of seroconversion in 145 chamois during 2000 and 2001 hunting seasons (95% CI: 0-2%). However, in 1999, in the High Valley of Suza, an Italian bordering valley of the Briançon district, 25.5% of 110 tested chamois were seropositive (95% CI: 17.6-34.6%) either with an ELISA test or with a VNT (Olde Rieckerink et al., 2005). Titers obtained in Suza valley are significantly lower than those obtained in our study (data not shown), which could indicate either that the origin of contamination are located in France, or that the apparent prevalence has increased since 1999. Another hypothesis is that the BDV strain used in the VNT was antigenically different from the circulating *Pestivirus*.

Haydon et al. (2002) defined a reservoir as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population". This study showed that wild ungulates are widely seropositive for *Pestivirus*. High number of mouflons was seropositive with high OD values. They were introduced in the Hautes-Alpes department between 1973 and 1977 from populations originated of Bauges Reserve; themselves coming from Corsican Mouflon (Gauthier, personal communication). In zoological classification, they are the closest species to sheep among all wild ungulates present in the study area. We can thus imagine that mouflons are playing an important role in the disease transmission. All others ungulates (cervids and *Capra ibex*) should also be added to improve the study design with special emphasis on mouflons. The first next step to this study is to associate a study of

domestic animals infection. To understand epidemiology of *Pestivirus* infections at the wild and domestic ruminant interface, further studies are needed to know the *Pestivirus* prevalence among domestic herds and to characterize the circulating strain in both wild and domestic ungulates.

5. Conclusion

A high seroprevalence against *Pestivirus* was shown among chamois, and, for the first time, mouflon in the South of French Alpine Mountains. These results were confirmed by VNT in favour of BDV circulation in wild ungulates in the French South Alps. However, no *Pestivirus* RNA was detected by reverse-transcriptase polymerase chain reaction in samples and no virus was isolated either. As mountain pastures are a place where cohabitations between wild and domestic ungulates are particularly high (especially sheep), there is a wide probability that the circulating strain clusters within the BDV genotype. The high seroprevalence associated with positive VNT and lack of isolation in wild ungulates could suggest a domestic origin of infection. To assess the importance of circulation among domestic animals and to know the role of each species in the transmission of the pathogen, further epidemiological (viral and serological) and transversal studies are needed.

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476 **Figures and Tables**

477

478 **Figure 1**

479 Title: Location of the study area (French South Alps)

480

481 **Figure 2**

482 Title: Phylogeny of ruminants presents in the study area

483 Legend:

484 Scientific names were taken from the NCBI Taxonomy Database

485 (http://www.ncbi.nlm.nih.gov/taxonomy ; consulted on November, 8th of 2009).

486

487

488 **Figure 3**

489 Title: Annual evolution of the standardized apparent prevalence rate ( ) in chamois (*Rupicapra*

490 *rupicapra*) originating from the French South Alps with 95% confidence interval (-)* of the *Pestivirus*

491 p80 antibodies.

492 Legend: The standardization of the apparent prevalence has been calculated in function of the age on

493 the chamois, considering 5 strates: 0-2 years, 2-4 years, 4-6 years, 6 to 8 years and more than 8 years ;

494 * exact 95% binomial confidence intervals

495

496 **Figure 4**

497 Title: Boxplot representation of the titer logarithm (Y axis) obtained against each viral strain (X axis)

498

499 **Table I**

500 Title: Estimations of population size of the chamois population in the Game and Wildlife National

501 Reserve of Ristolas

502 Legend: * Not realized for meteorological reasons

503

Table II

Title: Reproduction rate in the chamois population in the Game and Wildlife National Reserve of Ristolas

Legend: reproduction rate = kid/female rate

Table III

Title: Annual and species repartition of all blood samples

Legend: In bracket values are the ELISA positive samples for *Pestivirus* antibodies.

Table IV

Title: Virus neutralization titers on 15 chamois (*Rupicapra rupicapra*) originating from the French South Alps for 7 *Pestivirus* strains

Legend: F=female, M=male, n.d.: data not available.

Table V

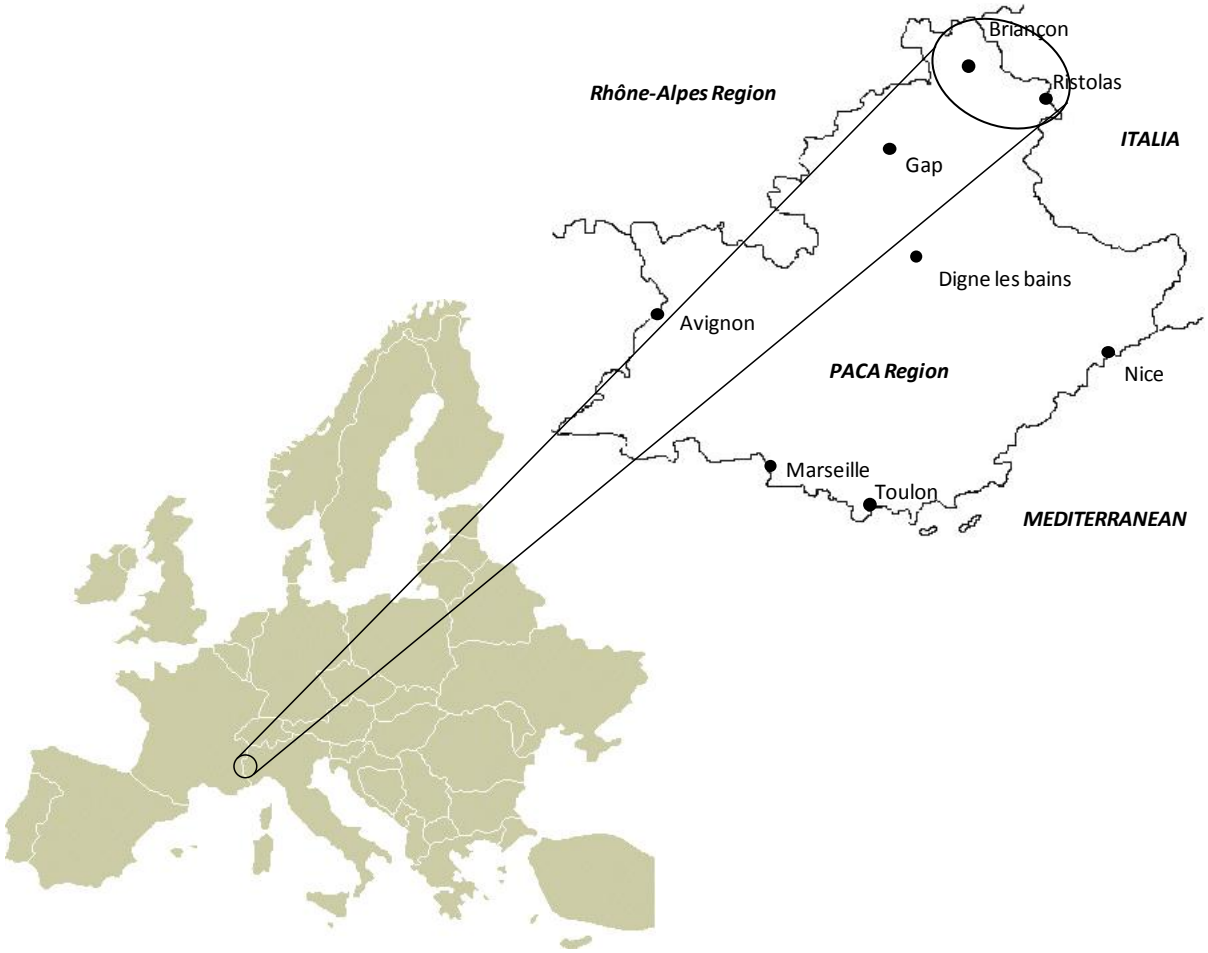
Title: Arithmetic mean and standard error of titers obtained for each virus strain and *P* value obtained with the Wilcoxon signed rank test

Legend: *Significant value after Bonferroni's correction ($P<0.002$)

524 **Figure 1**

525

526



527

528

529

Figure 2

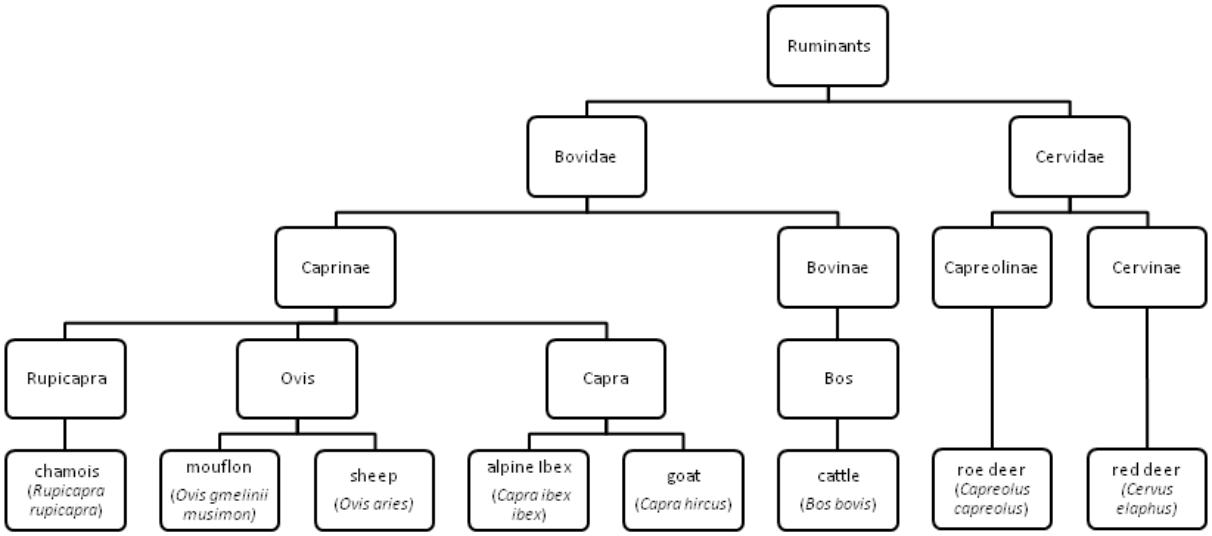

Ruminants
Bovidae
Cervidae
Caprinae
Bovinae
Capreolinae
Cervinae
Rupicapra
Ovis
Capra
Bos
chamois (Rupicapra rupicapra)
mouflon (Ovis gmelinii musimon)
sheep (Ovis aries)
alpine Ibex (Capra ibex ibex)
goat (Capra hircus)
cattle (Bos bovis)
roe deer (Capreolus capreolus)
red deer (Cervus elaphus)

Figure 3

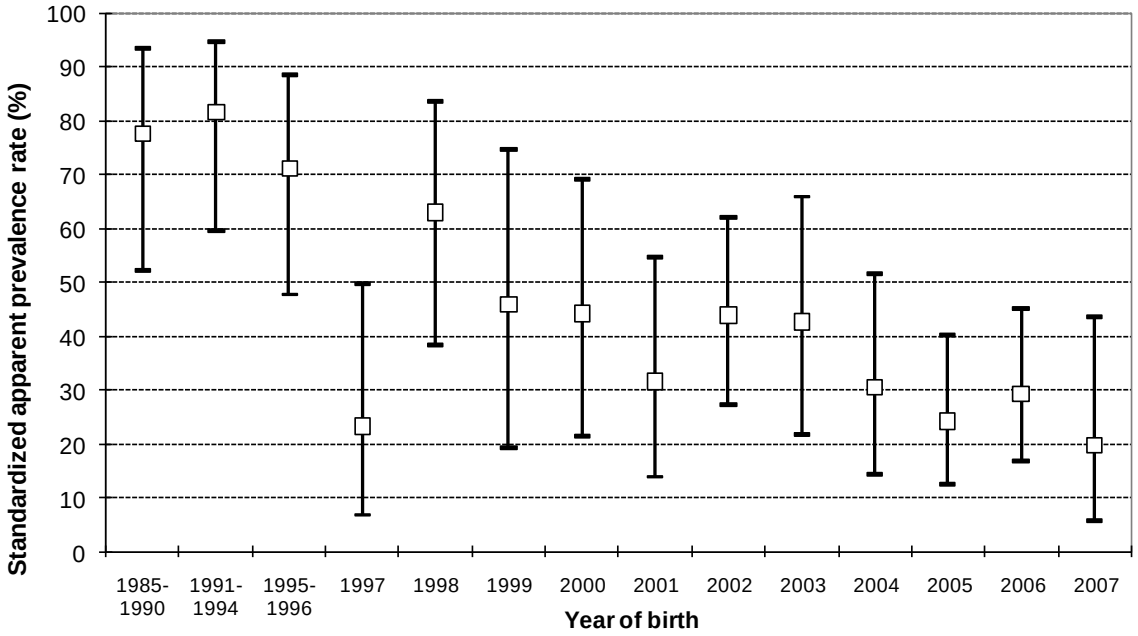

Standardized apparent prevalence rate (%)
100
90
80
70
60
50
40
30
20
10
0
1985-1990
1991-1994
1995-1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
Year of birth

Figure 4

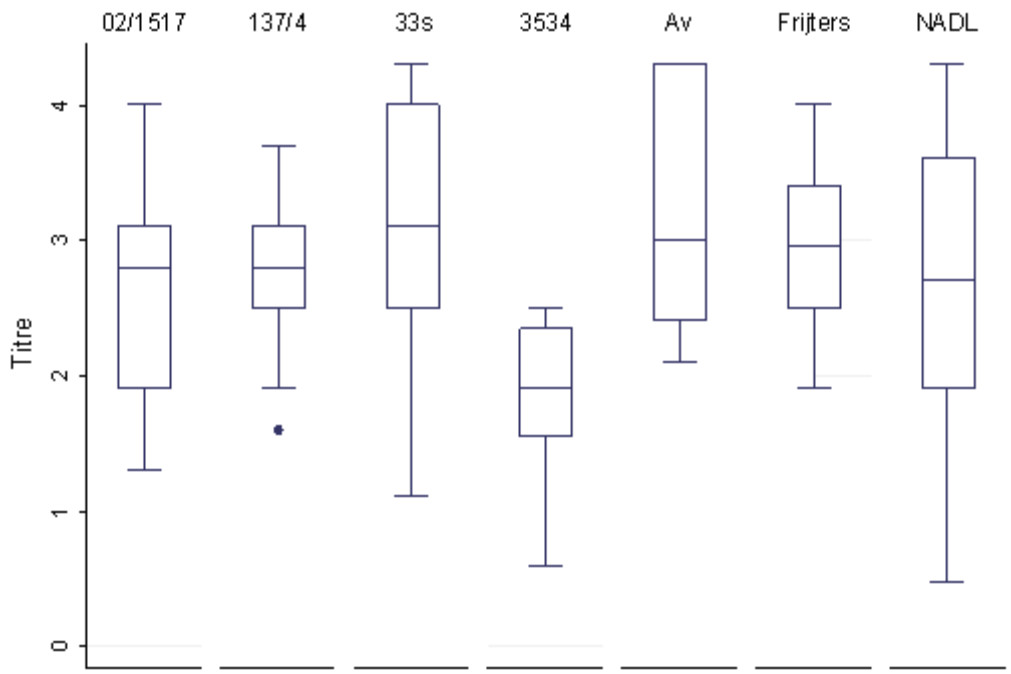


Table I

Year	Population size	
1986	415	
1989	469	
1992	510	
1995	-	*
1998	729	
2001	-	*
2004	344	
2006	268	

Table II

Year	Reproduction index
2002	0.61
2003	0.56
2004	0.56
2005	0.68
2006	0.76
2007	0.85

555 **Table III**

556

	2003	2004	2005	2006	2007	Total
Chamois	33 (18)	60 (35)	42 (13)	131 (53)	77 (26)	343 (145)
Mouflon	3 (0)	-	-	12 (11)	3 (0)	18 (11)
Roe deer	6 (2)	-	-	1 (0)	13 (0)	20 (2)

557

558

559

Table IV

Serum	Age (year)	Sex	Strain						
			Av	33s	02/1517	Frijters	137/4	NADL	3534
3.N.5	2	F	128	13	20	0	40	1024	0
3.N.25	14	M	1024	5120	640	2560	2560	2048	160
5.R.13	2	M	20480	10240	1280	2560	640	4096	320
5.R.27	10	M	512	320	80	160	320	512	80
5.R.30	6	M	256	320	160	1280	640	32	16
6.N.1	8	F	512	160	40	80	160	8	4
6.N.2	13	F	256	640	320	640	640	128	0
6.R.10	3	M	1024	1280	640	640	640	256	80
6.B.7	0	M	256	320	40	160	80	3	6
7.V.40	n.d.	n.d.	20480	10240	10240	5120	1280	5120	160
7.V.42	10	F	20480	20480	10240	10240	5120	20480	320
7.V.46	0	M	20480	5120	10240	2560	1280	5120	320
7.N.4	n.d.	n.d.	4096	5120	640	640	1280	320	80
7.N.13	n.d.	n.d.	20480	10240	1280	5120	320	2560	80
7.N.59	18	F	1024	640	320	320	320	80	0

Table V

Strain 1	(Mean ± SE)	*vs*	Strain 2	(Mean ± SE)	Wilcoxon signed rank test *P* value	
Av	(7432,53 ± 2125,47)	-	NADL	(2785,8 ± 1198,95)	0,02	
Av	(7432,53 ± 2125,47)	-	3534	(108,4 ± 29,78)	< 0,001	*
Av	(7432,53 ± 2125,47)	-	02/1517	(2412 ± 722,09)	< 0,001	*
Av	(7432,53 ± 2125,47)	-	33s	(4683,53 ± 1216,67)	0,28	
Av	(7432,53 ± 2125,47)	-	Frijters	(2138,67 ± 547,98)	0,028	
Av	(7432,53 ± 2125,47)	-	137/4	(1021,33 ± 322,90)	0,027	
NADL	(2785,8 ± 1198,95)	-	3534	(108,4 ± 29,78)	< 0,001	*
NADL	(2785,8 ± 1198,95)	-	02/1517	(2412 ± 722,09)	< 0,001	*
NADL	(2785,8 ± 1198,95)	-	33s	(4683,53 ± 1216,67)	0,009	
NADL	(2785,8 ± 1198,95)	-	Frijters	(2138,67 ± 547,98)	0,86	
NADL	(2785,8 ± 1198,95)	-	137/4	(1021,33 ± 322,90)	0,31	
3534	(108,4 ± 29,78)	-	02/1517	(2412 ± 722,09)	< 0,001	*
3534	(108,4 ± 29,78)	-	33s	(4683,53 ± 1216,67)	< 0,001	*
3534	(108,4 ± 29,78)	-	Frijters	(2138,67 ± 547,98)	< 0,001	*
3534	(108,4 ± 29,78)	-	137/4	(1021,33 ± 322,90)	< 0,001	*
02/1517	(2412 ± 722,09)	-	33s	(4683,53 ± 1216,67)	0,009	
02/1517	(2412 ± 722,09)	-	Frijters	(2138,67 ± 547,98)	0,227	
02/1517	(2412 ± 722,09)	-	137/4	(1021,33 ± 322,90)	0,819	
33s	(4683,53 ± 1216,67)	-	Frijters	(2138,67 ± 547,98)	0,003	
33s	(4683,53 ± 1216,67)	-	137/4	(1021,33 ± 322,90)	0,008	
Frijters	(2138,67 ± 547,98)	-	137/4	(1021,33 ± 322,90)	0,135	