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LES PESTIVIRUS À L'INTERFACE FAUNE SAUVAGE/FAUNE DOMESTIQUE : Pathogénie chez l'isard gestant et épidémiologie dans la région Provence-Alpes-Côte D'azur

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RESUME

Dans les Alpes du Sud de la France, des diminutions de populations de chamois (*Rupicapra rupicapra*) ont été rapportées. Or, depuis une dizaine d'année, des pestivirus ont causé de fortes mortalités dans des populations d'isards des Pyrénées (*Rupicapra pyrenaica*). Bien que les signes cliniques associés à cette infection aient été caractérisés chez cette espèce, la pathogénie chez les animaux gestants est peu étudiée. De plus, des transmissions inter-espèces ont régulièrement été incriminées dans l'épidémiologie des pestiviroses ; ceci particulièrement au niveau des alpages où des contacts fréquents sont décrits entre ruminants sauvages et domestiques.

Les objectifs de ce travail de thèse ont donc été, dans un premier temps, d'étudier la pathogénie de l'infection à pestivirus chez des isards et plus particulièrement ses effets sur la gestation. Dans un second temps, nous avons étudié l'épidémiologie de l'infection dans différentes zones de la région Provence-Alpes-Côte d'Azur (PACA), tout d'abord chez des ruminants sauvages, puis à l'interface entre les ruminants sauvages et domestiques partageant les mêmes alpages.

Trois femelles isards ont été inoculées, durant le deuxième tiers de leur gestation, avec une souche de BDV-4, préalablement isolée d'un isard sauvage dans les Pyrénées espagnoles. Un groupe témoin était constitué d'une quatrième femelle isard gestante, associée à une agnelle. Une virémie longue, de quatre jours post infection jusqu'à la mort des animaux, était associée à des profils de séroconversion variables et à des lymphopénies importantes. Bien qu'aucune gestation n'ait été menée à terme, la détection des ARN viraux dans tous les organes testés des fœtus de femelles inoculées suggéraient la naissance possible d'animaux infectés persistants immunotolérants (IPI) chez cette espèce.
Par ailleurs, dans la région PACA, une première étude séro-épidémiologique, longitudinale, a été réalisée dans le département des Hautes-Alpes sur les campagnes de chasse de 2003 à 2007. Des anticorps dirigés contre les pestivirus étaient présents chez 45,9% (Intervalle de confiance à 95% [IC95%] : 40,5-51,3) des chamois et 61,1% (IC 95% : 38,6-83,6%) des mouflons (*Ovis aries musimon*). Une deuxième étude épidémiologique transversale, conduite à la fois chez les ruminants sauvages et domestiques lors des saisons de chasse 2009 et 2010, a montré des séroprévalences élevées, atteignant 38,8% (IC95% : 74,3 – 78,8 %) chez les chamois et 25,9% (IC95% : 9,4 – 42,4 %) chez les chevreuils (*Capreolus capreolus*). Tous les cheptels ovins testés (n=37) présentaient une séroprévalence positive, atteignant 76,6 % (IC95%: 74,3 – 78,8 %) pour l'ensemble des 1383 sérums analysés. Dans le département des Alpes-Maritimes, deux souches ovines de pestivirus ont été isolées et classées respectivement dans le génogroupe BDV-3 (Border Disease Virus type 3) et dans le génogroupe BDV-Tunisien. Dans le département des Alpes de Haute-Provence, deux souches ont été isolées, d'une brebis avortée et, pour la première fois, d'un chamois (souche « Rupi-05 »). Les deux souches ont été classées parmi les virus du génogroupe BDV-6. Des séroneutralisations croisées ont montré que les chamois avaient des titres en anticorps supérieurs contre la souche « Rupi-05 », alors que les moutons réagissent de façon homogène envers les différentes souches ovines locales. De plus, les ovins ont des titres en anticorps neutralisants en moyenne plus élevés que les chamois pouvant laisser suspecter une circulation plus importante chez les moutons. Des anticorps neutralisant ont été détectés chez un seul chevreuil et étaient dirigés vers une souche de BVDV-1 (Bovine Viral Diarrhea Virus type 1).

En conclusion, une circulation active de pestivirus est présente dans la région PACA, chez les animaux sauvages comme domestiques. Dans les Alpes de Haute-Provence, les souches isolées des différentes espèces sont classées parmi le même génogroupe, montrant une continuité géographique dans la répartition des souches. Les résultats obtenus lors de l'infection expérimentale montrent des effets sur la gestation importants, avec une possible présence d'animaux IPI chez les chamois comme chez les ovins. Bien que nos résultats ne permettent pas d'établir de façon précise un sens de transmission entre les espèces de ruminants domestiques et sauvages, deux cycles épidémiologiques semblent être présents, caractérisés par une forte circulation intra-spécifique et connectés à des transmissions ponctuelles entre chamois et moutons.

ABSTRACT

In the French South Alps, diminutions of effectives of chamois (*Rupicapra rupicapra*) were recently reported. Besides, Pestivirus were shown to cause high mortalities in Pyrenean chamois (*Rupicapra pyrenaica*). While clinical signs associated to the infection were characterized in wild populations, the pathogeny in pregnant female has not been studied yet. Moreover, interspecies transmissions were recently incriminated in the epidemiology of Pestivirus infections, especially in alpine pastures, known for their high rate contact between wild and domestic ruminants.

The objectives of this doctorate were, in a first time, to study the pathogeny on pregnancy associated to the Pestivirus infection in Pyrenen chamois. Then, this survey was aimed to study the infection epidemiology in various areas of the Provence-Alpes-Côte d'Azur (PACA) region, firstly in wild ruminants and at the interface between wild and domestic ruminants sharing the same pastures.

Three pregnant female Pyrenean chamois were inoculated during the second third of gestation with BDV-4 strain, previously isolated from a diseased wild Pyrenean chamois. A group control was constituted by a fourth pregnant female Pyrenean chamois and a ewe. A long-lasting viremia from four days post inoculation to the death of animals was associated to different profiles of seroconversion and important lymphopenia. All pregnancies aborted. Viral RNA were detected in all organs tested of the three inoculated females, suggesting that persistently infected animals (PI) may be born in this species. Besides, in the PACA region, a first epidemiological study was performed in the Hautes-Alpes district during the hunting seasons of 2003 to 2007. Antibodies directed to Pestivirus were found in 45,9% (95% Confidence Interval [95% CI] : 40,5-51,3%) of chamois and 61,1% (95% CI: 38,6-83,6%) of mouflons (*Ovis aries musimon*). A second epidemiological study, transversal, performed during the hunting seasons 2009 and 2010 showed high seroprevalences, reaching 38,8% (95% CI: 74,3 – 78,8 %) in chamois and 25,9% (95% CI : 9,4 – 42,4 %) in roe deer (*Capreolus capreolus*). All ovine herds tested (n=37) presented positive seroprevalence, reaching 76,6 % (95% CI: 74,3 – 78,8 %) of the 1383 sera tested. In the Alpes-Maritimes district, two ovine strains were isolated and classified in the Border Disease Virus (BDV) type 3 and the BDV-tunisian genogroups, respectively. In the Alpes de Haute-Provence district, two viral strains were isolated: one from an aborted ewe, and for the first time, from a chamois (strain named "Rupi-05"). Comparative viral neutralization tests showed that chamois had neutralizing antibodies titers higher against the "Rupi-05" strain, and sheep had homogenous titers against all local ovine strains. Besides, sheep had mean titers in neutralizing antibodies higher than chamois, suggesting a circulation more important in sheep. Neutralizing antibodies were detected in only one roe deer, and were directed against a Bovine Viral Diarrhea Virus type 1.

In conclusion, an active circulation of Pestivirus is present in the PACA region, in both wild and domestic ruminants. In the Alpes de Haute-Provence district, isolated pestiviral strains from chamois and sheep clustered in the same genogroup, showing a geographical continuity in the strains repartition. Results obtained from the experimental infection showed important effects on the pregnancy, with a possible birth of PI animal in chamois and in sheep. Our results showed that two epidemiological cycles are present in chamois and sheep, respectively, characterized by an important circulation intra-species and connected by punctual transmission between animal species.

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On ne va jamais aussi loin que lorsque
l'on ne sait pas où l'on va.
Christophe Colomb

SOMMAIRE

LISTE DES ABREVIATIONS

ACERSA	Association pour la Certification en Santé Animale
Ag ELISA	Antigen Enzyme Linked Immunosorbent Assay
Anses	Agence nationale charge de la sécurité sanitaire de l'alimentation, de l'environnement et du travail
BD	Border Disease
BDV	Border Disease Virus
Bo-HV	Bovine Herpes Virus
BTV	Blue Tongue Virus
BVD	Bovine Viral Diarrhoea
BVDV	Bovine Viral Diarrhoea Virus
cDNA	complementary Desoxyribo-Nucleic Acid
CI	Confidence Interval
CSF	Classical Swine Fever
CSFV	Classical Swine Fever Virus
DNA	Desoxyribo-Nucleic Acid
dpi	Days post inoculation
DO	Densité Optique
EDTA	Acide Ethylène Diamine Tétracétique
EID	Emerging Infectious Disease
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agricultural Organization
FELASA	Federation of European Laboratory Animal Science Associations
ICTV	International Committee on Taxonomy of Viruses
IPCC	Intergovernmental Panel on Climate Change
IPMA	Immuno Peroxydase Monolayer Assay
IUCN	International Union of Conservation of Nature
NS-3	Non Structural (protein) -3
OD	Optical Densities
OIE	Office International des Epizooties
OMSA	Organisation Mondiale pour la Santé Animale
OR	Odds Ratio
ORF	Open Reading Frame
PACA	Provence Alpes Côte d'Azur
PBS	Phosphate Buffered Solution
PCR	Polymerase Chain Reaction
PI	Persistently Infected
RNA	Ribo Nucleic Acid
RNCFS	Réserve Nationale de Chasse et de Faune Sauvage
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
S.D.	Standard Deviation
$TCID_{50}$	50% tissue culture-infectious doses
UK	United Kingdom
UTR	Un-Translated Region
VNT	Virus Neutralization Test

INTRODUCTION ET OBJECTIFS

1. Présentation des espèces sauvages concernées par ce travail

L'ensemble des espèces sauvages concernées par cette étude est repris dans le Tableau I, en fonction de leur classification phylogénétique. Il ne s'agit que d'espèces de ruminants (classe des Mammifères, sous-classe des Euthériens, super-ordre des Ongulés, ordre des Artiodactyles, et sous-ordre des Ruminants).

Classement	Chamois/Isard	Chevreuil	Mouflon
Règne	Animal	Animal	Animal
Sous-règne	Métazoaires	Métazoaires	Métazoaires
Embranchement	Chordés	Chordés	Chordés
Sous-embranchement	Vertébrés	Vertébrés	Vertébrés
Classe	Mammifères	Mammifères	Mammifères
Sous-classe	Euthériens	Euthériens	Euthériens
Super-ordre	Ongulés	Ongulés	Ongulés
Ordre	Artiodactyles	Artiodactyles	Artiodactyles
Sous-ordre	Ruminants	Ruminants	Ruminants
Famille	*Bovidae*	*Cervidae*	*Bovidae*
Sous-famille	*Caprineae*	*Capreolinae*	*Caprineae*
Genre	*Rupicapra*	*Capreolus*	*Ovis*
Espèces	*rupicapra* (chamois)	*capreolus*	*gmelinii*
	pyrenaica (isard)		*musimon*

Tableau I. Classification phylogénétique des espèces concernées par cette étude (d'après Aulagnier et al., 2008).

a. Chamois/isard

Le genre *Rupicapra* serait apparu il y a plus d'un million d'années. Au cours du quaternaire, la progression des glaciers dans les régions montagneuses les oblige à descendre vers des régions de plus faible altitude. Ainsi, des restes osseux datant de la période de glaciation de Würm ont été mis en évidence dans le Massif Central, les Vosges, la Forêt-Noire, les Ardennes et jusqu'en Pologne. Après quelques millénaires passés en plaine, il regagne les territoires de plus forte altitude. Les différentes espèces et sous-espèces sont alors apparues, en fonction de leur répartition géographique (Bonneton, 2001). Actuellement, deux espèces sont reconnues, s'agissant du chamois (*Rupicapra rupicapra*) présent dans le massif Alpin et de l'isard (*Rupicapra pyrenaica*) présent dans les Pyrénées. Le mot chamois viendrait du grec *kemas*, qui désigne une chèvre sauvage de la Grèce ancienne. Le chamois

était appelé *camos* en provençal ancien, *stamou* en Savoie, *chamus* dans le Dauphiné. En Italie, les chamois sont appelés *camoscio*. Le mot isard a lui une origine plus incertaine. Pour certains auteurs, le mot *ysarus* en vieux français viendrait de *serre* et *sarrat* qui sont deux localités pyrénéennes. D'autres auteurs penchent plutôt pour une origine germanique, de *isern* ou *eisern*, qui évoquerait le pelage gris fer de l'isard, ou pour une origine de l'ancien allemand *isarn* (Wéber, 2001).

L'espèce *Rupicapra rupicapra* compte sept sous-espèces, alors que trois sont décrites pour l'espèce *Rupicapra pyrenaica*, tel qu'indiqué ci-dessous (Tableau II). Elles se distinguent d'une part par leurs aires de répartition mais également par des différences au niveau de la couleur du pelage, de la stature et de leurs comportements.

Par la suite dans ce manuscrit, le mot chamois sera utilisé pour les animaux présents dans les Alpes et isard pour ceux présents dans les Pyrénées. En France, le chamois est présent dans les Alpes (départements des Alpes de Hautes-Provence, des Hautes-Alpes, des Alpes-Maritimes, de la Drôme, de l'Isère, de la Savoie et Haute-Savoie, du Var et du Vaucluse), dans le Jura (Ain, Doubs et Jura) et dans les Vosges (Haut-Rhin, Vosges, Haute-Saône). Les isards sont présents quand à eux dans tous les départements des Pyrénées (Ariège, Aude, Haute-Garonne, Pyrénées-Atlantiques, Pyrénées Orientales, Hautes-Pyrénées).

Espèce	Sous-espèce	Localisation
Rupicapra rupicapra	*Rupicapra rupicapra asiatica*	Chaine Pontique, Mont Taurus et Anti-Taurus
	Rupicapra rupicapra balkanica	Péninsule des Balkans, Alpes Dinariques et Monts du Pinde
	Rupicapra rupicapra caucasica	Petit et grand Caucase
	Rupicapra rupicapra carpatrica	Carpates centrales et Alpes de Transylvanie
	Rupicapra rupicapra cartusiana	Massif de la Chartreuse
	Rupicapra rupicapra rupicapra	Reste de la chaîne alpine
	Rupicapra rupicapra tatrica	Tatras slovaques
Rupicapra pyrenaica	*Rupicapra pyrenaica ornata*	Abruzzes
	Rupicapra pyrenaica parva	Mont Cantabriques
	Rupicapra pyrenaica pyrenaica	Pyrénées

Tableau II. Espèces et sous-espèces du chamois et de l'isard (d'après Bonneton, 2001).

Le milieu de vie des chamois et des isards est essentiellement lié à la présence d'un relief accidenté. Il est présent sur les pelouses alpines en été, et dans des zones d'hivernage constituées de forêts ou de

couloirs déneigés en hiver. Il consomme une grande variété de plantes herbacées en été, des arbrisseaux, rameaux, aiguilles de conifères, écorces ou lichens en hiver.

A l'âge adulte, les mâles chamois pèsent entre 35 et 50 kilos contre 25 à 38 kg chez les femelles. Il existe un dimorphisme saisonnier important. En été, le pelage est assez clair, brun roux avec la bande dorsale, la queue, les membres et les bandes jugales brunes ou gris brunâtre. En hiver, le pelage est plus dense, brun foncé sauf le disque caudal et les parties claires de la tête qui restent beige clair.

Les cornes, l'allure générale et la présence du pinceau pénien sont des critères de différenciation sexuelle. Les cornes sont présentes chez les deux sexes et sont généralement épaisses et plus fermées (courbure plus importante) chez le bouc et plus fines et plus ouvertes chez la femelle. Il peut toutefois exister des exceptions telles que des femelles avec des crochets fermés ou mâles avec des crochets ouverts.

La période de rut a lieu à partir de la moitié du mois d'octobre jusqu'en moyenne vers la mi-décembre. Après cinq mois et demi (de 160 à 170 jours) de gestation, la femelle met bas un seul chevreau dans la période du 15 mai au 15 juin. Le chevreau est allaité jusqu'à l'âge de deux mois environ, au-delà desquels le sevrage a lieu progressivement. L'indice de reproduction (calculé à partir du nombre de jeunes présents en fin de période de naissance pour 100 femelles de 2 ans et plus) est variable en fonction des densités des populations, de leur structure, de leurs état sanitaire et des conditions météorologiques de l'année. Toutefois, un taux important de mortalité (de 30 à 50%) affecte la classe d'âge des chevreaux.

Sur les animaux vivants, la reconnaissance de l'âge se fait à l'aide de la taille des cornes (position par rapport à l'oreille), de la taille et de la silhouette générale des individus. Trois ou quatre classes d'âges peuvent être différenciées selon le moment de l'année. De mai à août, les chevreaux sont facilement reconnaissables, de par leur petite taille. Les éterlous ou éterles (individus de deuxième année) sont de taille légèrement supérieure avec des cornes ne dépassant pas le bout des oreilles. Enfin, il est possible de différencier les individus de troisième année des adultes à l'aide de leur silhouette plus fine, et de leurs cornes dépassant faiblement la pointe des oreilles. De septembre à avril, trois classes d'âges sont différenciables, les troisièmes années étant semblables aux adultes. La distinction des deux premières classes d'âge se fait essentiellement à l'aide de la taille générale et de la taille des cornes. L'examen des cornes d'un animal mort ou captif permet de déterminer de façon assez précise son âge. En effet, leur croissance est ralentie en hiver, et reprend au printemps suivant, permettant de différencier des anneaux d'âge. Il suffit dès lors de compter le nombre de segments délimités par ces anneaux d'âge pour connaître l'âge de l'animal. (Aulagnier et al., 2008) (ONCFS, 2011).

b. Chevreuil

Le chevreuil est un petit cervidé, dont la répartition est commune en Europe. Il pèse de 20 à 25 kilos, les mâles pesant 2 à 3 kilos de plus que les femelles. Son habitat est vaste, présent dans des forêts tempérées, des bosquets ou des prairies un peu boisées. En montagne, les chevreuils sont présents dans les forêts de feuillus ou de mélèzes. Les animaux sont essentiellement sédentaires, territoriaux et solitaires en été et au printemps. En hiver, les animaux sont plus grégaires, formant des groupes de composition fluctuante. C'est un herbivore sélectif, se nourrissant de bourgeons, fruits, feuilles, graines selon la saison et son milieu de vie. Le rut a lieu entre mi-juillet et mi-août. L'ovo-implantation différée (ou diapause embryonnaire) est une autre particularité de l'espèce. Après la fécondation, l'œuf cesse tout développement après être parvenu au stade blastula pendant environ 170 jours. La phase réelle de gestation commence entre la fin décembre et début janvier. Elle est de l'ordre de 130 jours. La durée de gravidité est de 300 jours environ. La mise-bas (de 1 à 3 faons) a lieu en mai ou en juin. Les jeunes faons restent tapis au sol les premiers jours, puis suivent la femelle vers l'âge de 6 à 8 semaines. Les sub-adultes quittent leurs mères en avril-mai, puis recherchent un domaine personnel durant une période erratique variant de 6 mois à un an. Les bois, présents seulement chez les mâles, tombent en automne. La détermination de l'âge dans des conditions naturelles est impossible à faire. L'observation des dents et de la taille permet de déterminer si l'animal est jeune ou adulte (Aulagnier et al., 2008) (ONCFS, 2011).

c. Mouflon

Les populations de mouflons continentales sont classées parmi les caprinés (famille des bovidés). La taxonomie du Mouflon a récemment fait l'objet d'une révision. La sous-espèce dénommée traditionnellement "Mouflon de Corse" (*Ovis ammon musimon*) prend la dénomination "*Ovis gmelini musimon*" avec trois variétés : Mouflon de Corse (var. *corsicana*), Mouflon de Sardaigne (var. *musimon*) et Mouflon de Chypre (var. *ophion*). Les Mouflons présents sur le continent ont été introduits ces derniers siècles, et leurs origines ne sont pas toujours clairement connues. De plus, beaucoup d'animaux sont plus ou moins hybridés avec des moutons domestiques, ou d'autres mouflons. Ils sont regroupés sous l'appellation « Mouflon méditerranéen (*Ovis gmelinii musimon* x *Ovis sp.*). En Europe, les mouflons sont présents sur des habitats variés, depuis les collines sèches méditerranéennes aux grandes forêts d'Europe Centrale. De régime alimentaire herbivore général, les mouflons consomment de nombreuses espèces végétales.

Le dimorphisme sexuel et saisonnier est très marqué. Les mâles, de 35 à 50 kg et d'environ 75 cm au garrot, présentent deux cornes imposantes et recourbées pouvant atteindre 90 cm de longueur. Certaines femelles sont cornées, mais présentent alors des cornes courtes et souvent dissymétriques.

Celles-ci sont plus petites, de 25 à 35 kilos, pour une hauteur au garrot d'environ 65 cm. La distinction de l'âge en quatre classes d'âge (1° année, 2° à 3° année, 4°, 5° et 6° année ou sujets plus âgés) est aisée chez les mâles, à l'aide de la taille, de l'allure générale, du profil de la tête et des cornes. Chez les femelles, les cornes étant absentes ou peu développées, seuls les critères de taille, d'allure générale, de forme de la tête permettent de distinguer les 4 classes d'âge (Aulagnier et al., 2008) (ONCFS, 2011).

Dans les Hautes-Alpes, les mouflons ont été introduits à partir de populations des Bauges entre 1973 et 1977.

2. Problématique des contacts en alpage

Les alpages, zones d'élevage extensif, constituent des zones privilégiées d'interaction entre ongulés sauvages et domestiques. Le rôle de ces interactions dans la problématique des transmissions d'infections entre les différentes espèces présentes n'est pas toujours clairement établi, mais est souvent incriminé dans l'épidémiologie de différentes maladies. En effet, la faune sauvage est régulièrement considérée comme un réservoir de diverses infections pour les troupeaux domestiques. Inversement, la présence de cheptels domestiques sur les zones peuplées par des espèces sauvages est fréquemment suspectée comme la cause de transmissions d'agents pathogènes (Pastoret et al., 1988) et de subséquentes diminutions de certaines populations de faune sauvage. D'importants conflits peuvent dès lors exister entre les éleveurs, les chasseurs, et les gestionnaires de milieu naturel. Il convient donc de connaître le mieux possible les éventuelles transmissions qui pourraient exister sur ces zones particulières.

3. Pestivirus des ruminants

a. Classification et phylogénie

Les genres *Pestivirus*, *Flavivirus* et *Hepacivirus* forment la famille des *Flaviviridae*. Quatre espèces de pestivirus sont officiellement acceptées par le comité international de taxonomie des virus (*International Committee on Taxonomy of Viruses* - ICTV) : le virus de la diarrhée virale bovine-1 (*Bovine Viral Diarrhea Virus* 1, BVDV-1), le virus de la diarrhée virale bovine-2 (*Bovine Viral Diarrhea Virus* 2, BVDV-2), le virus de la maladie des frontières (Border Disease Virus, BDV) et le virus de la peste porcine classique (*Classic Swine Fever Virus*, CSFV) (Becher et al., 1999). Trois souches virales, isolées d'une girafe (nommée « giraffe-1 ») (Becher et al., 2003), d'un renne (nommée « reindeer-1) (Avalos-Ramirez et al., 2001) et d'un sérum foetal de veau (souche « Hobi ») (Schirrmeier et al., 2004) sont classées comme des pseudo-espèces (« tentative species ») dans le genre Pestivirus (ICTV, 2004). Cette classification virale est entre autre basée sur l'espèce hôte dans laquelle

les souches virales ont été découvertes. Cependant, il n'existe pas de spécificité d'espèces, et de nombreuses infections croisées ont été décrites (Nettleton et Entrican, 1995).

Au sein de chaque espèce de pestivirus, de nombreux génogroupes (ou sous-espèces) sont décrits. Les virus classés parmi les BVDV-1 sont subdivisés en 11 sous-espèces (Vilcek et al., 2001) et en 2 dans le BVDV-2 (Vilcek et al., 2005). Les souches correspondant au génotype 1 sont présentes dans le monde. Le génotype 2 a été mis en évidence lors d'épisodes cliniques graves en Amérique du Nord. Ces souches hypervirulentes ont été associées à un syndrome hémorragique associé à une thrombocytopénie périphérique (Ridpath et al., 1994 ; Thiry, 2007). Toutefois, un grand nombre de souches de génotype 2, notamment celles circulant en Europe, ne sont pas associées à ce type de syndrome.

Les *Border Disease Virus* ne sont officiellement pas subdivisés en différentes sous-espèces (ICTV, 2004). Cependant, neuf sous-groupes ont été décrits, correspondants essentiellement à des souches isolées chez des moutons ou des petits ruminants sauvages européens. Les BDV-1 ont été décrits chez des ovins aux USA ou en Angleterre (Sullivan et al., 1997). Le deuxième groupe de virus (BDV-2) est constitué par des virus ayant été isolés chez différentes espèces de ruminants en Allemagne (Becher et al., 2003). Les BDV-3 ont été isolés en Suisse (Stalder et al., 2005) et en Autriche (Krametter-Froetscher et al., 2007), les BDV-4 en Espagne (Arnal et al., 2004; Valdazo-Gonzalez et al., 2007) Les BDV-5 et BDV-6 ont été isolés en France, en Aveyron et dans la région PACA, respectivement (Dubois et al., 2008). Différentes souches virales ont été isolées dans un lot de vaccins atténués contre les poxvirus ovins tunisien, et classées parmi les BDV-Tunisian (Thabti et al., 2005). De plus, deux souches ont été mises en évidence en Turquie, qui seraient classées dans un nouveau sous-groupe (appelé « BDV-Turkey ») (Oguzoglu et al., 2009). Enfin, des BDV-7 ont récemment été mis en évidence en Italie (Giammaroli et al., 2011)

Par ailleurs, deux biotypes sont décrits et peuvent être différenciés en culture de cellules par la présence ou l'absence d'un effet cytopathogène. Au niveau biochimique, cette distinction se traduit par un clivage de la protéine NS2-3 chez le biotype cytopathogène (cp).

b. Génome viral

Les pestivirus sont de petites particules sphériques enveloppées, de 40 à 60 nm de diamètre. Leur génome est constitué par une molécule d'ARN (Acide Ribo Nucléique) monocaténaire de polarité positive non polyadénylée de 12,3 kilo bases (kb) (Meyers et Thiel, 1996). Elle se compose d'un seul ORF (*Open Reading Frame*) d'approximativement 4000 codons encadrés par 2 régions non codantes,

la région 5'UTR (*untranslated region*) (d'approximativement 372 à 385 nucléotides) et la région 3'UTR (de 185 à 273 nucléotides) (Lindenbach et al.,2007) (Figure 2).

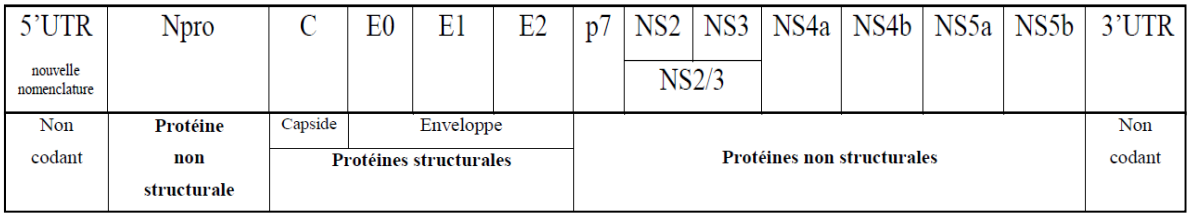

Figure 2. Génome des pestivirus indiquant les types de protéines codées par chaque partie du génome.

Dans des conditions expérimentales in vitro, ils peuvent survivre à une large gamme de pH mais sont inactivés par la chaleur, les solvants organiques et les détergents (Lindenbach et al., 2007). En milieu extérieur, leur survie est faible.

c. Cycle réplicatif

L'entrée dans l'organisme se fait au niveau des muqueuses oro-nasales, conjonctivales ou génitales. Le virus a un tropisme pour les cellules épithéliales et les cellules mononuclées sanguines. Une virémie transitoire apparaît deux à quatre jours post-infection ; le virus étant alors libre ou associé aux cellules mononuclées. Chez les ruminants, la protéine E2 est un déterminant majeur pour le tropisme et l'entrée dans la cellule. Le récepteur cellulaire « CD46 bovin» a été identifié comme étant le récepteur pour les souches de BVDV-1 et BVDV-2. L'internalisation se fait par un puits d'endocytose et est dépendante de la présence de la clathrine (protéine structurelle constituant l'enveloppe des vésicules) (Lindenbach et al., 2007). La réplication de l'ARN est associée à la membrane cytoplasmique et nécessite l'intervention des protéines NS3 et NS5B, ainsi que de certains composants cellulaires. Les protéines sont traduites en une seule longue polyprotéine d'environ 3400 acides aminés, clivée ultérieurement par des protéases virales et cellulaires. L'ordre du clivage de la polyprotéine du virus NADL est : NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Les protéines structurelles C, Erns, E1, et E2 sont encodées au niveau de la partie 5' de l'ORF (Lindenbach et al., 2007). Les titres maximaux de virus sont atteints dans les 12 à 24 heures après infection (figure 3).

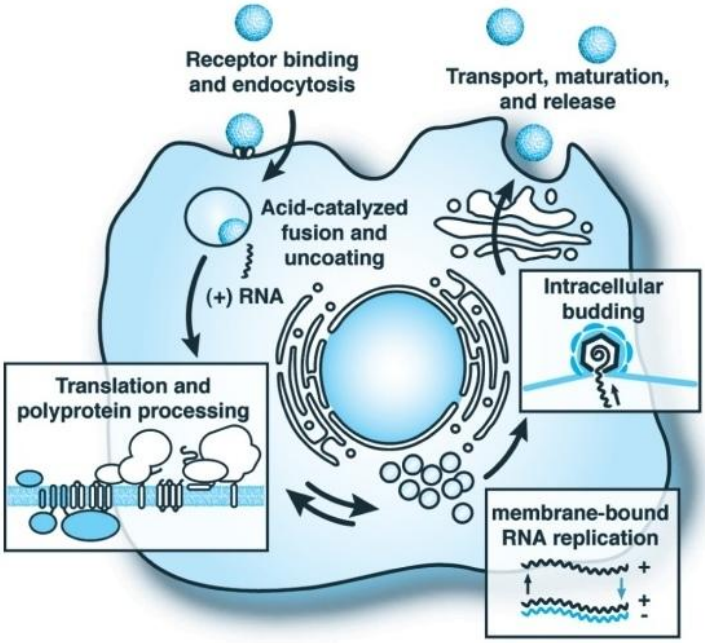

Figure 3. Cycle viral des pestivirus (d'après Fields Virology, 2007)

d. Maladies reconnues chez les espèces domestiques

L'expression clinique lors d'infection par un pestivirus est très variable, pouvant aller d'une simple virémie transitoire subclinique à une sévère atteinte multi-systémique (Evermann et Ridpath, 2002). Les signes cliniques généralement rencontrés incluent des anomalies de la reproduction (infécondité, avortement, mortalité fœtale), une immunodépression (leucopénie et neutropénie associés à une infection aiguë), des diarrhées, un dépérissement et, dans certains cas, un syndrome hémorragique sévère. Trois entités cliniques sont reconnues chez les animaux domestiques et sont associées aux espèces chez qui elles ont été découvertes. La diarrhée virale bovine (Bovine Viral Diarrhea) a été mise en évidence chez des bovins, la maladie des frontières ou Aveyronite (Border Disease) a été décrite chez des ovins, et la peste porcine classique (Classical Swine Fever) chez les porcs.

i. Diarrhée virale bovine

Chez des bovins adultes, l'infection est généralement subclinique ou peut parfois être associée à des signes cliniques respiratoires (surinfection bactérienne facilitée par l'immunodépression) ou digestifs (diarrhée). Chez les animaux reproducteurs, une baisse de la fécondité (inactivité ovarienne) est observée chez les femelles. Chez les mâles, une chute temporaire de la fertilité est souvent constatée et serait liée à un passage viral dans la semence (OMSA b, 2008).

Chez les femelles gravides, les conséquences de l'infection varient avec le stade de gestation (Figure 4a). Les animaux IPI (Infectés Persistants Immunotolérants) sont des animaux infectés *in utero* par une souche non cytopathogène (ncp) entre le 30ème et le 125ème jour de gestation (phase de reconnaissance du soi). Leur virémie est ainsi permanente et l'excrétion du virus considérable. L'identification de ces animaux est donc indispensable pour envisager un quelconque plan de lutte. Ils ne survivent généralement pas plus de 2 ans (Letellier et al., 2005 ; Thiry, 2007).

La forme « maladie des muqueuses » survient lors d'association chez un IPI de la souche initiale infectante et d'une souche ayant un biotype cytopathogène ; cette dernière provenant soit de surinfection (Bolin, 1995), soit de mutation du biotype présent ou de recombinaison entre biotypes non cytopathogènes (Loehr et al., 1998). Cette forme est toujours rapidement fatale, parfois accompagnée par une courte phase de diarrhée profuse et une perte importante de poids. Des érosions des muqueuses sont souvent visibles dans la cavité buccale, particulièrement au niveau de la marge gingivale. Ces cas de maladie des muqueuses sont sporadiques et rares (OMSA b, 2008).

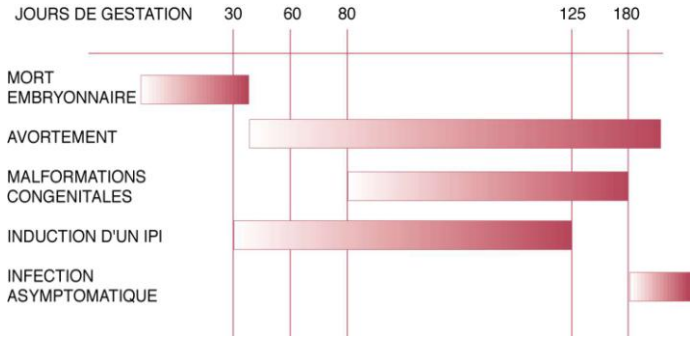

Figure 4a. Conséquences de l'infection par un BVDV en fonction du stade de gestation chez la vache (Thiry, 2007)

ii. Maladie des frontières / Border Disease / Aveyronite

La pathogénie des virus classés dans les BDV chez les ovins est assez proche de celle des BVDV (Krametter-Froetscher et al., 2007).

Après la naissance, l'infection est bénigne et quasiment inapparente : les agneaux peuvent présenter pendant une dizaine de jours après infection une légère leucopénie associée à une faible fièvre. Les mortalités peuvent être importantes dans les ateliers d'engraissement à forte densité d'animaux. Par contre, chez les femelles gestantes, les conséquences sont beaucoup plus graves et varient selon le moment de l'infection (Figure 4b) (Pastoret., 2003).

Les BDV provoquent essentiellement des problèmes reproducteurs, surtout détectés au moment de la mise-bas : les avortements sont rarement précédés d'autres signes cliniques et ne sont décelés que lors de l'agnelage. Des tremblements, accompagnés d'une conformation anormale (petits agneaux chétifs) et d'une toison hirsute sont fréquemment retrouvés chez les agneaux nouveau-nés malades (cette maladie est parfois appelée « hairy shaker disease » ; maladie des trembleurs hirsutes). La plupart de ces animaux meurent avant le sevrage (OMSA a, 2008).

Contrairement à ce qui est décrit pour les BVDV, quasiment toutes les souches de BDV sont non cytopathogènes en culture cellulaire (OMSA a, 2008). La souche Moredun possède un biotype cytopathogène (numéro GENBANK U65022.1) et un biotype non cytopathogène (numéro GENBANK U65023.1), ce qui une exception parmi les BDV.

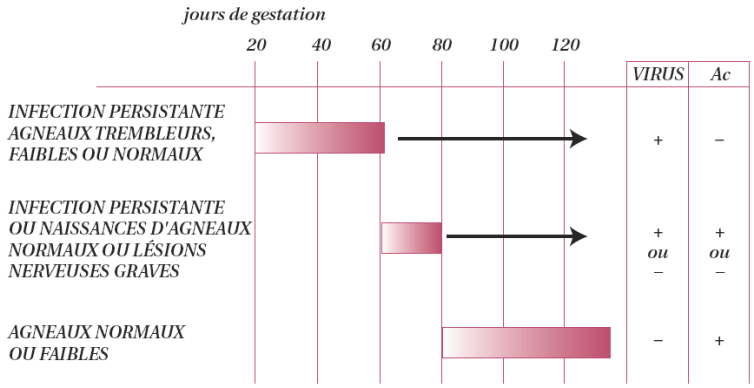

Figure 4b. Conséquences de l'infection par un BDV chez la brebis en fonction du stade de gestation (Thiry, 2007)

iii. Peste porcine classique

La gravité de la maladie est fonction de la virulence des souches présentes mais aussi de facteurs individuels tels que l'âge, la race, le statut immunitaire et les conditions environnementales (Moennig, 2000). Dans toutes les classes d'âge, l'infection est accompagnée de fièvre, d'inappétence, d'apathie, d'abattement, et/ou de pertes d'équilibre (OMSA c, 2008).

Lors d'infection post-natale aiguë par une souche de forte ou moyenne virulence, le taux de mortalité atteint 95 à 100% et est notamment associé à une forte hyperthermie, une thrombocytopénie, des pétéchies cutanées et une coloration violacée des oreilles, de l'abdomen et de la région postérieure (OMSA c, 2008).

L'infection post-natale chronique est définie lorsque la durée de survie après l'infection est supérieure à 30 jours (Thiry, 2004). Elle est provoquée par une souche de virulence moyenne. L'évolution de la maladie se fait en trois phases, à savoir une première phase d'abattement, d'anorexie accompagnée de leucopénie ; une deuxième phase pendant laquelle l'état de santé s'améliore mais avec persistance de la leucopénie ; puis une dernière période faite de rechutes successives avec la mort de l'animal, de un à trois mois après l'infection. Par ailleurs, il existe des souches hypovirulentes, qui n'occasionnent qu'un syndrome transitoire, suivi par la guérison complète de l'animal.

Comme pour les autres espèces de pestivirus, les troubles de la reproduction sont majoritaires et varient en fonction de la période de gestation (figure 4c). Les conséquences les plus importantes sont des mortalités embryonnaires et néonatales, des avortements, des anomalies congénitales, ainsi que des momifications fœtales. La formation d'animaux infectés immunotolérants est plus rare que chez les autres espèces et se fait lorsque la truie est infectée entre le 40ème et le 90ème jour de gestation (Thiry, 2004). Les porcins peuvent être atteints par des pestivirus de ruminants et la forme clinique développée n'est pas différentiable de celle développée lors de CSF (Vilcek and Belak, 1996).

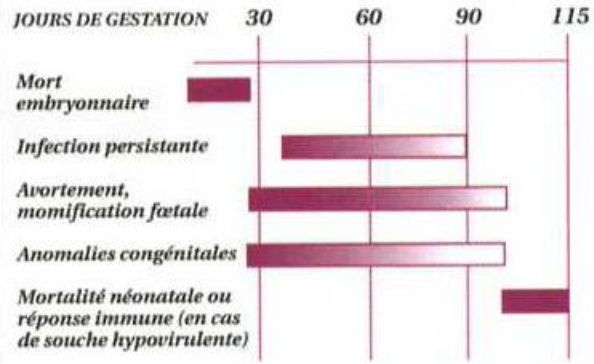

Figure 4c. Conséquences de l'infection par un CSFV en fonction du stade de gestation chez la truie (Thiry, 2004)

iv. Sources et transmission de l'infection

La transmission du virus s'effectue facilement par contact direct (oro-nasale ou génitale) entre les animaux réceptifs (n'étant plus sous protection colostrale). Lors de transmission horizontale, le virus pénètre au niveau oronasal, génital ou conjonctival, s'y mulpiplis puis est transporté par voie sanguine vers les différents organes cibles. Pour ces animaux infectés transitoirement, l'installation d'une réponse immunitaire de type humorale suffirait à éliminer le virus. Le virus peut être isolé dans les sécrétions rectales, vaginales et nasales.

Lors de transmission verticale, les conséquences de l'infection sur le fœtus sont variables en fonction du moment d'infection, comme décrit précédemment. Chez les animaux IPI, la charge virale est considérable, et le virus peut aisément être détecté dans de nombreux organes. Ces animaux IPI jouent

un rôle prépondérant dans l'épidémiologie des infections avec des pestivirus, constituant la principale source de propagation du virus au sein d'un troupeau. L'éradication de la maladie au sein d'un troupeau passe par l'élimination des animaux IPI éventuellement présents (Pastoret et al., 2003).

v. Plans de lutte

Plusieurs études de prévalence menées depuis les années 1970 ont montré que le BVDV est (ou a été) enzootique dans tous les pays où un contrôle systématique n'a pas été mis en place (Linberg et al., 2006). L'impact économique, de part sa forte prévalence, est très important. La première mesure de lutte à mettre en place est l'identification et l'élimination systématique des IPI (Letellier et al., 2005). A l'échelle du troupeau, lorsqu'aucun IPI n'est présent, le moyen le plus classique de contrôler le BVDV est la vaccination (Brock, 2004). Néanmoins, celle-ci est insuffisante à elle seule lorsque l'éradication du pathogène est recherchée dans un pays. Les mesures de prophylaxie adoptées varient en fonction des pays.

Actuellement, des plans de contrôle du BVDV, sur base volontaire, sont en place dans la plupart des pays Européens (Lindberg et al., 2006). Au Royaume-Uni, en Irlande, aux Pays-Bas et en Slovénie, seuls des vaccins inactivés sont mis sur le marché. Les pays Scandinaves (Danemark, Norvège, Finlande, Suède et Iles Shetland) et l'Autriche ont mis en place un plan d'éradication avec interdiction d'utilisation de vaccins, depuis plus de 10 ans pour les premiers et depuis 2004 pour l'Autriche. En Suisse, un plan national d'éradication des virus de la BVDV a été mis en place en 2008. Dans ce cadre, chaque bovin testé positif pour la recherche d'antigènes a été abattu, et des restrictions de mouvements d'animaux ont été appliquées. De plus, des efforts de contrôle sont réalisés dans certaines régions de France (Bretagne, Bourgogne) (Petit, 2005) et d'Italie (province de Rome), aux Pays-Bas, en Allemagne, en Grèce et en Galice espagnole.

Par ailleurs, un groupe de travail scientifique s'est constitué au niveau européen en Décembre 2002, visant à encourager et à évaluer les mesures actuelles de lutte contre le BVDV. Un modèle a été créé, concluant que « une approche systématique incluant comme éléments clés la biosécurité (nouvelle introduction de l'infection dans les troupeaux sains), l'élimination des IPI, et la surveillance (suivi des interventions et rapidité de détection de nouvelles infections) est nécessaire pour diminuer durablement et convenablement la prévalence et l'incidence du pathogène ». La vaccination est alors considérée comme une mesure supplémentaire, dont chaque tenant et aboutissant doivent être évalués avant son utilisation (Lindberg et al., 2006). A l'heure actuelle, aucun plan de lutte contre la border disease n'a été mis en place à grande échelle. En France, des réflexions sont actuellement en cours au sein d'un groupe de travail « avortements des petits ruminants » concernant la mise en place d'un programme de détection et de diagnostic différentiel des avortements chez les petits ruminants (Duquesne, communication personnelle).

La peste porcine classique est quant à elle une maladie à déclaration obligatoire. La vaccination est interdite dans les pays indemnes ou engagés dans un plan de lutte. En Europe, seuls des vaccins atténués sont autorisés, en cas d'urgence autour d'un foyer (OMSA c, 2008).

e. Infections avec des pestivirus reconnues au sein des ruminants sauvages

i. Dans le monde

Le Tableau III reprend les résultats d'études de prévalence ou de séroprévalence de pestivirus conduites sur différentes espèces d'ongulés sauvages sur tous les continents.

Famille	*Genre*	Espèce	Signes cliniques	Virologie	Test	Séroconversion	Test	Titre (si SN)	Souche/Espèce Pestivirus	Bibliographie
Antilocapridae	*Antilocapra*	Pronghorn *(Antilocopra Americana)*	-	-	-	3/14	SN	-	-	(Doyle et Heuschele, 1983)
			-	1/1	RT-PCR + IV	-	-	-	Pronghorn	(Vilcek et al., 2005)
Bovidae	*Alcephalinae*	Topi *(Damaliscus lunatus)*	-	-	-	6/16	SN	9/54	BVDV-1	(Hamblin et Hedger, 1979)
		Bubale roux *(Alcelaphus buselaphus)*	-	-	-	8	SN	1/7	BVDV-1	(Hamblin et Hedger, 1979)
		Gnou noir *(Connochaetes taurinus)*	-	-	-	4/64	SN	19/280	BVDV-1	(Hamblin et Hedger, 1979)
	Antilopinae	Springbok (*Antidorcas marsupialis)*	-	-	-	12/51	SN	8-178	BVDV-1	(Hamblin et Hedger, 1979)
			-	-	-	2/51	SN	-	-	(Doyle et Heuschele, 1983)
	Bovinae	Bison d'Amérique du Nord (*Bison bison)*	-	-	-	31/101	SN	8 à 16	-	(Taylor *et al*., 1997)
			-	2/2	IV	-	-	-	BVDV	(Deregt *et al*., 2005)
		Bison d'Europe *(Bison bonasus)*	-	-	-	5/28	SN	8	BVDV-1	(Frolich et Flach, 1998)
		Yak *(Bos poephagus grunniens)*	Avortement		RT-PCR	3/71	SN	320	BVDV-1	(Mishra *et al*., 2008)
		Buffle d'Afrique *(Syncerus caffer)*	-	-	-	605/1442	SN	4 à 708	BVDV-1	(Hamblin et Hedger, 1979)
		Eland *(Taurotragus oryx)*	-	-	-	33/61	SN	4 à 1400	BVDV-1	(Hamblin et Hedger, 1979)
			-	3/303	RT-PCR + IV	492/ 1539	ELISA	-	BVDV-1	(Vilcek *et al*., 2000)
		Nyala *(Tragelaphus angasi)*	-	-	-	1/1	SN	32	BVDV-1	(Hamblin et Hedger, 1979)
		Grand Koudou *(Tragelaphus strepsiceros)*	-	-	-	61/179	SN	4 à 2048	BVDV-1	(Hamblin et Hedger, 1979)
	Caprinae	Chèvre (*Capra aegagrus*)	Avortement	-	RT-PCR + IV	-	-	-	BDV (712/02)	(Mia *et al*., 2005)
			-	-	-	63/113	ELISA AND SN	>4	-	(Krametter-Froetscher *et al.*, 2007)
			None	0/10	RT-PCR	10/10	SN	6 to >256	BVDV-1	(Broaddus *et al*., 2007)
		Chèvre (*Capra hircus*)	Avortement	-	-	-	-	-	BVDV	(Doyle et Heuschele, 1983)
		Sérow Japonais (*Capricornis crispus*)	-	-	RTnested PCR	3/16			BVDV-1	(Harasawa *et al*., 2006)
		Isard (*Rupicapra pyrenaica)*	-	2/21	RT-PCR	33/200	ELISA		BDV-4	(Arnal *et al.*, 2004)
			Faiblesse	oui	RT-PCR	-			BDV	(Frolich *et al.*, 2005b)
				8/12	RT-PCR	Absence	SN		BDV	(Hurtado *et al*., 2004)
			Chronic wasting	3/3	RT-PCR	Absence	ELISA		BDV-4	(Alzieu *et al*., 2004)
			-	-	RT-PCR	Absence (0/5)	ELISA AND SN		BDV-4	(Marco *et al*., 2007)
		Chamois *(Rupicapra rupicapra)*	-	-	-	6/35	SN	4 à 8	-	(Gentile et al, 2000)
			-	-	-	28/110	SN	10 à 960	-	(Olde Riekerink et al., 2005)
	Cephalophinae	Céphalophe de Grimm *(Sylvicapra grimmia)*	-	-	-	10/36	SN	8-512	BVDV-1	(Hamblin et Hedger, 1979)
	Hippotraginae	Hippotrague noir *(Hippotragus niger)*	-	-	-	12/28	SN	4-355	BVDV-1	(Hamblin et Hedger, 1979)
		Oryx algazelle (*Oryx dammah*)	-	-	-	3/87	SN	-	-	(Doyle et Heuschele, 1983)
			-	-	-	7/37	SN	5-16	BVDV-1	(Frölich et Flach, 1998)
		Oryx gazelle *(Oryx gazella)*	-	-	-	9/16	SN	6-355	BVDV-1	(Hamblin et Hedger, 1979)
		Oryx d'Arabie (*Oryx leucoryx*)	-	-	-	6/294	SN	9 to 298	BVDV-1	(Frolich *et al*., 2005a)
Bovidae	*Reduncinae*	Defassa waterbuck (*Kobus defassa)*	-	-	-	7/8	SN	128-2048	BVDV-1	(Hamblin et Hedger, 1979)
		Cobe à croissant (*Kobus ellipsiprymnus*)	-	-	-	13/19	SN	6-512	BVDV-1	(Hamblin et Hedger, 1979)
		Cobe de Lechwe (*Kobus leche*)	-	-	-	9/14	SN	4-64	BVDV-1	(Hamblin et Hedger, 1979)
		Grand cobe des roseaux (*Redunca arundinum*)	-	-	-	7/10	SN	90-2048	BVDV-1	(Hamblin et Hedger, 1979)
Camelidae	*Camelus*	Dromadaire (*Camelus dromedarius*)	Diarrhée	-	-	-	-	-	BDVD-2	(Evermann, 2006)

	Lama	Lama (*Lama glama*)	avortement, amaigrissement	2/3	IV	0/136	SN	<8	BVDV-1	(Belknap et al., 2000)
		Alaga (*Lama pacos*)	Avortement	1/1	RT-PCR	-	-	-	BVDV-1	(Goyal et al., 2002)
Cervidae	*Odocoileinae*	Elan (*Alces alces*)	-	-	-	35/1794	SN	4 à ≥128	BVDV-1	(Lillehaug et al., 2003)
			-	-	-	4/22	SN	5 à 100	BVDV-1	(Thorsen et Henderson, 1971)
		Chevreuil (*Capreolus capreolus*)	-	-	(serology)	12/123	SN	5 à 125	BVDV	(Frolich, 1995)
			-	-	(serology)	78/635	SN	8 à ≥128	VDV-1	(Lillehaug et al., 2003)
			2/113	2/113	RT-PCR +IV	-	-	-	BVDV-1	(Fischer et al., 1998)
		Hydropote (*Hydropotes inermis*)	-	-	-	10/144	SN	5 à 11	BVDV-1	(Frolich et Flach, 1998)
		Cerf hémione (*Odocoileus hemionus*)	Amaigrissement	4/4	-	4/4	SN		NADL+ NY-1	(Van Campen et al., 1997)
			Faiblesse	1	-	74/124	SN	≥32	BVDV-1 ncp	(Van Campen et al., 2001)
		Cerf de Virginie (*Odocoileus virginianus*)	absence	1/1	-	1/1	SN		NADL+ NY-1	(Van Campen et al., 1997)
			-	5/8	IV	5/8	SN	"titres élevés"	BVDV-1&2	(Ridpath et al., 2007)
			-	-	(sérologie)	1/165	SN	8	BVDV-1	(Passler et al., 2007)
		Pudu (*Pudu puda*)	Ulcère + congestion	1/1	RT-PCR	-	-	-	BVDV-1	(Pizarro-Lucero et al., 2005)
		Caribou (*Rangifer tarandus*)	-	-	-	38/58	SN	4 à 512	-	(Elazhary et al., 1981)
			-	(sérologie)	-	35/1794	SN	8 à 128	BVDV-1	(Lillehaug et al., 2003)
	Cervinae	Cerf élaphe (*Cervus elaphus*)	-	-	(sérologie)	3/60	SN		BVDV	(Frölich, 1995)
			-	-	-	8/136	SN	5 à 125	BDV	(Olde Riekerink et al., 2005)
			-	Absence	RT-PCR	1/59	ELISA+SN		BVDV-1	(Krametter et al, 2004)
			-	-	-	3/77	SN		-	(Nielsen et al, 2000)
			1 mort subite	9/10	IV	11/12	SN	16 à 45	Singer & 24515	(Tessaro et al., 1999)
			-	-	(sérologie)	7/658	SN	4 à 64	BVDV-1	(Lillehaug et al, 2003)
		Wapiti (*Cervus elaphus canadensis*)	-	-	-	247/456	SN	-	-	(Aguirre et al., 1995)
		Daim (*Dama dama*)	-	-	(sérologie)	1/87	SN	5 à 125	BVDV	(Frölich, 1995)
		Cerf du Père David (*Elaphurus davidianus*)	-	-	-	11/49	SN	5 à 10	BVDV-1	(Frölich et Flach, 1998)
Giraffidae	*Giraffa*	Girafe (*Giraffa camelopardalis*)	-	-	-	3/24	SN	32-90	BVDV-1	(Hamblin et Hedger, 1979)
			-	1/1	IV	-	-		Giraffe	(Avalos-Ramirez et al., 2001)
Tragulidae	*Tragulus*	Mousedeer (*Tragulus javanicus*)	Absence	3/4	RT-PCR + IV	1/4	ELISA+SN	1280	BVDV-1	(Grondahl et al., 2003)

Tableau III. Infections avec des pestivirus déjà décrites chez des ruminants sauvages (d'après Martin, 2007).

ii. En Europe

Dans les pays scandinaves, des études de séroconversion réalisées sur des cervidés montrent que des souches de pestivirus circulent au sein de ces populations. En effet, en Norvège, les pestivirus sont enzootiques chez les rennes (*Rangifer tarendus*) et chez les chevreuils (*Capreolus capreolus*) : des séroneutralisations réalisées sur la souche NADL puis sur une souche norvégienne cytopathogène (souche MD 2157/66, isolée d'un veau atteint de maladie des muqueuses) révèlent une présence d'anticorps chez 12,3% des chevreuils [78/635] et chez 4,2% des rennes [34/810] (Lillehaug et al., 2003). En revanche, au Danemark, les chevreuils semblent faiblement infectés puisque seuls 3 cervidés sur 476 testés ont montré une séroconversion lors de 2 études menées sur une période de 3 ans (Nielsen et al., 2000).

En Allemagne, sur 355 cervidés testés, 17 chevreuils ont développé une réaction immunitaire contre un BVDV et 1 contre un BDV (Frölich, 1995). Deux souches cytopathogènes ont été isolées sur 2 chevreuils (Frölich et Hofmann, 1995) et ont été classées dans l'espèce BVDV-1 (Fischer et al., 1998). Plus récemment, un dépistage sérologique réalisé dans 6 Parcs Nationaux a révélé l'absence d'anticorps dirigés contre un BVDV chez 158 animaux testés (chevreuils et cerfs) (Frölich et al., 2006).

En Autriche, seul un cerf élaphe présentait des anticorps contre un BVDV-1 parmi 145 animaux testés (cervidés et caprinés). Dans cette zone d'étude, la séroprévalence du BDV et du BVDV était de 1,1% chez les moutons et de 70-80% chez les bovins (Krametter et al., 2004). Or, un fort taux de contact existe entre les espèces sauvages testées et les ruminants domestiques, notamment au niveau des dépôts de sel en alpages. Le sens de transmission semble alors s'être fait d'un bovin IPI vers le cerf (Krametter et al., 2004).

iii. En Espagne

Un cas particulier semble être celui des populations d'isards (*Rupicapra pyrenaica*) dans les Pyrénées. Depuis 2001, de nombreux épisodes de fortes mortalités ont été constatés dans plusieurs populations d'isards dans la principauté d'Andorre et quatre réserves de chasse en Aragon, en Espagne (réserve de Benasque, Los Circos, Viñamala, et Los Valles) (Arnal et al., 2004), dans la réserve Alt Pallars, en Catalogne (Hurtado et al., 2004 ; Marco et al., 2007), ainsi que dans la réserve d'Orlu, dans le département de l'Ariège, en France (Frölich et al., 2005). Ces mortalités étaient associées à des signes cliniques non spécifiques, tels qu'une diminution de la mobilité et une absence de fuite lors de présence humaine, différents degrés d'alopécie au niveau du tronc et de la nuque, une rétention du duvet hivernal au niveau des membres et de la face, et une hyperpigmentation de la peau. A l'examen clinique, ces isards étaient cachectiques et anémiés (Arnal et al., 2004 ; Hurtado et al., 2004 ; Frölich et al., 2005 ; Marco et al., 2007).

Des lésions histologiques ont été observées au niveau de la peau (alopécie sévère avec atrophie folliculaire et regroupement des follicules pileux, accompagnée d'hyperplasie et de mélanose de l'épiderme) et du cerveau (œdème cérébral, spongiose de la substance blanche et dégénérescence neuronale) (Marco et al., 2007). Le virus responsable de ces affections a été séquencé par plusieurs équipes (Hurtado et al., 2004 ; Frölich et al., 2005) et a été classé parmi les BDV, sous-type BDV 4 (Arnal et al., 2004).

iv. En France

Une étude épidémiologique réalisée de 1995 à 2004 dans la RNCFS d'Orlu (Pyrénées Françaises) a montré que des souches classées parmi les BDV-4 circulaient au sein des isards. Une séroprévalence élevée a pu être montrée, atteignant 70,3% des 323 isards testés. Les auteurs ont montré que l'infection était endémique au sein de la population d'isard de la réserve d'Orlu, sans être associée à des mortalités massives ni à des signes cliniques particuliers (Pioz et al., 2007).

Par ailleurs, durant le printemps 2005, une femelle isard infectée expérimentalement entre le 91ème et le 101ème jour de gestation par une souche locale (souche « Ariège 2002) a mis bas un jeune suspecté comme IPI (Vautrain et Gibert, 2008). Il est né sans malformation et avec un examen clinique à la naissance normal. Jusqu'à deux jours avant sa mort, il a eu une croissance normale et n'a présenté aucun signe clinique. Il est mort à 84 jours, après avoir présenté un état fébrile sévère accompagné d'une diarrhée profuse. L'analyse par RT-PCR du sang prélevé à la naissance et de sa rate (prélevée lors de l'autopsie) a montré deux résultats positifs à plus de deux mois d'intervalle (Vautrain et Gibert, 2008). Ces résultats laissent suspecter un statut immunotolérant persistant, par ailleurs déjà décrit dans d'autres espèces de ruminants sauvages (Passler et al., 2009).

Un programme de recherche « espaces protégés » a été mis en place au début des années 2000 dans les Réserves Nationales de Chasse et de Faune Sauvage (RNCFS) gérées par l'Office National de Chasse et de Faune Sauvage (ONCFS) (RNCFS des Bauges, du Caroux et d'Orlu) visant à étudier le statut sanitaire des espèces d'ongulés présentes sur les alpages et à évaluer les risques de transmission inter-espèces. Ce programme s'est plus particulièrement attaché à l'étude de cinq pathologies abortives non règlementées (la chlamydophylose abortive liée à *Chlamydophila abortus*, la fièvre Q liée à *Coxiella burnetii*, la salmonellose ovine provoquée par *Salmonella abortus ovis* ; la toxoplasmose liée à *Toxoplasma gondii* et les pathologies liées aux pestivirus, la BVD et la Border Disease) (Jourdain, 2003). Au sein de la réserve nationale de chasse et de faune sauvage des Bauges (département de Savoie), 423 chamois ont été testés pour la présence d'anticorps dirigés contre les pestivirus (correspondant à des prélèvements effectués entre 1980 et 2001). Seuls huit individus ont montré une sérologie positive. Sur 18 mouflons testés, aucun animal n'était séropositif (Jourdain, 2003). Dans la

RNCFS du Caroux-Espinouse (située au sein du Parc naturel régional du Haut-Languedoc), 427 sérums de mouflons ont été prélevés entre 1990 et 2001. Sur l'ensemble de ces sérums, 3 étaient positifs, et 5 étaient douteux pour la recherche d'anticorps dirigés contre les pestivirus (Dupraz, 2004).

Par ailleurs, en 2006, dans le département des Hautes-Alpes, une chute des effectifs avait été observée sur plusieurs populations de chamois, allant jusqu'à 17% au sein de la réserve naturelle du Combeynot (briançonnais). Plusieurs suivis sanitaires ont été successivement mis en place en partenariat avec le laboratoire Départemental Vétérinaire et d'Hygiène Alimentaire des Hautes-Alpes et les différentes sociétés de chasse concernées. La mise en place de ces suivis sanitaires a montré une faible séroprévalence des pathologies abortives. Aucun animal testé sur 300 n'a présenté d'anticorps contre la fièvre Q (provoquée par *Coxiella burnetii*), la chlamydophilose (*Chlamydophila abortus*), la brucellose (*Brucella abortus, B. melitensis et B. suis*), la paratuberculose (*Mycobacterium paratuberculosis*), et l'epididymite ovine (testée seulement en 2004) (*Brucella ovis*). Dix sérums sur 262 étaient positifs en séroneutralisation dirigée contre la salmonellose (*Salmonella abortus ovis*), la rhinotrachéite infectieuse bovine (IBR), la toxoplasmose. Une séroprévalence faible (égale à 7,6%) contre le CAEV-Visna Maedi avait pu être mise en évidence sur les 262 sérums testés. En revanche, une séroprévalence élevée associée dans certains cas à une antigénémie positive élevée contre des pestivirus a été démontré dans le département des Hautes-Alpes (Martin, 2007).

4. Objectifs et présentation des travaux

Les objectifs principaux de cette étude ont été d'identifier si des pestivirus peuvent être associés aux cas de mortalités observés sur le terrain, tant chez les ruminants domestiques que sauvages ; d'évaluer le pouvoir pathogène des souches de pestivirus en terme d'effet sur la prolificité (nombre de naissances par rapport au nombre de gestations) et sur la mortalité néonatale chez les espèces sauvages et d'évaluer la possibilité de création d'un réservoir de pestivirus chez la faune sauvage (capacité de survie des animaux infectés et cinétiques d'excrétion virale). Dès lors, deux principaux objectifs ont conduit les travaux menés lors de cette thèse.

En premier lieu, nous avons étudié la pathogénie de l'infection par des pestivirus chez des isards, en s'attardant plus particulièrement sur les troubles de la gestation. En collaboration avec le Service de faune Sauvage de la Faculté de Médecine Vétérinaire de Barcelone, une infection expérimentale de femelles isards gestantes a été réalisée avec une souche virale préalablement isolée d'un isard dans les Pyrénées espagnoles dans le but d'évaluer ses effets potentiels sur la gestation et de savoir si des animaux IPI pouvaient naître dans cette espèce. Les résultats ont été comparés à ceux obtenus sur des animaux témoins.

Parallèlement, nous avons étudié l'épidémiologie de l'infection dans différentes zones de la région Provence-Alpes-Côte d'Azur (PACA), tout d'abord chez des ruminants sauvages, puis à l'interface entre les ruminants sauvages et domestiques partageant les mêmes alpages. Des études séro-épidémiologiques ont été mises en place dans le but initial de décrire la diversité des pestivirus circulant en montagne et la comparer aux souches connues, ainsi que d'identifier si des souches virales sont spécifiques d'espèces animales ou partagées notamment entre ruminants domestiques et sauvages.

Dans la logique du travail effectué, ce mémoire s'articule en deux grandes parties principales, et est basé sur des publications acceptées, soumises ou en préparation dans des journaux à comité de lecture.

La première concerne la partie expérimentale du travail : une première étude, bibliographique, visait à étudier les méthodologies utilisées lors des expérimentations animales déjà publiées réalisées avec des pestivirus de ruminants. Par la suite, l'infection expérimentale d'isard gestant réalisée au sein de la station expérimentale de l'Anses Sophia-Antipolis fait l'objet de la seconde étude présentée.
La seconde partie aborde la partie épidémiologique du travail. De la même façon, un premier travail bibliographique fait état des différentes infections décrites sur les ruminants sauvages en Europe, en l'associant notamment à une étude des facteurs de risque associés. Les deux études suivantes concernent le travail mis en place dans la région PACA. Le premier décrit une première étude

longitudinale, réalisée sur les animaux sauvages de 2003 à 2007. La seconde concerne une étude épidémiologique transversale, réalisée dans plusieurs sites de la région PACA lors des saisons 2009 et 2010. Une dernière partie reprend l'ensemble des résultats dans une discussion générale et en présente les perspectives. Enfin, en annexe, sont exposées les présentations orales et posters réalisées au cours des différentes années de doctorat.

PARTIE I

ETUDE EXPERIMENTALE

Cette première partie du travail se décompose en deux études.

La première est une étude bibliographique (prête à être soumise dans *Veterinary Research*) qui a eu pour objectif d'étudier et de comparer les méthodologies utilisées dans des infections expérimentales réalisées avec des pestivirus de ruminants (BVDV et BDV). La grande diversité de souches et de voies d'inoculation employées, d'espèces animales inoculées et de procédures utilisées ont rendu difficile une comparaison « simple » des méthodes et des résultats obtenus. Nous avons dés lors développé une méthode d'évaluation quantitative basée sur un score, permettant la comparaison de 157 études expérimentales déjà publiées. Les études ont été groupées et comparées en fonction de leur principal objectif, dans le but de développer des recommandations pour les scientifiques préparant une infection expérimentale et d'harmoniser les différentes méthodes utilisées afin de pouvoir comparer les résultats plus facilement.

Par la suite, une seconde étude (acceptée avec modifications mineures dans *Journal of Wildlife Diseases*) présente des travaux d'infection expérimentale de femelles isards gestantes réalisés avec un Border Disease Virus type 4. Ces travaux, réalisés en collaboration avec le service de faune sauvage de la faculté de médecine vétérinaire de Barcelone (SeFAS), ont eu pour objectif d'étudier la pathologie induite par une souche virale préalablement isolée d'un isard en Espagne, et plus particulièrement ses effets sur la gestation. Dans ce cadre, trois femelles ont été inoculées pendant le deuxième tiers de gestation, dans la station expérimentale de l'Anses située à Sclos de Contes. En accord avec les recommandations fournies par la première étude, un suivi clinique, un suivi de la virémie, de la sérologie, et de la formule sanguine ont été réalisés, et comparés à ceux des deux animaux témoins.

ARTICLE 1. ARTICLE DE REVUE

Critical review of experimental infections performed with ruminant pestivirus in both wild and domestic ruminants

Prêt à être soumis dans Veterinary Research

ARTICLE 1. Resumé

Revue critique des infections expérimentales réalisées avec des pestivirus de ruminants sur des ruminants sauvages et domestiques

Les pestivirus sont des virus à ARN d'importance majeure pour les ruminants domestiques et sauvages. Leur grande variété est reflétée par leur diversité génétique, leur pathogénie, les différentes espèces animales infectées et leur comportement en culture de cellules. Actuellement, malgré une bonne connaissance de leur pathogénie pour les espèces domestiques, beaucoup de questions restent en suspens et de nombreuses infections expérimentales sont encore conduites. Les objectifs de cette étude ont été d'investiguer et de comparer les méthodologies utilisées lors des infections expérimentales réalisées avec des pestivirus de ruminants. Un total de 157 infections expérimentales a été évalué à l'aide d'une évaluation quantitative originale basée sur une liste de 37 critères, préalablement validée par sept experts internationaux. Les expérimentations ont été évaluées en fonction de leur objectif principal. Les scores obtenus par les études de pathogénie et d'effets vaccinaux ont été analysés et comparés à l'aide d'une analyse statistique. Les méthodes utilisées lors des expérimentations animales ont été analysées en fonction de leur évolution dans le temps. Ce travail nous a permis de développer des recommandations (i) pour avoir une structure « modèle » pour des scientifiques préparant une infection expérimentale et (ii) pour harmoniser les pratiques utilisées afin de permettre une meilleure comparaison des différentes études.

Title

Critical review of experimental infections performed with ruminant pestivirus in both wild and domestic ruminants

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PLAN

ABSTRACT

Pestiviruses are RNA viruses of major concern for both wild and domestic ruminants. Their huge variety is reflected in their genetic diversity, pathogenesis, range of animal species infected and behavior in cell cultures. Nowadays, despite a better knowledge of their pathogenesis in livestock, many questions remain and numerous experimental infections are still conducted. The objectives of this study were to investigate and compare the methodologies used in experimental infections performed with ruminant pestiviruses. A total of 157 selected experimental studies were evaluated through an original quantitative assessment based on a list of 37 criteria previously approved by seven international experts. Experimentations were evaluated with reference to their main objective. The scores obtained by pathogenesis and vaccine studies were analyzed and compared through statistical analysis. We drew up a picture of all the methods used in animal experimentations and their evolution over time, in order to develop experimental guidelines that will enable scientists preparing an experimentation (i) to have a framework for their future studies and (ii) to harmonize experimentation practices in order to improve comparison of studies.

Keywords: Pestivirus, Ruminants, Experimental infection, Experimental model, Quantitative assessment.

1- INTRODUCTION

Pestiviruses are present worldwide (Zimmer *et al*., 2002) and are of major concern for both domestic and wild ruminants. Four species of pestiviruses are officially accepted by the International Committee on Taxonomy of Viruses (ICTV): (i) Bovine Viral Diarrhea Virus 1 (BVDV-1); (ii) Bovine Viral Diarrhea Virus 2 (BVDV-2); (iii) Border Disease Virus (BDV) and (iv) Classical Swine Fever Virus (CSFV). Three distinct genotypes isolated from giraffes (giraffe-1 strain), deer (reindeer-1 strain) (Avalos-Ramirez *et al*., 2001) (Becher *et al*., 2003) or fetal calves (Hobi strain) (Schirrmeier *et al*., 2004) have been proposed as new species. Clinical entities were first described in the 1950s (Richards, 1956). Nowadays, pestivirus pathogenesis is well described among domestic ruminants. The consequences of infections vary depending on the reproductive status of infected animals. Most infections in adults or immunocompetent animals are asymptomatic, or may cause mild diarrhea: the animal develops short-lived viremia, seroconverts and eliminates the infection within 10-15 days. On the other hand, the infection of pregnant females may result in abortion, congenital abnormalities or the birth of persistently infected animals (PI) if the infection occurs during the second third of gestation (Brownlie *et al*., 1995). Descriptions of PI animals often include neurologic signs, abnormal body conformation with a poor growth rate and viability, and abnormally-developed hairy fleeces. The presence of PI animals is of major concern due to transmission of the infection. Besides, the immunosuppressive effects of acute infection might increase the severity of other opportunistic infections in immunologically competent animals (Letellier et al., 2003).

Experimental infections have played an important role in disease research (Sun, 2011). They have clarified pathogenesis, epidemiology and immunology associated with pestivirus infections. Numerous experimentations (more than 150) have been carried out by different scientific teams in various countries throughout the world. These experiments were guided by three main objectives. Studies have mainly focused on (i) viral pathogenesis, (ii) determining vaccine effects and (iii) testing diagnostic laboratory methods. However, in most cases, the experimental procedures followed by scientists differ from study to study, even when objectives are similar. Studies have not been standardized and it is thus difficult to compare results. There is no official reference or guideline concerning experiment design, whether for small mammals or ruminants. To the authors' knowledge, there are neither reference textbooks nor review papers to this end.

The main goal of this review is therefore to compare the methodologies used in experimental studies of pestivirus infection in wild and domestic ruminants through a quantitative assessment in order to propose experiment guidelines for the objectives sought. These guidelines will (i) harmonize experimentation practices, (ii) provide a framework for future studies and (iii) improve inter-study comparisons.

2- MATERIAL AND METHODS

2.1. Selection of publications in peer-reviewed journals

Databases available on the Internet (Pubmed, ISI Web of knowledge, Scopus, Science Direct, Web of Science, Current Contents®) were consulted, in addition to archives of the Anses Laboratory of Sophia-Antipolis for articles published before 1995. Keywords used were [experimental infection], [experimentation], [experimental] or [animal experimentation] combined with [pestivirus], [ruminant pestivirus] or [pestiv*]. Searches performed are shown in Table I depending on the database consulted and the keywords used. They were mostly carried out from February 2010 to November 2010, and were updated until July 2011.

To be included in this study, publications had to comply with the following parameters: (i) English was the only language accepted, (ii) only experimental infections conducted with BVDV or BDV were considered (infections using CSFV were excluded), (iii) experimental infections could be applied to any animal species, (iv) infections had to involve live animals (infections performed only in cell cultures were rejected), (v) reports of "natural" infection were not included: only clinical cases reported following experimental infections were included, (vi) the full text had to be available, and (vii) no date limits were imposed.

Figure 1 represents the publication short listing process.

2.2. Quantitative assessment

Given the diversity of pestivirus strains used for inoculation, of ruminant species used in experiments and of methods used for experiment follow-up, we used a quantitative assessment to standardize comparison of the 157 publications selected. This assessment was based on a list of criteria permitting an objective evaluation of the materials and methods used by scientists. We first established a list of all the critical points to be checked for a good animal experimentation monitoring plan. Then we submitted the list to seven international experts for confirmation and approval. These experts were chosen on the basis of three points: (i) they had to be category C persons responsible for directing animal experiments, qualified by the Federation of European Laboratory Animal Science Associations (FELASA) (Felasa, 1995); (ii) they had to be experienced in carrying out animal experimentation (at least three experimental trials each) and (iii) the experiments performed could be with pestivirus, but other pathogens were also required to have a larger overview of experiment requirements.

Once the list was approved by all seven experts, we attributed a score for each criterion. A binary score was attributed (presence: 1; absence: 0) for most criteria, the exceptions being sample size, clinical monitoring and temperature measurements, for which a numerical score from 0 to 1 was attributed. The list of criteria and the scoring system used is shown in Table II. To limit risks of subjectivity or bias, it was decided to give the same weight to all criteria.

The 157 selected publications were then encoded in an Excel® database, taking into account the country (determined by the address of the majority of authors), date of publication, animal species concerned and the strain inoculated. All criteria were completed for each publication. Six experimentations were the subject of more than one publication, but were encoded in the database only once.

2.3. Statistical analysis

Statistical analysis was performed for research concerning pathogenesis studies and vaccine studies (see paragraph 3.2.). A linear regression analysis was used to check the relationship between the score and the year of publication (Statacorp, 2007). A Wilcoxon rank sum test (Dagnelie, 1998) was used to compare the mean scores for each criterion and groups of criteria for pathogenesis and vaccine studies. This test was chosen because scores were quantitative, neither paired nor normally distributed and, furthermore, variances were unequal. A Spearman rank correlation test (r_s in the text) was chosen to compare use of an ethical commission, sample size, clinical monitoring and hematology analysis scores for pathogenesis and vaccine studies. The limit of statistical significance for the tests was defined as $P \leq 0.05$.

3- DESCRIPTIVE RESULTS OF SELECTED PUBLICATIONS

3.1. Number of publications selected and objective pursued

A total of 157 publications were included in this review (Table III). We divided them up according to their objective. Three main objectives were identified. Most publications (n=134) focused on viral pathogenesis. Several (n=18) investigated the effects of vaccines, and five addressed laboratory methods. Out of the 134 papers addressing viral pathogenesis, there were 127 different experiments. Six of these were covered by two to three published papers but were considered only once. Studies on diagnostic methods had to be excluded from statistical analysis because of their small number (n=5), and will instead be presented descriptively.

3.2. Species, time and geographic distribution of studies

3.2.1. Species concerned

Most studies were carried out on cattle (50.9%) or sheep (31.2%). Some were designed to compare pestivirus pathogenesis between species, so involved more than one species (n=4; 2.6% of studies). Three surveys used pigs and seven used goats. Only a few studies have been carried out on wild ruminants (n=11; 7.1%). These concerned North American wild ruminant species (mule deer – *Odocoileus hemionus;* white-tailed deer - *Odocoileus virginianus;* or elk – *Cervus elaphus*) or Pyrenean chamois (*Rupicapra pyrenaica*). In these cases, the objectives were mainly to reproduce the clinical course of pestiviral infection in wild species in order to determine whether or not they could

serve as a reservoir for farm animals. To investigate the susceptibility of deer and the clinical course of BVDV infection, for example, four mule deer and one white-tailed deer were infected by a BVDV strain (Van Campen *et al*., 1997). Ridpath and coauthors (2007) used two BVDV strains previously isolated from white-tailed deer (Ridpath *et al*., 2006) to infect eight white-tailed deer fawns. Nine pregnant white-tailed deer were infected by BVDV to determine whether PI fawns could be born. Histopathologic and immunohistochemical lesions associated with BVDV persistent infection in two white-tailed deer fawns were then studied by Duncan *et al* (2008). Two groups of five elk were inoculated with BVDV-1 or BVDV-2 strains respectively and mixed with control cattle to investigate the clinical presentation of the infection in elk and to determine whether they can act as a reservoir for cattle.

3.2.2. Time distribution

The disease was first described as being associated with pestiviruses in ruminants in 1956 by Richards *et al*. The first experimental infections were carried out in 1969 using tissue homogenate from diseased animals. Figure 2 illustrates the evolution of the distribution of experimentations involved in the present study per year depending on the objective pursued. Ruminant pestivirus pathogenesis has been studied since 1969. The first published studies on vaccination protocol and effects appeared in the eighties. Except one study published in 1978, all the surveys designed to study diagnostic methods were carried out between 1997 and 2006 (Figure 2).

3.2.3. Geographic distribution

Finally, most studies (60.5%) were conducted in Europe, especially in the United Kingdom (n=39) or Germany (n=12). Several took place in the United States (28.9%), but only 16 were performed in other parts of the world.

3.3. Studies of reproductive status

A total of 19 surveys were carried out in pregnant females in order to investigate the consequences of pestivirus during pregnancy and viral pathogenesis in PI animals. Three surveys were designed to investigate semen production and infection after inoculation.

3.4. Studies aimed to assess diagnostic methods

Most studies investigating a diagnostic method used samples from previous animal experimentations (and were not taken into account twice in this survey) or from naturally infected animals. There are very few (five) experimentations with this single objective. The first one, conducted in 1978, aimed to detect the viral antigen of pestiviruses in the organs of naturally and experimentally infected sheep using a direct fluorescent technique (Terpstra, 1978). This method was tested because it was proved to detect non cytopathic strains of BDV. Antigens were detected in many organs or affected tissues. For the first time, the authors showed viral dissemination in the respiratory, digestive and urogenital tracts

as well as in skin. These conclusions were of great importance because they advanced knowledge of how the virus spread in the organism (Terpstra, 1978).

Sandvik *et al*. (1997) studied an enzyme linked immunosorbent assay (ELISA) to detect viral antigens in blood leukocytes of animals with acute infection. The test was developed with samples from PI animals but the objective was to evaluate its presumed diagnostic capacity in transient infected animals. The authors obtained lower optical density values in transient infected animals than in PI animals. Viral isolations carried out at the same time showed that the viremia was short-lived and with low virus titers.

In 2004, a study was designed to compare the sensitivity of an animal test (experimental infection of six calves) and a tissue culture test for the detection of BVDV-1 strains in a contaminated BHV-1 vaccine. No significant difference in susceptibility was observed between the *in vitro* and *in vivo* tests (Antonis *et al*., 2004).

An antigen ELISA and a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) were compared with the viral isolation test for the diagnosis of PI animals with maternal antibodies. Calves were tested at birth then at seven days of life by antigen ELISA, RT-PCR, viral isolation and virus neutralization tests (to ensure the presence of maternal antibodies). At birth, all 25 PI calves tested positive by viral isolation, 24 by antigen ELISA and 14 by RT-PCR. On day seven, only four out of 25 tested positive by viral isolation, 10 by antigen ELISA and 19 by RT-PCR. Therefore, the authors highly recommended the use of RT-PCR for calves under 3 months of age (Zimmer *et al*., 2004).

In the fifth study on evaluating a diagnostic method, an immunochromatographic test was developed using anti-NS3 monoclonal antibodies for a rapid detection of BVDV. Animal experimentation was only used to produce samples from acute infected animals and to complete the PI samples obtained from the field (Kamayema *et al*., 2006).

Therefore, for studies designed to evaluate a diagnostic method, it is difficult to propose an experimental model, as these methods may be very specific. Hence, the experiment design has to be guided by the method. Many diagnostic methods were evaluated using samples from previous experimentations or *in vitro* viral cultures. To limit experimentations, samples collected during previous experimentations should be used as a priority or should be combined with other objectives.

4. ANALYTIC RESULTS AND DISCUSSION

4.1. Limits of quantitative assessment

It is difficult to produce a score to evaluate the general procedures used by scientists during their experiments. Indeed, as mentioned above, the list of criteria was established by the authors and approved by international experts involved in animal experimentation. Even if it was done as objectively as possible, it is important to bear in mind that this score does not constitute a grade

designed to rank experimentations depending on their score but to objectively and quickly compare procedures used.

The distribution of publications depending on their score is shown in Figure 3. The distribution mode is situated between the scores of 11 and 14. In the oldest publications, most material and methods were poorly described or simply not described at all. In some cases, the methods used were only briefly mentioned in the results. For example, the clinical monitoring performed by Barlow *et al.* in 1983 was only mentioned in the results section. Although we tried to collect all the information available in all parts of the publication, there was a risk of underestimating the score associated with these papers. Moreover, some of the oldest publications referred to other articles for their material and methods. Unfortunately, three of these articles were unavailable to the present authors and the publications had to be excluded from the study (e.g. Vantsis *et al*., 1976).

The quantitative assessment used in this study is based on a list of criteria established in cooperation with seven international experts involved in animal experimentation. This list is thus a standardized list applied to publications classified according to any of the three main objectives selected. However, some studies have a specific objective, such as the study of interferon response in cattle (Charleston *et al*., 2001), or the study of morphologic effects of pestiviruses in lambs (Terlicki *et al*., 1973). For those studies, many criteria had a score of 0 whereas the methodology used was appropriate for the objective in question. It could have been useful to assess the relevance of publications by comparing their score with the number of citations reported (for instance in PubMed central). Unfortunately, we could not perform such a comparison for lack of time.

The database generated could have been better exploited through more probing statistical analysis, but we preferred to limit the analysis as presented so as not to overestimate data.

4.2. Evolution of scores over time for pathogenesis and vaccine studies

Since the appearance in 1969 of experimentation with pestiviruses, there has been a significant increase in scores for both pathogenesis (R^2= 0.54, P<0.001) and vaccine studies (R^2=0,24, P=0,04) (Figure 4). The lowest score (score = 5) was found in 1973 and the highest (score = 27.66) in 2011. This evolution of scores was expected. Indeed, the list of criteria was drawn up in 2011, thus taking into account all current methodologies and knowledge. In the paragraphs below, we present and discuss the main evolutions of experimental procedures driving this development in both pathogenesis and vaccine studies.

Animal welfare

The first study presented to an ethical commission was published in 1999. Table IV shows the percentage of studies presented to an ethical commission between 1999 and 2011. This percentage is increasing over time, but does not yet concern all studies (r_s = 0.68, P = 0.01). The first directive of the European Union (EU) on the use of animals for scientific purposes (Directive 86/609/EEC) was

published in the Official Journal in 1986. On 22 September 2010, the EU adopted Directive 2010/63/EU. This directive updates and replaces the 1986 directive, considering changes in methods. It is aimed to consolidate legislation, improve animal welfare and to anchor the principle of the Three Rs—to Replace, Reduce and Refine the use of animals—in EU legislation (European Commission Environment, 2011). Both the Council of Europe (Convention ETS 123, Article 26) and the European Union (Council Directive 86/609/EEC, Article 14) require an appropriate education and training of all those engaged in the use of live vertebrate animals for scientific purposes (FELASA, 1995). These requirements were drawn up by the Federation of the European Laboratory Animal Society Association (FELASA). Four categories of qualifications exist: Category A - persons taking care of animals; Category B - persons carrying out animal experiments; Category C - persons responsible for directing animal experiments; Category D - laboratory animal science specialists. Training programs mainly focus on animal behavior, physiology or welfare and mostly on the management of small mammals such as rabbits or mice.

Objectives and experiment preparation

Defining the objective is the first requisite prior to designing a scientific study. However, as studies included in the assessment were published from 1969 on, we included definition of the objective in the criteria. In all studies, the objective is clearly defined and formulated in the text.

For experiment preparation, we focused on the preparation of animals prior to inoculation. We looked, for example, at sample size, presence or not of a control group, pre-experiment serologic and virologic status. The mean score of sample size equals 0.81 (Standard Deviation, S.D.=0.29) for pathogenesis studies and 0.89 (S.D.=0.20) for vaccine studies. This means that the mean sample size lies between six and 10 animals per experiment. The sample size has been decreasing significantly since 1969 for pathogenesis studies only (mean score by year: $r_s = -0.53$; $P = 0.0004$). Statistics were used for the first time in 1985, but really came into their own from 1999 on (23 out of 50 studies published between 1999 and 2011 included statistical analysis). A control group was used in 72% (S.D.=0.45) of pathogenesis studies and 83% (S.D.=0.38) of vaccine studies.

The sample is not randomly chosen as scores reach only 0.01 (S.D.=0.09) for pathogenesis studies and zero in vaccine studies (mean=0.00 ; S.D.=0.000). Serologic status was analyzed prior to the experiment in 81 % and 83 % of pathogenesis and vaccine studies respectively. A prior virologic status analysis was performed significantly more in vaccine studies (83% of surveys) than in pathogenesis studies (57%) (Wilcoxon signed rank test, $p<0.005$).

Virus inocula

Because of the wide variety of means available depending on the period of experimentation, we did not grade the inoculum source: a score of one was given if the source was cited and zero if not. Indeed, it is interesting to note that the viral inocula have changed over time. Between 1969 and 1973,

scientists used tissue homogenate of diseased animals as inocula. 1974 saw the first study using a virus grown in cell culture. Since 1980, viral cultures have been the main inoculate employed. Some published experiments used other sources of inocula, depending on their main objectives. For instance, some used PI animal or contaminated/inoculated semen to evaluate direct or genital transmission of the virus. The GENBANK reference number was noted in 41% (S.D.=0.49) and 44% (S.D.=0.51) of pathogenesis and vaccine studies respectively. Dosage scores reached 0.76 (S.D.=0.43) and 0.89 (S.D.=0.31) for pathogenesis and vaccine studies respectively and the inoculation route 0.78 for both types of study.

Clinical, laboratory and hematology monitoring

The clinical monitoring score has increased significantly over time for both pathogenesis studies (mean score by year: $r_s = 0.70$; $P < 0.0001$) and vaccine studies (mean score by year: $r_s = 0.58$; $P = 0.046$). The same trend is observed with hematology monitoring, again for both pathology studies (mean score by year: $r_s = 0.60$; $P < 0.0001$) and vaccine studies (mean score by year: $r_s = 0.61$; $P = 0.034$). Scientists have become aware of the need to monitor animals carefully.

Virology, serology and post mortem investigations

The main evolutions in the monitoring of experimental infections concern the evolution of methods used to investigate virology and serology. Figure 5 presents the development of the cumulative use of methods depending on the year of publication.

During the first years of experimentation (between 1969 and 1974), scientists only performed *post mortem* investigations, and did not study viremia or serology evolutions. The first viral isolations were carried out in 1974 (French *et al*., 1974) and were complemented by the use of antigen ELISA in 1992 and conventional RT-PCR in 1994 (Hewicker-Trautwein *et al*., 1994). The first description of PCR was published by Mullis and coauthors in 1986. Until 1994, not even one out of 64 studies used this methodology. Real time RT-PCR was used for the first time in 2007 (Ridpath *et al*., 2007). The ELISA test for the detection of pestivirus antigens is rarely used: the slope in Figure 5 is lower than the viral isolation slope. For serologic investigations, two main methods are used: the virus neutralization test and ELISA to detect antibodies. The first one has been used since 1974 and ELISA since 1989. For *post mortem* investigations, scientists first used necropsy and histology (from 1969 on), with immunohistochemistry from 1975. It is noteworthy that for each investigation, new methods have never completely replaced old ones. Virus isolation, for instance, has never been supplanted by other methods. Even today, it is the only one that can detect infectious particles. PCR and ELISA only detect genomes or external proteins of viruses. Likewise, the virus neutralization test is more specific than ELISA tests and accurately quantifies antibodies.

Moreover, it is important to note that the trends in score evolution (which increase over time) are the same, whatever the animal species considered (Figure 6).

4.3. Comparison between pathogenesis and vaccine studies

Figure 7 shows the mean score for each criterion for pathogenesis studies compared to vaccine studies. Significant differences exist (Wilcoxon signed rank test, $p<0.005$) for several criteria. The scores were significantly higher in vaccine studies for the following criteria: (i) random distribution into groups, (ii) prior analysis of virology status, (iii) vaccination protocol, (iv) score of clinical signs, (v) virus neutralization tests and (vi) use of statistics. The scores were significantly higher in pathogenesis studies for the following criteria: (i) inoculum source, (vii) necropsy and (iii) histologic examination.

4.4. Pre- and post-inoculation periods

Criteria were assembled into two logical groups. The first one was related to the experiment design and comprised the criteria relating to (i) animal welfare (ethical commission, acclimatization period, environment enrichment), (ii) the objective (if the objective was clearly identified and specified), (iii) experiment preparations (sample randomly chosen, sample size, presence of group control, random distribution in groups, prior analysis of serology negativity, prior analysis of virology negativity, and vaccination protocol), and (iv) the virus inocula (molecular characterization, purity checked, inoculum source, dosage known, route of inoculation known). The second group focused on experiment follow-up and included criteria relating to (i) clinical monitoring (interval of clinical monitoring, score of clinical signs, body weight measurement, temperature) (ii) laboratory monitoring (sampling of blood, excretion measurements), (iii) hematology analysis (white blood cell counts), (iv) virologic analysis (real time RT-PCR, RT-PCR and/or nested RT-PCR, virus isolation, Ag ELISA), (v) serologic analysis (virus neutralization test, ELISA or other techniques), (vi) *post mortem* evaluation (necropsy, sampling of organs, histologic examination, detection of viral antigen via immunologic method, virus isolation in organs) and finally (vii) the use of statistics. There is no significant difference in the time taken for the experiment either for pathogenesis studies or vaccine studies (Wilcoxon signed rank test, data not shown). We may thus conclude that scientists in charge of research lent the same importance to preparation as to monitoring the experiment.

4.5. Prospects

Pestiviruses are now well-known viruses. However, there are still many experimental infections with various pestivirus strains. Indeed, since the first study was published, there have been more than 150 publications on the subject and, in the last five years, articles have reported 29 new experimental infections. The number of studies is still increasing and raises many questions.

First of all, pestiviruses are RNA viruses, which mean they have high mutation rates. There are always new descriptions of novel strains and genotypes (Vilcek *et al*. 2010). The BVDV-2 genotype was first described in 1994 by Ridpath *et al.* for example, and was then shown to cause an acute form of BVDV named hemorrhagic syndrome. To investigate the pathogenesis associated with this new BVDV

species, 11 studies were published between 1999 and 2009. Furthermore, the recent interest in the role of wildlife in the epidemiology of diseases (Martin *et al*., 2011) has raised other questions and opened up new research areas. Indeed, in the United States, free-ranging Cervidae are in close contact with domestic cattle, and have been shown to be susceptible to pestivirus infections. Various surveys have thus been conducted in order to determine viremia, serologic response, and their possible role in the transmission of BVDV between domestic cattle and wild populations (Tessaro *et al*., 1999; Ridpath *et al*., 2007). In Europe, a recent Border disease outbreak has been described in Pyrenean chamois and has been associated with a high mortality rate in this species. In order to understand lesions, viral dynamics and humoral immune response in both non pregnant and pregnant chamois, two experimental infections were performed on this species with a BDV-4 strain previously isolated from a naturally infected chamois (Cabezon *et al*., 2011, Martin *et al*., submitted). The effects of this specific strain were also assessed in sheep (Cabezon *et al*., 2010a) and pigs (Cabezon *et al*., 2010b).

Nevertheless, many questions remain. Viral transmission has been investigated in different ways, including (i) semen inoculation (in three studies: Gardiner *et a*l., 1981; Meyling *et al*., 1988 and Niskanen *et al*. 2002), and (ii) study of direct transmission from PI animals to sensitive animals (in seven surveys: Carlsson *et al*., 1991; Mars *et al*., 1999; Uttenthal *et al*., 2005; Lamm *et al*., 2009: Broaddus *et al*., 2009 and Passler *et al*., 2009, 2010). Indirect transmission between PI and naïve animals via horn flies (*Haematobia irritans)* has not been shown to be an efficient means of BVDV transmission (Chamarro *et al*., 2011). However, interspecies transmission remains unclear and the sensitivity of one species to different strains still needs to be assessed.

The humoral immune response has been the main response studied. Cellular response (interferon evolution, cytokines) was only the subject of one article (Charleston *et al*., 2001).

5. TOWARDS AN EXPERIMENTAL MODEL AND GUIDELINES

In 2002, Thabti *et al*. proposed an experimental model of acute infection to study ovine pestiviruses. They compared three ovine pestivirus strains (AV strain, which was isolated during an acute form of Border Disease in Aveyron district, France and SN3G and Lot21, two strains isolated from two Tunisian batches of live sheep pox vaccines produced on Tunisian sheep kidney cells) (Thabti *et al*., 2002). To determine the inoculation route and dosage for their model, they first conducted experimental infections in lambs of various ages until they obtained "a significant panel of clinical, hematologic or virologic observations". They considered their final model "finished" as it enables researchers to "(i) reproduce, with the three strains tested, three major symptoms of the disease in immunologically competent animals: raised body temperature, leukopenia and specific seroconversion, and (ii) distinguish the virus strains on the basis of frequency, delay and duration of these symptoms in the inoculated groups of lambs" (Thabti *et al*., 2002).

Pestiviruses are single-stranded RNA viruses, with a genome 12.5 kb in length containing a single Open Reading Frame (ORF) (Meyers and Thiel, 1996). Although their genomic organization is simple, they are actually very complex viruses (Vilcek *et al*., 2010). This complexity is first reflected in their genetic diversity: four species are officially recognized but at least 20 subgroups have been described (11 subgroups for the BVDV-1 species—Vilcek *et al*., 2001; two subgroups for the BVDV-2 species—Vilcek *et al*., 2005; and seven subgroups for the BDV species—Giammaroli *et al*., 2011) and three genotypes have been proposed as novel species. Interactions with their hosts vary according to the moment of infection (persistent or transient infection) (Coria *et al*., 1978). Transmission is possible through reproductive or respiratory routes. For all of these reasons, it was difficult to design an experimental model geared to all possible cases. We therefore preferred to give guidelines specifically designed to monitor animals as well as possible and which may be adapted by researchers to their study objectives.

Seven criteria initially defined had a mean score lower than 0.1 for both objectives: (i) sample randomly chosen, (ii) environment enrichment, (iii) score of clinical signs, (iv) body weight measurement, (v) inocula purity check (vi) use of real time RT-PCR and (vii) use of ELISA for antigen detection. For the criterion "samples randomly chosen", authors often have difficulties in finding suitable animals, mainly due to health requisites (animals often have to be virologically and serologically negative). FELASA does not require enrichment of the environment. For mainly practical reasons, body weight measurements are not often taken, or not taken properly. However, such measurements may provide important information on the animal's clinical status.

Their relevance in the evaluation of an experimental infection is thus limited and can be excluded from the model. The correct monitoring of an animal experimentation with pestiviruses would have to take into account all the criteria proposed in this survey minus the seven abovementioned criteria. If all the parameters listed are taken into account, the animal's overall status should be well defined. However, there is a significant difference between the maximum theoretical score and the real score (36 to 26.6). This difference may be explained by the presence of criteria which are not used very often. Seven criteria have a mean value under 0.1 for both objectives. All of these criteria should be addressed to improve the value of experimental models.

6. CONCLUSION

This review used a quantitative assessment to compare reported experimental infections with ruminant pestiviruses. This original method, based on the opinion of seven international experts, allowed us to draw a picture of the methods used to monitor experimental infections and their evolution over time. Finally, we proposed experiment guidelines to ensure a comprehensive monitoring of animals in future animal experimental infections. Although these guidelines were developed for pestiviruses, they may be used for infections with other viruses or pathogens.

Acknowledgments

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7. LIST OF FIGURES AND TABLES

Table I. Results of database searches, specifying the search date, database consulted and keywords used

Legend: ®, Registered trademark; *, Truncated word

Table II. Criteria used and associated score

Table III. List of publications selected and attributed score.

Table IV. Number and percentage of studies with an ethical commission since 1999

Legend: Studies without an ethical commission (before 1999) are not shown in the table.

519 **Figure 1. Selection of available publications: number and limits used**

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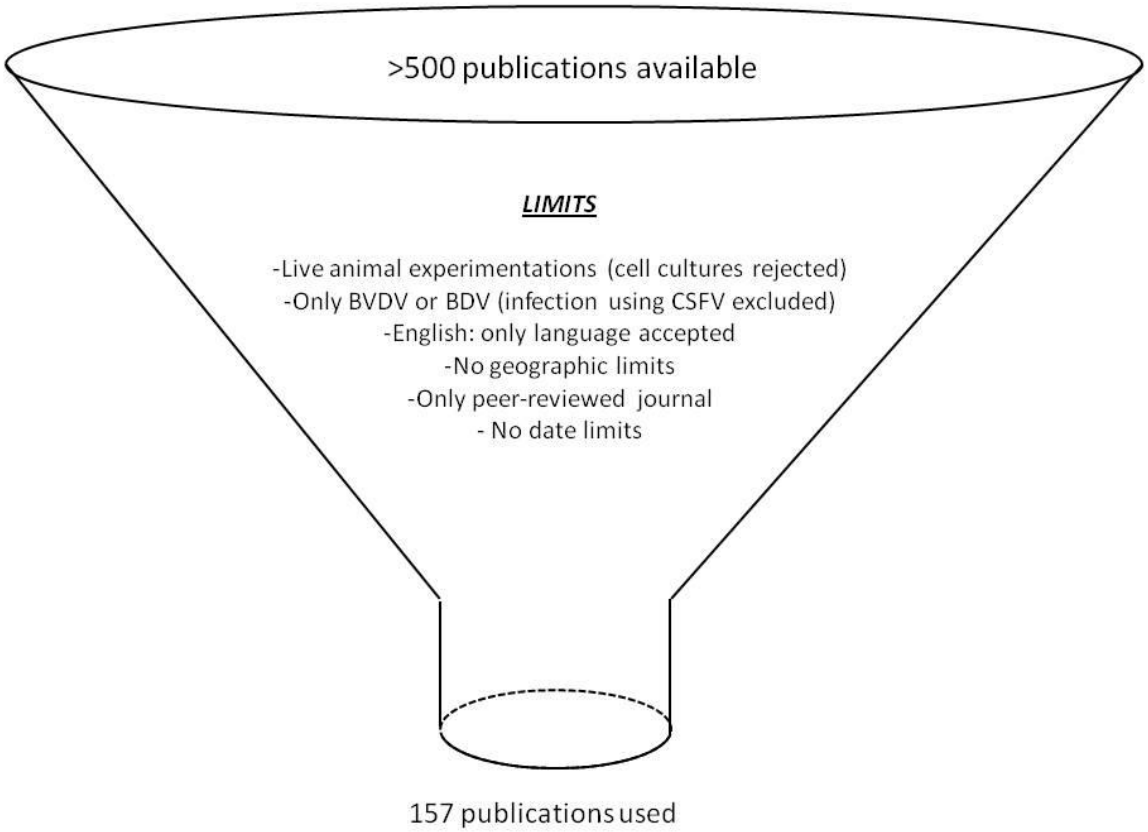

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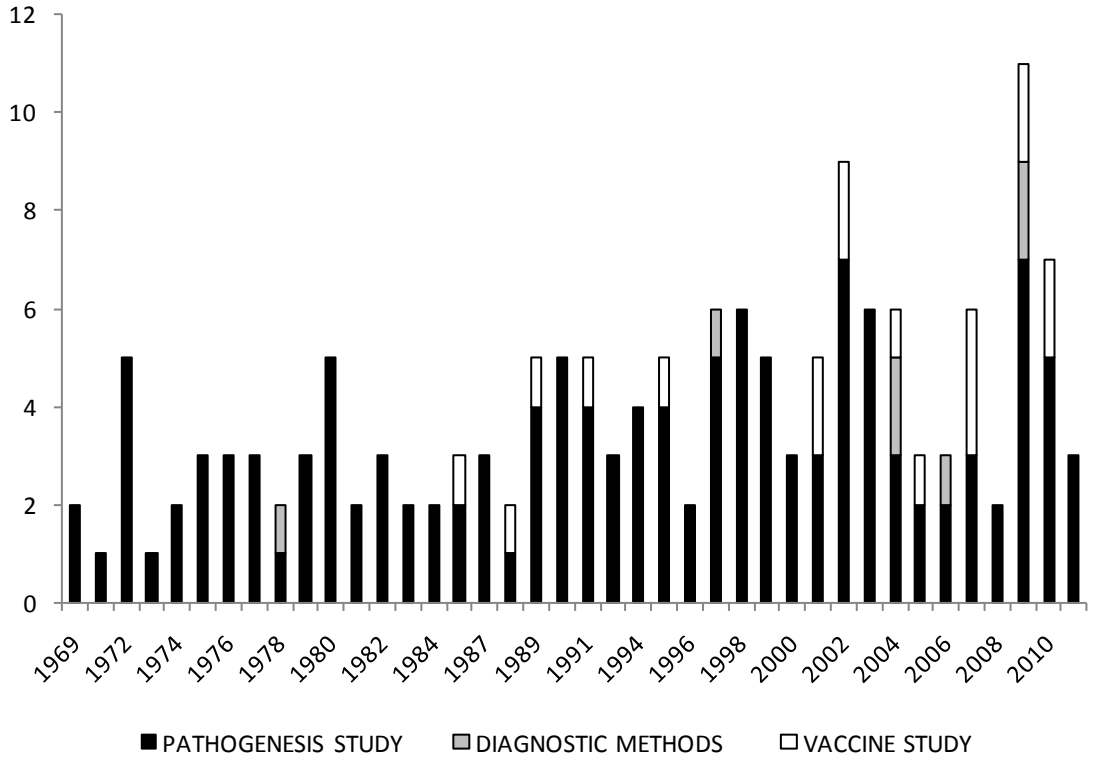
Figure 2. Annual distribution of published experimentations with ruminant pestivirus depending on the main objective (n = 157; X-axis: time in year; Y-axis: Frequency)



527 **Figure 3. Distribution of publications depending on their score (n = 157)**

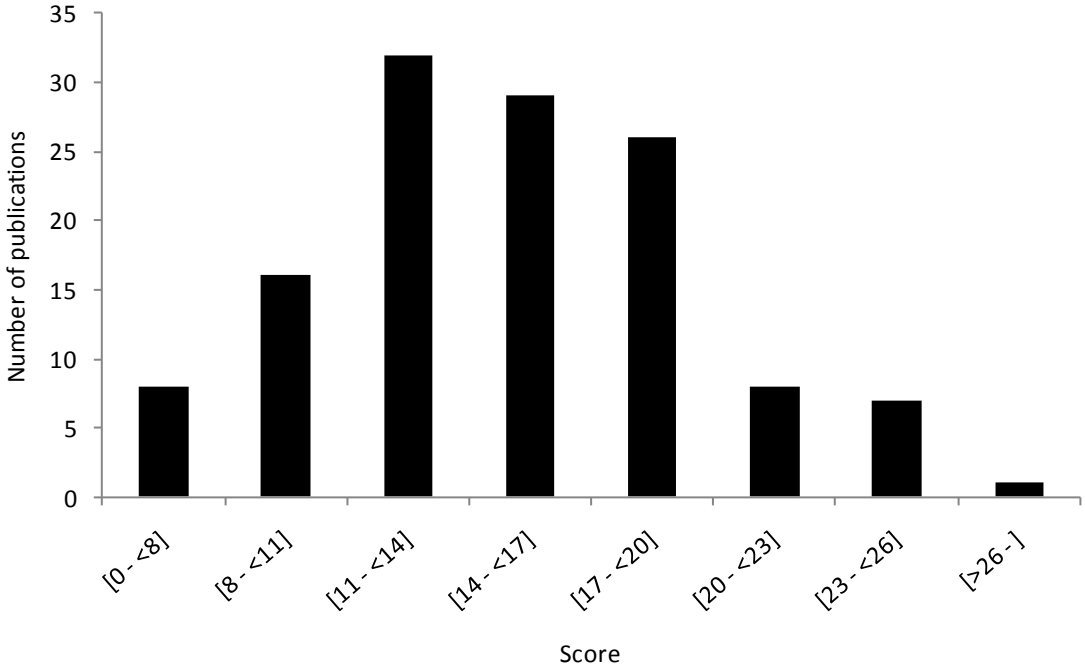


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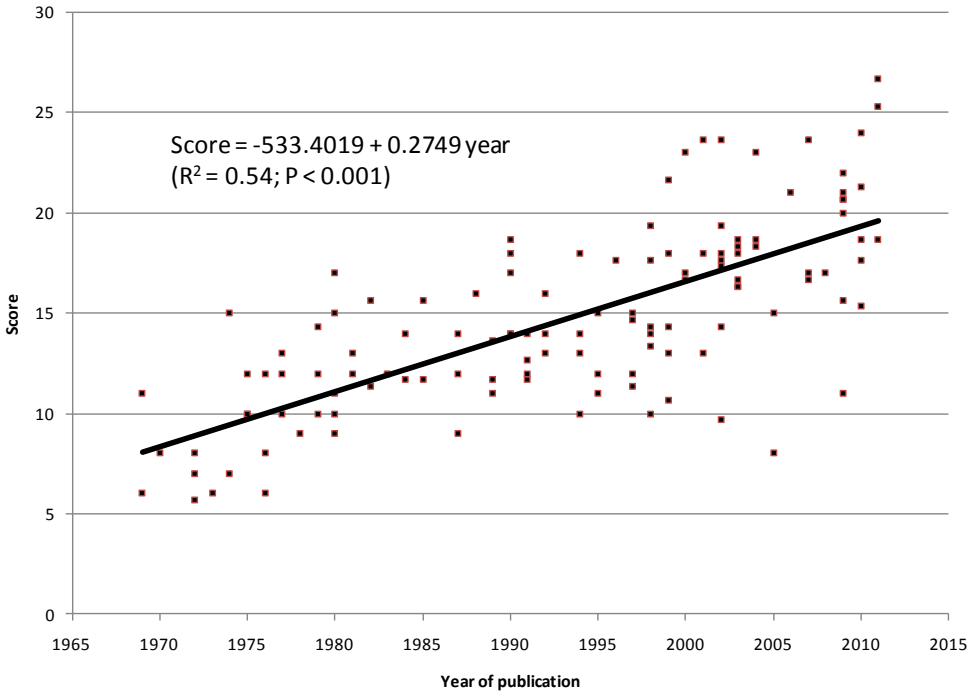
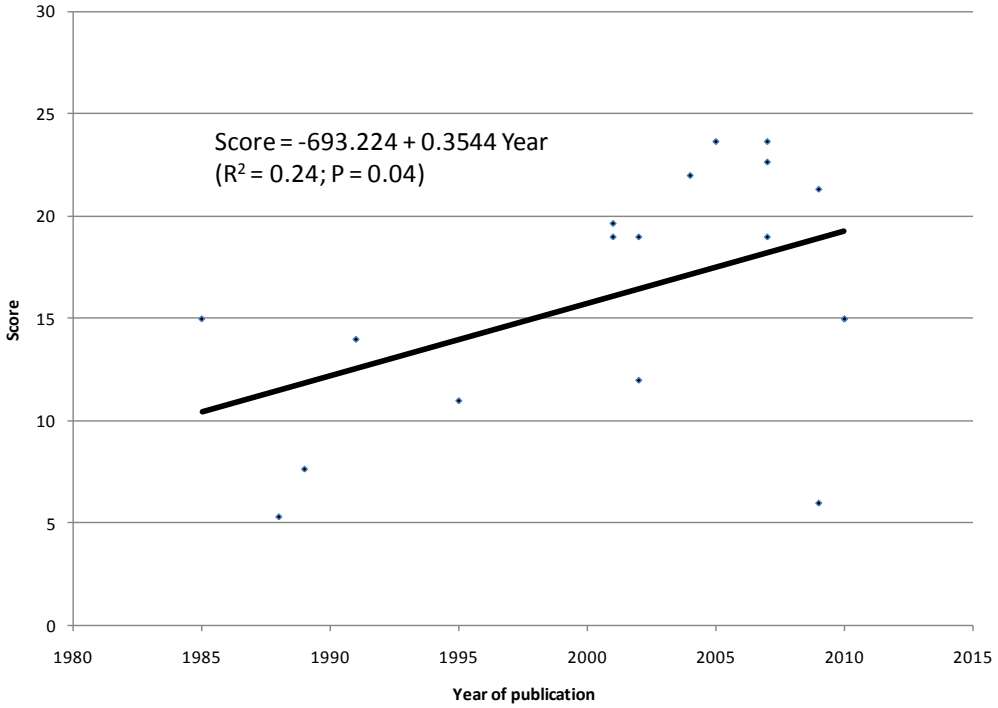
Figure 4. Evolution of scores over time for pathogenesis (n = 127) [A] and vaccine (n = 18) [B] studies

[A]

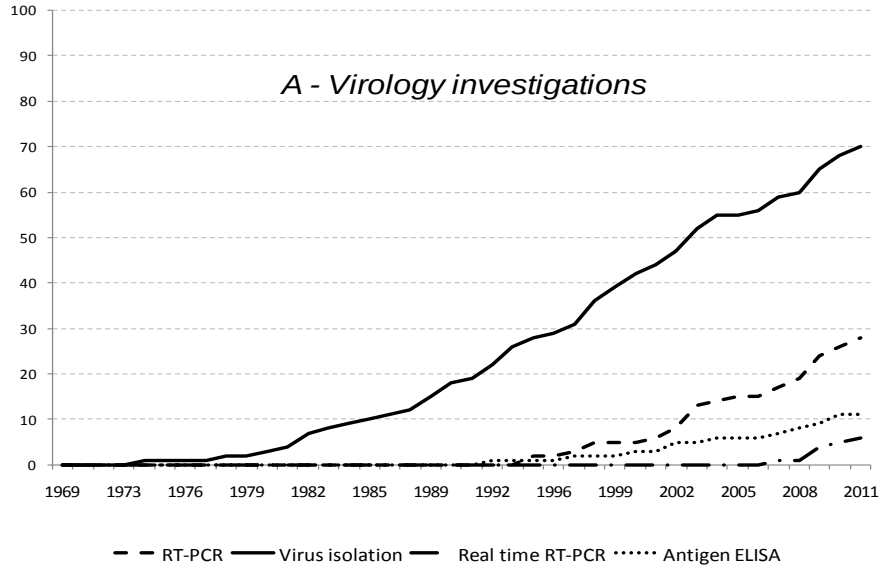


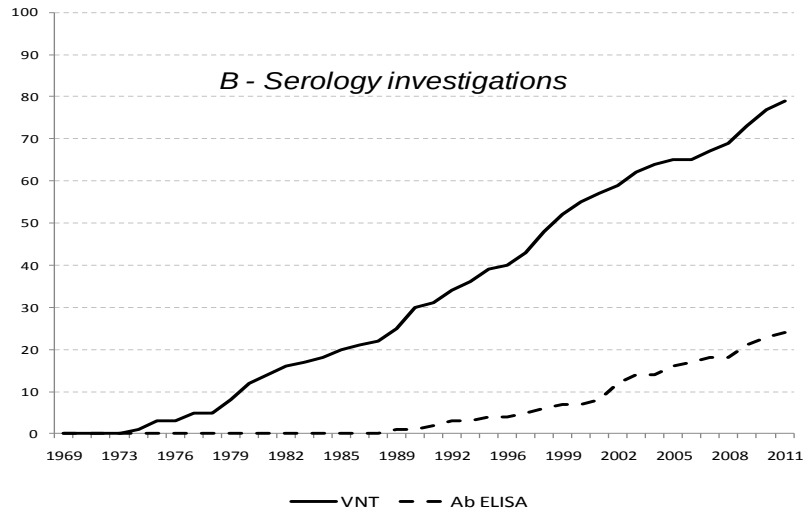
[B]



Legend: for [A], the linear regression equation for scores is the following: -693.2 + 0.354 year (R^2 = 0.24; P = 0.04); for [B], the linear regression equation for scores is the following: -533.4 + 0.275 year (R^2 = 0.54; P < 0.001).

Figure 5. Appearance of diagnostic methods over time (X-axis: time in year; Y-axis: cumulative frequency)





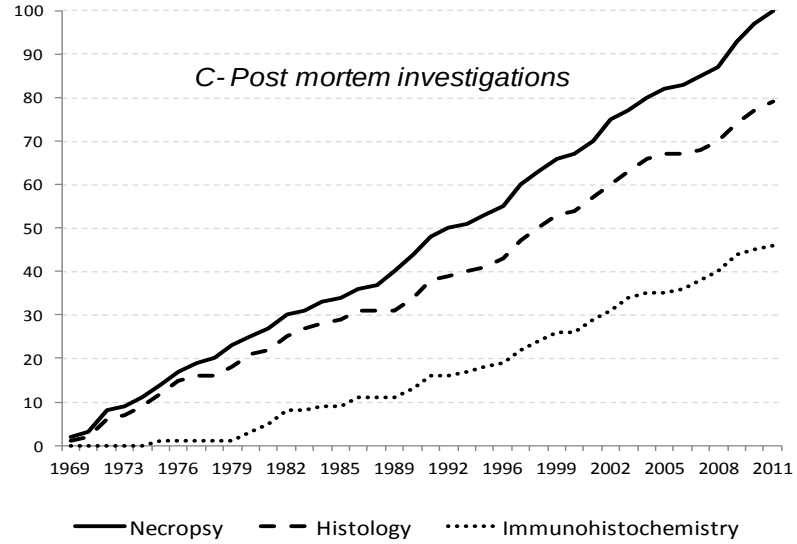


Figure 6. Evolution of score depending on animal species and year of publication

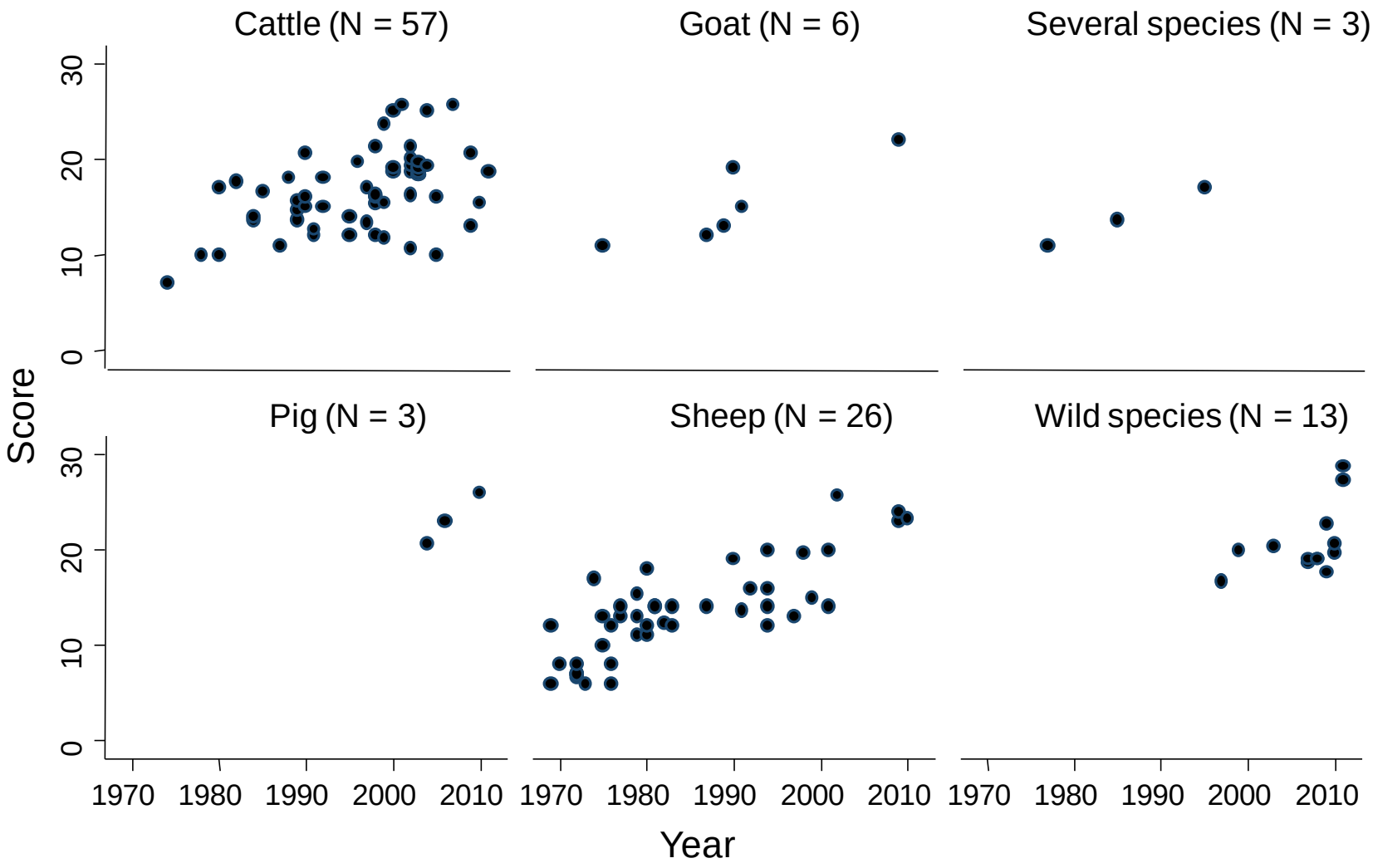
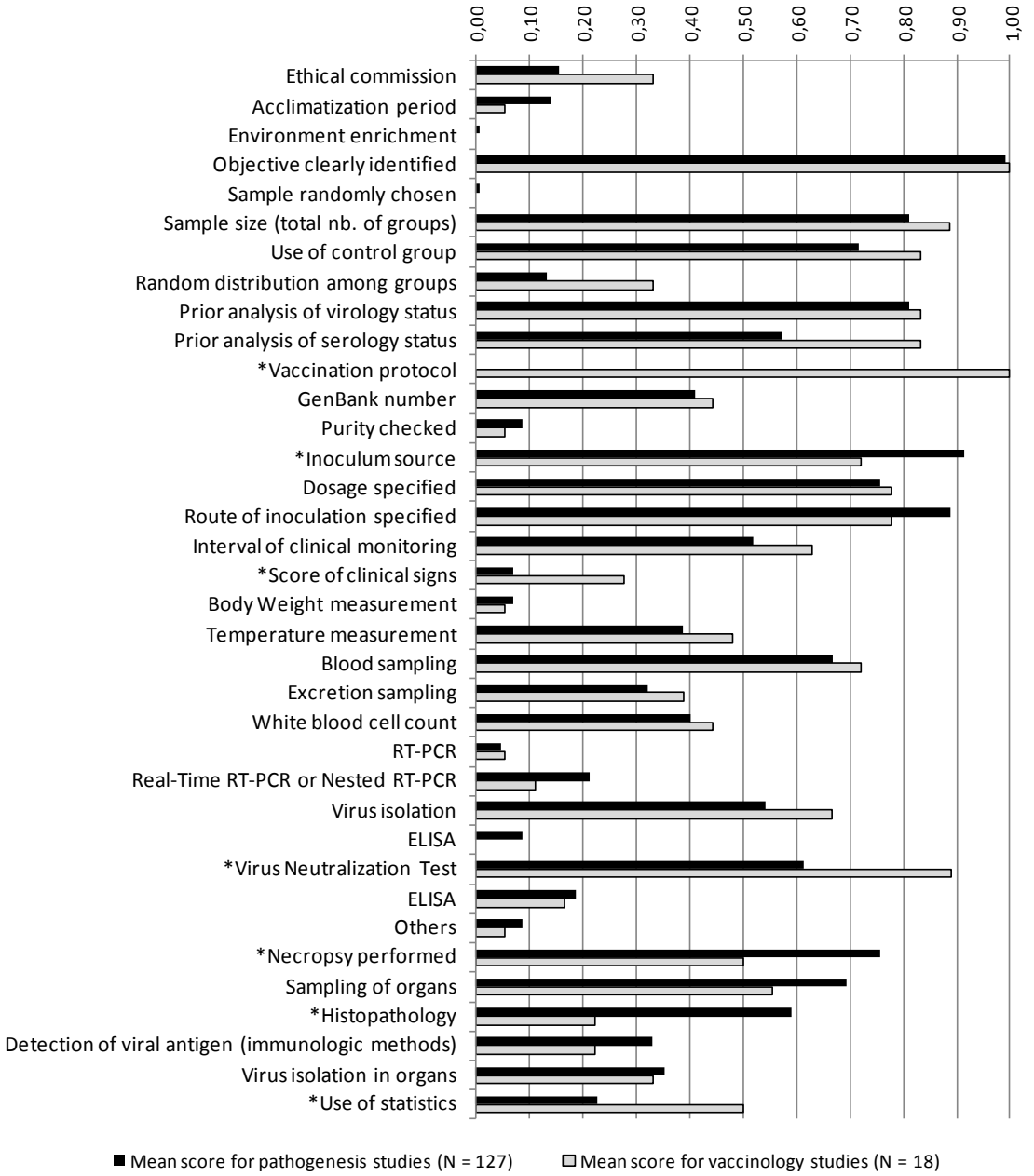


Figure 7. Comparison of mean score (X-axis) for each criterion between pathogenesis and vaccine studies



Legend: * significant difference between the two types of studies (Wilcocon rank sum test; P<0.05)

552 Table I: Results of database searches, specifying the search date, the database consulted and the
553 keywords used
554

Date of search	Database consulted	Keywords and Boolean operators	Results	Effective results#
28/07/2010	Pubmed	Experimental infection AND ruminant pestivirus	106	75
23/06/2011	Pubmed	Experimental infection AND pestiv*	35	19
29/07/2010	Pubmed	Animal experimentation AND pestivirus	2	0
02/08/2010	ISI Web of knowledge	Experimental infection (topic) AND ruminant Pestivirus (topic)	5	3
02/08/2010	ISI Web of knowledge	Experimental infection (topic) AND Pestivirus (topic) NOT swine (topic)	271	16
12/10/2010	Scopus	Experimental infection AND ruminant pestivirus	2	0
12/10/2010	Scopus	Experimental infection AND pestiv*	128	38
12/10/2010	Science direct	Experimental infection AND ruminant pestivirus	305	17
13/10/2010	Scopus	Animal experimentation AND pestivirus	1	0
13/10/2010	Scopus	Experimentation AND pestivirus	1	0
13/10/2010	Pubmed	Experimentation AND pestivirus	0	0
13/10/2010	Pubmed	Experimental AND pestivirus	214	73
20/10/2010	Laboratory archives	Experimentation AND pestivirus	50	15
25/10/2010	ISI Web of knowledge	Pestivirus (topic) AND Experimentation (topic)	5	0
25/10/2010	Web of science	Pestivirus (topic) AND Experimentation (topic)	0	0
25/10/2010	Current contents ®	Pestivirus (topic) AND Experimentation (topic)	0	0
25/10/2010	Web of science	Experimental infection (topic) AND ruminant pestivirus(topic)	1	0
25/10/2010	Current contents ®	Experimental infection (topic) AND ruminant pestivirus(topic)	0	0
25/10/2010	Web of science	Experimental infection (topic) AND pestiv* (topic)	50	2
25/10/2010	Current contents ®	Experimental infection (topic) AND pestiv* (topic)	41	22

555
556
557 Legend: ®, Registered mark; *, Truncated word; #, Effective results were publications selected for this
558 review according to inclusion criteria. In all, 250 publications were found but after elimination of
559 duplicates, 157 were finally selected.
560

561 **Table II. Criteria used and associated scores**

Topic	Criteria	Scoring
ANIMAL WELFARE	Ethical commission	0/1
	Acclimatization period	0/1
	Environment enrichment	0/1
OBJECTIVE	Objective clearly identified	0/1
EXPERIMENT PREPARATION	Sample randomly chosen	0/1
	Sample size (total nb. of groups)	0: from 1 to 2 animals 0.33: from 3 to 5 animals 0.66: from 6 to 10 animals 1: more than 10 animals
	Use of control group	0/1
	Random distribution among groups	0/1
	Prior analysis of virology status	0/1
	Prior analysis of serology status	0/1
VIRUS INOCULUM	GenBank number	0/1
	Inoculum source	0/1
	Purity checked	0/1
	Dosage specified	0/1
	Route of inoculation specified	0/1
VACCINATION/ CHALLENGE	Vaccination protocol described	0/1
	Challenge protocol described	0/1
CLINICAL MONITORING	Frequency of clinical monitoring	0: no clinical monitoring 0.33: weekly 0.66: two to three times per week 1: daily
	Score of clinical signs	0/1
	Temperature measurement	0: no temperature measurement 0.33: weekly 0.66: two to three times per week 1: daily
	Body Weight measurement	0/1
SAMPLING	Blood sampling	0/1
	Excretion sampling	0/1
SEROLOGIC ANALYSIS	Serology analysis	0/1
	Virus Neutralization Test	0/1
	ELISA	0/1
HEMATOLOGY ANALYSIS	White blood cell count	0/1
VIROLOGIC ANALYSIS	Viremia analysis	0/1
	Real-Time RT-PCR or Nested RT-PCR	0/1
	RT-PCR	0/1
	Virus isolation	0/1
	Detection of viral antigen (immunologic methods)	0/1
NECROPSY	Necropsy performed	0/1
	Sampling of organs	0/1
	Histopathology	0/1
	Virus isolation in organs	0/1
	RT-PCR in organs	0/1
DATA ANALYSIS	Use of statistics	0/1

562 **Table III. List of publications selected and attributed score.**

FIRST AUTHOR	YEAR	FIRST PAGE	JOURNAL	TITLE	SCORE
Acland	1972	70	Aust.Vet.J.	Infection of sheep with a mucosal disease virus	5.66
Anderson	1987	499	Am.J.Vet.Res.	Experimentally induced ovine border disease: Extensive hypomyelination with minimal viral antigen in neonatal spinal cord	13
Antonis	2004	131	Vet.Microbiol.	Comparison of the sensitivity of in vitro and in vivo tests for detection of the presence of a bovine viral diarrhoea virus type 1 strain	17
Archambault	2000	215	Vet.Res.	Clinical response and immunomodulation following experimental challenge of calves with type 2 noncytopathogenic bovine viral diarrhea virus	24
Barlow	1969	397	J.Comp.Pathol.	Experiments in Border Disease. I. Transmission, Pathology and some serological aspects of the experimental disease	11
Barlow	1970	635	J.Comp.Pathol.	Experiments in Border Disease. II. Some aspects of the disease in the fœtus	7
Barlow	1972	151	J.Comp.Pathol.	Experiments in Border Disease. IV Pathological changes in ewes	7
Barlow	1975	291	J.Comp.Pathol.	Experiments in border disease. VII. The disease in goats	10
Barlow	1979	334	Vet.Rec.	The definition of border disease: Problems for the diagnostician	10
Barlow	1983	451	J.Comp.Pathol.	The pathology of a spontaneous and experimental mucosal disease-like syndrome in sheep recovered from clinical border disease	11
Baule	2001	146	J.Clin.Microbiol.	Pathogenesis of Primary Respiratory Disease Induced by Isolates from a New Genetic Cluster of Bovine Viral Diarrhea Virus Type I	24.66
Blanchard	2010	128	J.Vet.Diagn.Invest.	An outbreak of late-term abortions, premature births, and congenital deformities associated with a Bovine viral diarrhea virus 1 subtype b that induces thrombocytopenia	14.33
Bolin	1985	573	Am.J.Vet.Res.	Severe clinical disease induced in cattle persistently infected with noncytopathic bovine viral diarrhea virus by superinfection with cytopathic bovine viral diarrhea virus	15.66
Bolin	1985	2467	Am.J.Vet.Res.	Response of cattle persistently infected with noncytopathic bovine viral diarrhea virus to vaccination for bovine viral diarrhea and to subsequent challenge exposure with cytopathic bovine viral diarrhea virus	16
Bolin	1988	40	Am.J.Vet.Res.	Viral and viral protein specificity of antibodies induced in cow persistently infected with noncytopathic bovine viral diarrhea virus after vaccination with cytopathic bovine viral diarrhea virus	6.33
Bolin	1989	817	Am.J.Vet.Res.	Specificity of neutralizing and precipitating antibodies induced in healthy calves by monovalent modified-live bovine viral diarrhea virus vaccines	9.66
Bolin	1992	2157	Am.J.Vet.Res.	Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves	17
Broaddus	2009	45	Vet.Pathol.	Bovine Viral Diarrhea Virus Abortion in Goats Housed with Persistently Infected Cattle	21
Brock	2001	354	Vet.Therap.	Experimental fetal challenge using type II bovine viral diarrhea virus in cattle vaccinated with modified-live virus vaccine	21.66

Brock	2007	88	Vet.Therap.	Onset of protection from experimental infection with type 2 bovine viral diarrhea virus following vaccination with a modified-live vaccine	23.66
Brownlie	1980	371	Vet.Immunol.Immunopathol.	Experimental infection of calves with two strains of bovine virus diarrhoea virus: certain immunological reactions	9
Brownlie	1984	535	Vet.Rec.	Experimental production of fatal mucosal disease in cattle	12.66
Brownlie	1989	307	Res.Vet.Sci.	Experimental infection of cattle in early pregnancy with a cytopathic strain of bovine virus diarrhoea virus	13.66
Brownlie	1995	58	Vet. Rec.	Protection of the bovine fetus from bovine viral diarrhoea virus by means of a new inactivated vaccine	13
Brownlie	1998	141	Clin.Diagn.Virol.	Maternal recognition of foetal infection with bovine virus diarrhoea virus (BVDV)—the bovine pestivirus	11
Byers	2010	591	Vaccine	Evaluation of a commercial bovine viral diarrhea virus vaccine in nonpregnant female alpacas (Vicugna pacos)	16.99
Cabezón	2010	360	J.Vet.Diagn.Invest.	Experimental infection of pigs with Border disease virus isolated from Pyrenean chamois (Rupicapra pyrenaica)	25
Cabezón	2010	619	Vet.Rec.	Experimental infection of lambs with Border disease virus isolated from a Pyrenean chamois	22.32
Cabezón	2011	In press	J.Virol.Methods	Experimental infection with chamois Border Disease Virus causes long-lasting viraemia and disease in Pyrenean chamois (Rupicapra pyrenaica)	27.66
Caffrey	1997	245	Res.Vet.Sci.	Morphometric analysis of growth retardation in fetal lambs following experimental infection of pregnant ewes with Border Disease virus	12
Carlsson	1991	145	Vet.Rec.	Border disease in sheep caused by transmission of virus from cattle persistently infected with bovine virus diarrhoea virus	14
Carlsson	1991	577	Vaccine	Protective effect of an ISCOM bovine virus diarrhoea virus (BVDV) vaccine against an experimental BVDV infection in vaccinated and non-vaccinated pregnant ewes	16
Castrucci	1990	41	Comp.Immun.Microbiol.Infect.Dis.	A study of some pathogenic aspects of bovine viral diarrhea virus infection	15
Castrucci	1992	163	Comp.Immun.Microbiol.Infect.Dis.	An experimental contribution to the study of the pathogenesis of bovine viral diarrhea virus infection	14
Charleston	2002	923	J.Virol.	Alpha/Beta and Gamma Interferons Are Induced by Infection with Noncytopathic Bovine Viral Diarrhea Virus In Vivo	10.66
Collins	2009	289	Vet.Microbiol.	Infectivity of pestivirus following persistence of acute infection	13
Corapi	1989	3934	J.Virol.	Severe Thrombocytopenia in Young Calves Experimentally Infected with Noncytopathic Bovine Viral Diarrhea Virus	15.66
Dahle	1985	Brussels	Proc.EEC.Conf.Pestivirus	Interspecies transmission of pestiviruses: Experimental infections with bovine viral diarrhea virus in pigs and hog cholera virus in cattle	12.66
Derbyshire	1976	557	J.Comp.Pathol.	Experiments in border disease. IX. The pathogenesis of the skin lesion	6
Donis	1987	1549	Am.J.Vet.Res.	Molecular specificity of the antibody responses of cattle naturally and experimentally infected with	11

				cytopathic and noncytopathic bovine viral diarrhea virus biotypes	
Duncan	2008	289	J.Vet.Diagn.Invest.	Histopathologic and immunohistochemical findings in two white-tailed deer fawns persistently infected with Bovine viral diarrhea virus	18
Edwards	1995	181	Br.Vet.J.	Comparative studies of border disease and closely related virus infections in experimental pigs and sheep	16
Elvander	1998	251	Acta Vet.Scand.	An experimental study of a concurrent primary infection with bovine respiratory syncitial virus (BRSV) and Bovine viral diarrhoea virus (BVDV) in calves	15
Fairbanks	2004	1898	JAVMA	Evaluation of fetal protection against experimental infection with type 1 and type 2 bovine viral diarrhea virus after vaccination of the dam with a bivalent modified-live virus vaccine	24
Falcone	2003	577	Vet.Res.Comm.	Experimental infection of calves with Bovine Viral Diarrhoea Virus Type-2 (BVDV-2) isolated from a contaminated vaccine	17.33
Fernandez	2009	86	Rev.Arg.Microbiol.	Evaluation of experimental vaccines for bovine viral diarrhea in bovines, ovines and guinea pigs	7
Fray	1998	608	Vet.Rec.	Prolonged nasal shedding and viraemia of cytopathogenic bovine virus diarrhoea in experimental late-onset mucosal disease	1633
Fray	2002	281	Reprod.	Modulation of sex hormone secretion in cows by acute infection with bovine viral diarrhoea virus	21.33
French	1974	45	Aust.Vet.J.	Infection of pregnant ewes with mucosal disease virus of ovine origin	16
Fritzemeier	1995	285	Vet.Microbiol.	Experimentally induced "late-onset" mucosal disease - characterization of the cytopathogenic viruses isolated	13
Fritzemeier	1997	1335	Arch.Virol.	The development of early vs. late onset mucosal disease is a consequence of two different pathogenic mechanisms	12.33
Galav	2007	364	Res.Vet.Sci.	Pathogenicity of an indian isolate of bovine viral diarrhea virus 1b in experimentally infected calves	24.66
Ganheim	2003	183	J.Vet.Med. B	The acute phase response in calves experimentally infected with Bovine Viral Diarrhoea Virus and/or Mannheimia haemolytica	17
Ganheim	2005	380	J.Vet.Med. B	Changes in peripheral blood leucocyte counts and subpopulations after experimental infection with BVDV and/or Mannheimia haemolytica	18
Garcia-Perez	2009	331	J.Vet.Diagn.Invest.	Detection of Border disease virus in fetuses, stillbirths, and newborn lambs from natural and experimental infections	19
Garcia-Perez	2009	345	Res.Vet.Sci.	Clinical and laboratorial findings in pregnant ewes and their progeny infected with Border disease virus (BDV-4 genotype)	27
Gardiner	1972	29	J.Comp.Pathol.	Experiments in Border Disease. III. Some epidemiological considerations with reference to the experimental disease	6
Gardiner	1972	159	J.Comp.Pathol.	Experiments in border disease.V. Preliminary investigations on the nature of the agent	6
Gardiner	1980	469	J.Comp.Pathol.	Periartritis in experimental border disease of sheep III Immunopathological observations	10
Gardiner	1981	467	J.Comp.Pathol.	Vertical transmission of border disease infection	13
Gardiner	1983	463	J.Comp.Pathol.	Virology and immunolgy of a spontaneous and experimental mucosal disease-like syndrome in sheep recovered from clinical border disease	13

Gibbons	1974	357	Br.Vet.J.	Pathogenicity of the border disease agent for the bovine fœtus	6
Givens	2003	428	Am.J.Vet.Res.	Detection of bovine viral diarrhea virus in semen obtained after inoculation of seronegative postpubertal bulls	17.65
Givens	2009	975	Therio.	Safety and efficacy of vaccination of seronegative bulls with modified-live, cytopathic bovine viral diarrhea viruses	23.33
Gruber	1995	443	J.Vet.Med. B	Brain malformation in ovine fetuses associated with the cytopathogenic biotype of bovine viral-diarrhoea virus	15
Hadjisavvas	1975	237	Res.Vet.Sci.	The demonstration by interference tests of an infective agent in fetuses from ewes inoculated with border disease tissue	12
Hamers	2000	250	Vet.J.	Differences in experimental virulence of bovine viral diarrhoea viral strains isolated from hoemorrhagic syndromes	18
Harkness	1977	71	Vet.Rec.	Border disease of sheep: Isolation of the virus in tissue culture and experimental reproduction of the disease	12
Hashiguchi	1978	118	Nat.Inst.Anim.Hlth Quart.	Bovine Virus Diarrhea - Mucosal Disease. II. Isolation and characterization of a cytopathogenic virus and experimental production of the disease	9
Henningson	2009	1117	Am.J.Vet.Res.	Effect of the viral protein Npro on virulence of bovine viral diarrhea virus and induction of interferon type I in calves	19.66
Hewicker-Trautwein	1994	264	J.Vet.Med. B	Virological and pathological findings in sheep fetuses following experimental infection of pregnant ewes with cytopathic-bovine-virus diarrhoea virus	18
Hurtado	2009	189	Virol.J.	Detection and quantification of pestivirus in experimentally infected pregnant ewes and their progeny	20
Hussin	1994	201	Res.Vet.Sci.	Effects of experimental infection with border disease virus on lymphocyte subpopulations in the peripheral blood of lambs	13
Jewett	1990	1640	Am.J.Vet.Res.	Comparative pathogenicity of selected bovine viral diarrhea virus isolates in gnotobiotic lambs	18
Johnson	2010	66	Small Rum.Res.	Comparison of clinical, hematological, and virological findings in alpacas (Lama pacos) inoculated with bovine viral diarrhea virus isolates of alpaca or bovine origin	19.66
Kameyama	2006	140	J.Virol.Methods	Development of an immunochromatographic test kit for rapid detection of bovine viral diarrhea virus antigen	11.33
Kelling	2005	1785	AJVR	Characterization of protection from systemic infection and disease by use of a modified-live noncytopathic bovine viral diarrhea virus type 1 vaccine in experimentally infected calves	25.66
Kelling	2007	788	AJVR	Characterization of protection against systemic infection and disease from experimental bovine viral diarrhea virus type 2 infection by use of a modified-live noncytopathic type 1 vaccine in calves	25.66
Kirkland	1991	587	Vet.Rec.	Replication of bovine viral diarrhoea virus in the bovine reproductive tract and excretion of virus in semen during acute and chronic infections	12.65
Kommisrud	1996	41	Acta Vet.Scand.	Bovine virus Diarrhea virus in semen from acutely infected bulls	18.66
Lamm	2009	46	Vet.Pathol.	Distribution of Bovine Viral Diarrhea Virus Antigen in Aborted Fetal and Neonatal Goats by Immunohistochemistry	19

Liebler	1996	93	Vet.Immunol.Immunopathol.	Experimental mucosal disease in cattle: Changes in the number of lymphocytes and plasma cells in the mucosa of the small and large intestine	11
Liebler-Tenorio	1997	339	J.Comp.Pathol.	Experimental mucosal disease in cattle: Changes in cell proliferation in lymphoid tissues and intestinal epithelium	10
Liebler-Tenorio	2002	1575	Am.J.Vet.Res.	Distribution of viral antigen and development of lesions after experimental infection with highly virulent bovine viral diarrhea virus type 2 in calves	18.66
Liebler-Tenorio	2003	221	J.Vet.Diagn.Invest.	Distribution of viral antigen and development of lesions after experimental infection of calves with a BVDV 2 strain of low virulence	18.66
Loehr	1998	667	Arch.Virol.	Experimental induction of mucosal disease: consequences of superinfection of persistently infected cattle with different strains of cytopathogenic bovine viral diarrhea virus	14.32
Loken	1987	85	J.Comp.Pathol.	Experimentally-induced border disease on goats	11
Loken	1991	123	J.Comp.Pathol.	Experimental Pestivirus infections in newborn goat kids	18
Loken	1990	277	J.Comp.Pathol.	Experimental Pestivirus infections in pregnant goats	14
Makoschey	2001	3261	Vaccine	An inactivated bovine virus diarrhoea virus (BVDV) type 1 vaccine affords clinical protection against BVDV type 2	21
Manktelow	1969	245	New Zeal.Vet.J.	Hairy shaker disease of lambs	5
Mars	1999	197	Vet.Microbiol.	Airborne transmission of BHV1, BRSV, and BVDV among cattle is possible under experimental conditions	10.66
Marshall	1998	428	Aust.Vet.J.	Severe disease following experimental exposure of calves to noncytopathic bovine viral diarrhoea virus isolate New York-1	20.33
Martin	2011	In press	J.Wild.Dis.	Experimental study of border disease virus infection in pregnant Pyrenean chamois (*Rupicapra rupicapra*)	26.33
Meehan	1998	277	J.Comp.Pathol.	Acute pulmonary lesions in sheep experimentally infected with bovine viral diarrhoea virus	18.66
Meyling	1988	97	Vet.Microbiol.	Transmission of bovine virus diarrhoea virus (BVDV) by artificial insemination with semen from a persistently infected bull	17
Moennig	1990	200	Vet.Rec.	Reproduction of mucosal disease with cytopathogenic bovine viral diarrhoea virus selected in vitro	11
Nettleton	1992	179	Comp.Immun.Microbiol. Infect.Dis.	The production and survival of lambs persistently infected with Border Bisease virus	15
Niskanen	2002	171	Reprod.Dom.Anim.	Insemination of Susceptible Heifers with Semen from a Non-Viraemic Bull with Persistent Bovine Virus Diarrhoea Virus Infection Localized in the Testes	18.33
Niskanen	2002	251	Vet.J.	Failure to spread bovine virus diarrhoea virus infection from primarily infected calves despite concurrent infection with bovine coronavirus	19
Nuttal	1980	91	Res.Vet.Sci.	Experimental infection of calves with two strains of bovine virus diarrhoea virus: virus recovery and clinical reactions	16
Odeon	1999	221	J.Vet.Diagn.Invest.	Experimental infection of calves with bovine viral diarrhea virus genotype II (NY-93)	14.33
Ohmann	1982	357	Can.J.Comp.Med.	Experimental fetal infection with bovine viral diarrhea virus. I. Virological and Serological studies	16.66

Ohmann	1982	363	Can.J.Comp.Med.	Experimental fetal infection with bovine viral diarrhea virus. I. Morphological reactions and distribution of viral antigen	16.66
Passler	2007	350	Vet.Microbiol.	Experimental persistent infection with bovine viral diarrhea virus in white-tailed deer	17.66
Passler	2009	362	Vet.Microbiol.	Cohabitation of pregnant white-tailed deer and cattle persistently infected with Bovine viral diarrhea virus results in persistently infected fawns	16.66
Passler	2010	41	Vet.Res.	Transmission of bovine viral diarrhea virus among white-tailed deer (*Odocoileus virginianus)*	18.66
Patel	2002	2453	Arch.Virol.	Prevention of transplacental infection of bovine fœtus by bovine viral diarrhoea virus through vaccination	13
Paton	1999	185	Vet.Microbiol.	Foetal cross-protection experiments between type 1 and type 2 bovine viral diarrhoea virus in pregnant ewes	14
Plant	1976	57	Aust.Vet.J.	A mucosal disease virus as a cause of abortion hairy birth coat and unthriftiness in sheep	11
Plant	1977	574	Aust.Vet.J.	Transmission of a mucosal disease virus infection between sheep	13
Polak	2000	141	Comp.Immun.Microbiol.Infect.Dis.	Experimental inoculation of calves with laboratory strains of bovine viral diarrhea virus	17.66
Potgieter	1984	1582	Am.J.Vet.Res.	Experimental production of bovine respiratory tract disease with bovine viral diarrhea virus	13
Potts	1982	1464	Am.J.Vet.Res.	Border disease: experimental reproduction in sheep, using a virus replicated in tissue culture	11.33
Raizman	2009	653	J.Wild.Dis.	Experimental infection of white-tailed deer fawns (Odocoileus virginianus) with bovine viral diarrhea virus type-1 isolated from free-ranging white-tailed deer	21.66
Ridpath	2007	653	J.Wild.Dis.	Febrile response and decrease in circulating lymphocytes following acute infection of white-tailed deer fawns with either a BVDV1 or a BVDV2 strain	18
Ridpath	2010	691	Vet.Res.Commun	Evaluation of three experimental bovine viral diarrhea virus killed vaccines adjuvanted with combinations of Quil A cholesterol and dimethyldioctadecylamminium (DDA) bromide	18
Salt	2007	616	Vet.J.	Efficacy of a quadrivalent vaccine against respiratory diseases caused by BHV-1, PI3V, BVDV and BRSV in experimentally infected calves	22
Sandvik	1997	583	J.Vet.Med. B	Level of viral antigen in blood leucocytes from cattle acutely infected with Bovine viral diarrhoea virus	11
Scherer	2001	285	Vet.Microbiol.	Experimental infection of pregnant ewes with bovine viral diarrhea virus type-2 (BVDV-2): effects on the pregnancy and fetus	19
Semrau	2008	124	J. Zoo Wild. Med.	Experimental superinfection of a lesser Malazan mousedeer (Tragalus javanicus) persistently infected with bovine viral diarrhea virus	18
Shimizu	1989	13	Vet.Microbiol.	Serological characterization of viruses isolated from mucosal disease	12.66
Shimizu	1989	207	Vet.Microbiol.	Experimental infection of pregnant goats with swine fever virus	12
Spagnuolo-Weaver	1997	287	J.Vet.Diagn.Invest.	Distribution of cytopathic and noncytopathic bovine viral diarrhea virus antigens in tissues of calves following acute experimental infection	16
Spilki	2006	699	Arq.Bras.Med.Vet.Zootec.	Co-infections with bovine herpesvirus type 5 and bovine viral diarrhoea virus	15
Stokstad	2002	494	J.Vet.Med. B	Pestivirus in Cattle: Experimentally Induced Persistent Infection in Calves	15.33

Stokstad	2003	424	J.Vet.Med. B	Experimental infection of Cows with Bovine Viral Diarrhoea Virus in Early Pregnancy – Findings in Serum and Foetal Fluids	17.33
Stokstad	2004	571	Arch.Virol.	The role of the defective interfering particle DI9c in mucosal disease in cattle	18.33
Storey	1972	163	J.Comp.Pathol.	Experiments in border disease. VI. Lipid and enzyme histochemistry	6
Swasdipan	2001	275	Vet.Pathol.	Rapid Transplacental Infection with Bovine Pestivirus Following Intranasal Inoculation of Ewes in Early Pregnancy	13
Sweasey	1979	447	Vet.Rec.	Border disease: A sequential study of surviving lambs and an assessment of its effect on profitability	14.33
Taylor	1977	249	Trop.Anim.Health.Prod	Experimental infection of Nigerian sheep and goats with bovine virus diarrhoea virus	10
Terlicki	1973	310	Res.Vet.Sci.	Morphology of experimental border disease of lambs	6
Terlicki	1980	602	Br.Vet.J.	Pathogenicity for the sheep fœtus of bovine virus diarrhoea-mucosal disease virus of bovine origin	17
Terpstra	1978	350	Res.Vet.Sci.	Border disease: virus persistence, antibody response and transmission studies	10.33
Terpstra	1981	185	Res.Vet.Sci.	Detection of Border disease antigen in tissue of affected sheep and in cell cultures by immunofluoresence	13
Tessaro	1999	671	J.Wild.Dis.	Viremia and virus shedding in Elk infected with type 1 and virulent type 2 bovine viral diarrhea virus	19
Thabti	2002	35	Vet.Res.	Experimental model of Border Disease Virus infection in lambs: comparative pathogenicity of pestiviruses isolated in France and Tunisia	24.66
Tsuboi	2011	174	Res.Vet.Sci.	Experimental infection of early pregnant cows with bovine viral diarrhea virus: Transmission of virus to the reproductive tract and conceptus	17.66
Uttenthal	2005	87	Prev.Vet.Med.	Persistent BVDV infection in mousedeer infects calves - Do we know the reservoirs for BVDV?	9
Van Campen	1997	567	J.Wild.Dis.	Experimental infection of deer with bovine viral diarrhea virus	15.66
Vantsis	1976	111	J.Comp.Pathol.	Experiments in Border Disease. VIII Propagatio of a cytopathic virus	7
Vantsis	1979	331	J.Comp.Pathol.	Experimental challenge infection of ewes following a field outbreak of border disease	12
Vantsis	1980	39	J.Comp.Pathol.	The effects of challenge with homologous and heterologous strains of border disease virus on ewes with previous experience of the disease	11
Walz	1999	505	J.Vet.Diagn.Invest.	Experimental model of type II bovine viral diarrhea virus induced thrombocytopenia in neonatal calves	22.66
Walz	2004	191	J.Vet.Med. B	Experimental inoculation of pregnant swine with type 1 bovine viral diarrhoea virus	19.66
Welsh	1995	195	Vet.Immunol.Immunopathol.	Effect of BVD virus infection on alveolar macrophage functions	11
Wentz	2003	223	JAVMA	Evaluation of bovine viral diarrhea virus in New World camelids	19.33
Wieringa	2006	26	Vet.Microbiol.	Limited BVDV transmission and full protection against CSFV transmission in pigs experimentally infected with BVDV type 1b	22
Wilhelmsen	1990	235	Vet.Pathol.	Experimental primary postnatal bovine viral diarrhea viral infections in six-months-old calves	19.66

Wilhelmsen	1991	269	Am.J.Vet.Res.	Lesions and localization of viral antigen in tissues of cattle with experimentally induced or naturally acquired mucosal disease, or with naturaly acquired chronic bovine viral diarrhea	11
Woldehiwet	1994	201	Vet.Immunol.Immunopathol.	Cytotoxic T cell responses in lambs experimentally infected with border disease virus	12
Woldehiwet	1994	389	Vet.Immunol.Immunopathol.	Distribution of Border disease virus antigen in lymphocyte subpopulations in the peripheral blood of experimentally infected lambs	15
Wood	2004	42	Can.J.Vet.Res.	Effect on hematopoietic tissue of experimental infection of calves with noncytopathic type 2 bovine viral diarrhea virus	24
Zakarian	1975	453	J.Comp.Pathol.	Periarteritis in experimental border disease of sheep	9
Zimmer	2002	255	Vet.Microbiol.	Failure of foetal protection after vaccination against an experimental infection with bovine virus diarrhea virus	21
Zimmer	2004	145	Vet.Microbiol.	The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples	17

Table IV. Number and percentage of studies with an ethical commission since 1999

Year	Number of studies per year	Percentage of studies with an ethical commission (%)
1999	5	20
2000	3	0
2001	3	0
2002	7	28.6
2003	6	16.7
2004	3	33.3
2005	2	0
2006	1	0
2007	3	100
2008	2	50
2009	7	71.4
2010	5	80
2011	3	66.7

Legend: Studies without an ethical commission (before 1999) are not shown in the table.

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ARTICLE 2. ARTICLE ORIGINAL

Experimental infection of pregnant pyrenean chamois (*Rupicapra pyrenaica*) with border disease virus subtype 4

Accepté avec modifications mineures dans Journal of Wildlife Diseases

ARTICLE 2. Resumé

Infection expérimentale d'isards gestants (*Rupicapra pyrenaica*) avec un virus de la Border Disease type 4

Les virus de la Border Disease (BDV) ont récemment causé de fortes mortalités dans les populations d'isards (*Rupicapra pyrenaica*) des Pyrénées françaises et espagnoles. Le but de cette étude a été d'investiguer la pathologie induite par un BDV sur des femelles isards gestantes à travers une infection expérimentale. Trois femelles ont été inoculées pendant le deuxième tiers de gestation avec une souche de BDV-4 préalablement isolée d'un isard durant une épidémie aiguë de border disease dans les Pyrénées. Une quatrième femelle gestante était associée à une agnelle non gestante pour former le groupe témoin. Des suivis cliniques, hématologiques, virologiques et sérologiques ont été mis en place pendant toute la durée de l'expérimentation. Des examens post-mortem ont inclus des autopsies, des examens histopathologiques et une quantification de l'ARN viral dans les organes. Aucune des gestations n'a pu être menée à terme. Une femelle est morte à 24 jours post inoculation (jpi) sans montrer de signe clinique précurseur. Le second animal a eu une diarrhée profuse de 13 jpi jusqu'à sa mort à 51 jpi. La troisième femelle a avorté à 46 jpi et a été euthanasiée à 51 jpi. Une virémie positive a été mise en évidence à partir de 4 jpi et est restée positive jusqu'à la mort des animaux. Des anticorps neutralisants ont été détectés à partir de 12 jpi. Lors des autopsies, des lymphadénomégalies généralisées ont été mise en évidence, associée dans un cas à des pétéchies disséminées sur l'ensemble du tractus digestif. Sur 79 organes testés (provenant des adultes inoculés et de leurs fœtus), 78 étaient positifs en RT-PCR en temps réel. Les principales lésions histologiques constatées chez les femelles inoculées étaient une encéphalite lymphohistiocytique associée à une déplétion lymphoïde modérée ou sévère. Pour les animaux témoins, la virémie, la sérologie et les examens post-mortem sont restés négatifs. Pour conclure, l'infection par un BDV pendant la gestation de femelles isards cause une maladie sévère amenant à un avortement et secondairement à la mort des animaux. Les résultats obtenus lors de cette infection expérimentale montrent que l'interprétation des statuts infectieux d'animaux sauvages doit être réalisée de façon prudente lorsque des tests développés sur des animaux domestiques sont utilisés. Ils contribuent de plus à expliquer les diminutions de populations de chamois et d'isards rapportées dans diverses zones.

Running heading:

Martin, Duquesne, Guibert, Pulido, Gilot-Fromont, Gibert, Velarde, Thiéry, Marco, Dubois

Abbreviated title:

Experimental infection of pregnant Pyrenean chamois with pestivirus

Full title:

EXPERIMENTAL INFECTION OF PREGNANT PYRENEAN CHAMOIS (*RUPICAPRA PYRENAICA)* WITH BORDER DISEASE VIRUS SUBTYPE 4

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Abstract

Border Disease Virus (BDV) has been shown to cause high mortality in Pyrenean chamois (*Rupicapra pyrenaica*) on both French and Spanish sides of the Pyrenean mountains. The aim of this study was to investigate the pathology induced by BDV in pregnant chamois through an experimental infection. Three females were inoculated during the second third of pregnancy with a Border Disease Virus subtype 4 (BDV-4) strain previously isolated from a wild Pyrenean chamois during an acute epizootic disease outbreak. A fourth pregnant chamois and one non-pregnant ewe were kept as negative controls. All animals were monitored to assess clinical signs, hematology, viremia and serology. Post mortem examinations included necropsy, histopathology and quantification of viral Ribo Nucleic Acid (RNA) in organs. Pregnancy was unsuccessful in all inoculated animals. One died on 24 days post

inoculation (dpi) without showing any precursory clinical signs. The second animal had profuse diarrhea from 13 dpi to its death on 51 dpi. The third aborted on 46 dpi and was euthanized on 51 dpi. Viremia started on 4 dpi in all animals and remained positive until their death. Neutralizing antibodies against BDV-4 were detected from 12 dpi. Necropsies showed generalized lymphadenomegaly, associated in one case with disseminated petechial hemorrhages in the digestive tract. Seventy-eight of 79 organs from inoculated adults and their fetuses tested positive for viral RNA. The main histologic lesions found in adults were mild lymphohistiocytic encephalitis associated with moderate or moderately severe lymphoid depletion. Control animals remained negative for viral presence (in blood and organs), seroconversion and lesions upon post mortem examination. BDV infection during pregnancy in Pyrenean chamois causes severe disease leading to abortion then death. Finally, results from this experimental study show that great care must therefore be taken when interpreting infection status for wildlife studies in the field and may help explain the reported decrease in chamois populations in several areas.

Keywords

Border disease virus, Experimental infection, pathogenicity, pestivirus, pregnancy, Pyrenean chamois, *Rupicapra pyrenaica*

INTRODUCTION

Pestiviruses (family *Flaviviridae*) are single-stranded Ribo Nucleic Acid (RNA) viruses, officially classified into four species by the International Committee on Taxonomy of Viruses (ICTV). Bovine Viral Diarrhea Virus type 1 (BVDV-1) and Bovine Viral Diarrhea Virus type 2 (BVDV-2) mainly affect bovines; Border Disease Virus (BDV) is commonly isolated from sheep, and Classical Swine Fever Virus (CSFV) from pigs. To date, eleven subgroups or subspecies have been described for the BVDV-1 species (Vilcek et al., 2001); two subgroups for the BVDV-2 species (Vilcek et al., 2005) and at least seven subgroups for the BDV species (Giammaroli et al., 2011). In Europe, BDV-1 were isolated from sheep in United Kingdom (Vilček et al.,1997), BDV-2 in Germany (Becher et al., 2003),

BDV-3 in Switzerland (Stalder et al., 2005), and Austria ((Krametter-Froetscher et al., 2007), BDV-4 in Spain (Arnal et al., 2004; Valdazo-Gonzalez et al., 2007) and BDV-5, BDV-6 and BDV-Tunisian in France (Dubois et al., 2008). Besides, three distinct genotypes have been proposed as novel pestivirus species, isolated from a giraffe (giraffe-1 strain), a deer (reindeer-1 strain) (Avalos-Ramirez et al., 2001) (Becher et al., 2003) or from fetal calf serum (Hobi strain) (Schirrmeier et al., 2004). Pestivirus infections have been found in many wild ungulated species (Vilcek and Nettleton, 2006). Cross-contamination between species has been widely described (Passler et al., 2009) and the infection of wild boar (*Sus scrofa*) by CSFV represents a major risk for pig farms. In domestic flocks, the main clinical signs associated with Border Disease are reproductive failure such as abortion, stillbirth or lower fertility. Furthermore, the immunosuppressive effects of infection increase the severity of opportunistic infections. The birth of persistently infected (PI) animals is a critical point when considering infection epidemiology (Letellier and Kerkhofs, 2003). In wild ruminants, pestivirus pathology is relatively unknown. Several experimental infections with BVDV have been carried out in white-tailed deer (*Odocoileus virginianus*) (Duncan et al. 2008, Raizman et al., 2009; Ridpath et al., 2007), elk (*Cervus elaphus*) (Tessaro et al., 1999) and mule deer (*Odocoileus hemionus*) (Van Campen et al., 1997). In most of these studies, no clinical signs were reported, while abortion, fever and lymphocyte depletion were described in others (Duncan et al., 2008; Raizman et al., 2009).

Recent descriptions of pestivirus infection have been reported in Pyrenean chamois (*Rupicapra pyrenaica*) in Spain (Marco et al., 2007, 2009) and France (Pioz et al., 2007). In France, the virus (classified BDV-4) seems to have become endemic from at least 1995 (Pioz et al., 2007). In Spain, outbreaks were associated with high mortality (between 42 and 86 %) and clinical signs such as behavioral changes and alopecia, with skin hyperpigmentation. Histologic changes were found in the brain with mainly edema, gliosis, spongiosis and neuronal multifocal necrosis. Moderate hyperplasia with orthokeratotic hyperkeratosis was also observed in skin (Marco et al., 2007). BDV-4 strains were isolated during these outbreaks (Arnal et al., 2004; Frölich et al., 2005). A retrospective study carried out on archived sera and spleen showed that the populations studied had been infected by BDV-4 strains from at least 1990 on. The emergence of the disease in 2001 may thus be due to other factors such as viral mutation (Marco et al., 2011). Experimental infections with BDV-4 from chamois

(CADI-6 strain) have already been carried out in sheep (Cabezón et al., 2010c) and pigs (Cabezón et al., 2010a). Although these species developed short-lived viremia and seroconversion within 15 days post inoculation (dpi), no clinical signs were observed. To understand the infection and pathogenicity in chamois, it was thus important to inoculate the target species and assess its consequences on pregnancy.

The aim of this study was to investigate (i) the pathology of a BDV-4 strain named CADI-6 which had been previously isolated from a naturally-infected Pyrenean chamois during an acute epizootic disease outbreak and (ii) the consequences of infection on pregnancy (whether fetuses may be persistently infected) in semi-domesticated Pyrenean chamois.

MATERIAL AND METHODS

Animals

Four Pyrenean chamois were obtained from a game park in the French Pyrenean mountains. While animals were sedated, pregnancy was confirmed by trans-abdominal ultrasound echography. All animals were negative for BDV and BVDV, both by antigen and antibody detection (ELISA BVD Ag Mix®, Pourquier, Eragny, France; and ELISA BVD/MD/BD P80®, Pourquier, Eragny, France, respectively). They were also negative for Blue Tongue Virus 1 and 8. Age ranged from 4 to 14 years old. They were transferred to the experimental station (ANSES Sophia Antipolis laboratory) in level 2 confinement facilities under negative pressure. Animals were acclimatized for 10 days prior to inoculation, and randomly separated into two groups. Three individuals were assigned to the inoculated group (chamois A, B and C), and the last one (chamois D) was placed with a ewe to form the control group. The pre-Alp ewe originated from a specific pathogen-free herd.

Cells and virus

The CADI-6 viral strain (GENBANK accession number AM905923) was kindly provided by I. Marco (Sefas, Universitat Autònoma de Barcelona). Three virus subcultures were produced to obtain the inoculum. ETM 52 cells were grown in minimum essential medium (MEM-Gibco ®, Invitrogen,

Carlsbad, Mexico) as described in Thabti et al (2002) and used for both virus production and virus neutralization (VN) tests. Cells culture and were controlled for pestivirus infection fetal calf serum

Experimental design

The Veterinary Services Directorate of the Alpes-Maritimes Department approved the care and use of chamois in this experiment. The main author had the chamois farming capacity certificate n2010-2671. The experiment was conducted in the experimental station of the ANSES Sophia Antipolis laboratory, under agreement no. A 06-018-2 (agreement delivered by the local Direction of Veterinary Services). Animals were inoculated under sedation by an intratracheal injection of 5 ml of cell culture supernatant containing 10^6 50% tissue culture-infectious doses ($TCID_{50}$) of the CADI-6 strain. They were inoculated during the second third of gestation, as estimated by echography. All surviving animals were euthanized at the end of the experiment period. Samples of nine different organs (brain, cotyledon, kidney, liver, lung, mesenteric node, myocardium, skin and spleen) were collected from both adults and fetuses (when available) and frozen until use.

Clinical monitoring was realized daily by visual inspection. The two same persons realized the clinical monitoring during all the experiment after an initial formation. Besides, numerical pictures were taken each sample day, to visualize change in animal aspects. To standardize clinical monitoring, we used a clinical score taking into account the presence or absence of clinical signs specific to a pestivirus infection (Table I). Assessments consistent with good health were assigned a score of 0, abnormalities a score of 1. A score of 10 was attributed to death.

Sample collection

Samples of blood and swabs were performed ten and five days before inoculation, on the day of inoculation (day 0), and on days 1, 2, 4, 6, 8, 10, 12, 14, 17, 21, 28, 35, 42 and 49 post inoculation. Blood samples were taken from the jugular vein and collected in silicone-coated and spray-coated Vacutainer K_2EDTA® blood sampling tubes (BD, Franklin Lakes, USA). For each blood sample, a complete hemogram was performed manually (Veterinary diagnostic laboratory, Sophia Antipolis, France). To consider individual variations, values obtained for each hematologic parameter during the

167 acclimatization period were normalized to 100 (Martinelle et al., 2011). Nasal, rectal, vaginal and oral
168 swabs were taken using swabsticks composed of a sterile cotton plug (Single plastic swab, COPAN,
169 Italia). Swabs were resuspended in 1 mL of sterile phosphate buffered saline (PBS) and frozen at -80
170 C until use.
171
172 **Viral RNA extraction**
173 Total RNA was extracted from 100 µL of whole EDTA blood and from 100µL of PBS (for swabs)
174 using the NucleoSpin 8 Virus®(Macherey Nagel, Düren, Germany) and an automated extractor
175 (TECAN Evo® 75, Männedorf, Switerland). For organs, total RNA was extracted from 100 µL of
176 10%-homogenate (w/v) of tissue in PBS using the RNeasy mini kit® (Qiagen, Courtaboeuf, France)
177 according to the manufacturer's instructions. RNA was suspended in 30 (blood) or 100 µL (organs) of
178 RNase-free water and kept at -80 C until use.
179
180 **Virus detection**
181 Conventional and quantitative real-time reverse transcription-polymerase chain reactions (respectively
182 RT-PCR and real-time RTq-PCR) were performed on each whole blood sample. For swabs and
183 organs, only real-time RT-PCR was performed. For conventional PCR, the 5' untranslated region
184 (5'UTR) was amplified using primers 324 and 326 to amplify a 249 base pair fragment using a
185 previously described method (Dubois et al., 2008).
186 A one step real-time RT-PCR on the 5'UTR region was performed as follows. The primer pair R-
187 BD4-237 (5'-GCCCTCGTCCACGTAGCAT-3'), F-Orlu-171 (5'-
188 AGTACAGGGCAGTCGTCAGTAGTTC-3') and the FAM-Orlu 200 probe (FAM-
189 CTAACTCGGTTTAGTCTCG-MGB) (Applera, Villeron sur Yvette, France) were designed
190 specifically for the detection and quantification of CADI-6. The Applied Biosystems ABI 7500 Real
191 Time PCR system (Applied, Courtaboeuf, France) was used. Each PCR reaction was run in 25µL
192 containing 12.5 µL 2x Quantifast Probe RT-PCR Master Mix (w/o ROX), 10 µM of both primers, 10
193 µM of fluorescent probe, 0.25 µL of Quantifast RT Mix and 50x of ROX Dye Solution (Quantifast kit
194 ®, Qiagen, Courtaboeuf, France) and 2 µL of purified RNA. PCR conditions were as follows: a

starting period of 10 min at 55 C and 5 min at 95 C was followed by 45 cycles of 10 sec at 95 C and of 30 sec at 60 C. Fluorescent measurements were carried out during the elongation step.

The 5'UTR PCR amplicon was cloned using pGEMT Easy vector® (Promega, Charbonnières-les-Bains, France). After *in vitro* transcription with the Promega ribomax large scale RNA production system® (Promega, Charbonnières-les-Bains, France), RNAs were purified and quantified in order to obtain a standard dilution of synthetic RNA ($2x10^{6}$ to $2x10^{-1}$ copies of RNA per µL). Standard curves were used to estimate the quantity of RNA detected in blood or swabs.

Virus titration

Ten µL of each sample were diluted in 90 µL of MEM-Gibco and twofold serial dilutions were inoculated onto five wells on 96-well microtiter plates containing ETM52 cells. After 1h of incubation at 37C in 5% CO_2, the wells were drained and a growth medium supplemented with antibiotics (Penicillin 100000UI/l, Streptomycin 50mg/l) was added. Cultures were incubated for five days at 37C in 5% CO_2. After cell fixation with cold acetone, the virus was detected by immunofluorescence-assay using a poly pestivirus monoclonal antibody (Synbiotics, Lyon, France). Titers were expressed as the reciprocal of the highest blood dilution yielding 50% virus growth and calculated with the Spearman-Kärber method (Thrusfield, 1986).

Serologic analyses

Pestivirus-specific antibodies against the p80 protein (also known as NS3) were detected using a blocking enzyme linked immunosorbent assay (ELISA) (Synbiotics, Lyon, France) according to the manufacturer's recommendations.

For the VN test, strain CADI-6 was used to perform specific neutralizations. A previously described method was used (Martin et al., 2011) with minor modifications concerning incubation (1h at 37 C). The virus was detected as in the titration method and titers were expressed as the reciprocal of the highest serum dilution yielding 50% virus growth neutralization.

Necropsy and post mortem examinations

A necropsy was performed within 24 hours of the animal's death (the carcass being stored in a 5 C room at the meantime) or immediately after euthanasia. Adult and fetal brains, cotyledon, kidney, liver, lung, mesenteric node, myocardium, skin, and spleen were collected whenever possible. One hundred and eighty mg of tissue were sampled and ground in 600 µl of PBS. Of these 600 µL, 100 µL were used for extraction and subsequently analyzed by real-time RT-PCR, as described above.

Tissue samples collected during necropsy for histopathologic analyses were fixed in a 10% buffered formalin then trimmed, paraffin-embedded, sectioned at 3-4 µm and stained with hematoxylin and eosin.

RESULTS

Clinical signs and scores

Chamois A did not show any clinical signs until its sudden death on 24 dpi. During necropsy, signs of hemorrhagic diarrhea were found and the fetus was mummified. Chamois B showed clinical diarrhea from 14 dpi and weakness from 35 dpi until its death on 51 dpi. Clinical examination suggested weight loss during the experiment period. The third animal (C) aborted on 46 dpi. Except for abortion and weight loss, it did not present any clinical signs related to the pestivirus infection. As it had aborted, it was euthanized on 51 dpi for animal welfare considerations (not to keep it alone). The two control animals were euthanized at 52 dpi. They did not show any clinical signs throughout the experimentation. The fetus of the control chamois had nearly reached full term by the end of the experiment. The total sum of clinical scores came to ten for chamois A (corresponding to the death score), 63 for B, seven for C and zero for the two controls.

Hematology

No significant variation was observed in red blood cell count, hematocrit, mean corpuscular volume or mean corpuscular hemoglobin concentration in any individual (data not shown). Figure 1 shows the evolution of total leucocyte counts. All Pyrenean chamois (including control) first showed a decrease in the total leucocyte count, reflected in both lymphocyte and neutrophil counts from the inoculation

day to 5 dpi (data not shown). After 5 dpi, the total leucocyte count decreased again only for inoculated animals. It is noteworthy that chamois A died three days after its total leucocyte count decreased to 1200 cells/mm^3 and chamois B died five days after its total leucocyte count equaled 1500 cells/mm^3, whereas chamois C did not drop below 2000 cells/mm3 and did not die.

Viremia

Pestivirus RNA and viruses were detected in all inoculated animals on 4 dpi. Control individuals remained negative throughout the experimentation. Figure 2 shows viremia kinetics by both viral titration and RTq-PCR. Viremia remained positive for chamois C until its euthanization on 51 dpi, with the highest titers on 17 dpi ($2.2x10^6$ $TCID_{50}$/mL). For animal B, maximum values increased to $3x10^6$ $TCID_{50}$/mL during a 23-day period between 28 and 51 dpi. On the contrary, chamois A had a very low viremia from 4 to 17 dpi reaching only 2.10^5 $TCID_{50}$/mL. RNA was detected by real time RT-PCR in blood on 2 dpi for animals A and B and on 4 dpi for C. For chamois A, the total RNA count reached $1.1x10^6$ RNA copies/mL on 12 dpi and decreased thereafter. For the other two inoculated animals, maximum values were obtained on 35 dpi with values higher than $2.5x10^7$ copies of RNA/mL, and remained stable until their death on 51 dpi. Control animals remained negative throughout the experiment.

Viral excretion

All animals were tested by oral, vaginal, nasal and rectal routes. Viral detection was positive from 12 dpi in all vaginal, rectal and nasal samples. Animal A showed only a significant rectal excretion whereas the other two inoculated chamois had high excretion rates for nasal, rectal and vaginal routes (figure 3). Two increases in excretion were observed for chamois C: one around 12 dpi, and the other around 25 dpi. Chamois B increased strongly on 12 dpi for nasal and rectal excretions and on 17 dpi for the vaginal route. Oral excretion was very low for all inoculated animals. No excretion was measured for control animals.

Antibody response

Seroconversion was observed by virus neutralization on 12 dpi for animals A and B and on 14 dpi for C. No seroconversion was detected in control animals throughout the experiment. Figure 4 presents the kinetics of the pestivirus-neutralizing antibodies for inoculated animals. The three animals showed three different profiles for the evolution of antibody titers. Chamois B had a low and short positive serology, reaching only $4.4x10^2$ Dose Neutralizing $_{50}$/ml (DN_{50}/ml) on 21 dpi. Antibody titers were higher for animal C between 28 and 35 dpi, with the highest value of $3.6x10^3$ DN_{50}/mL, and reached $5.1x10^3$ DN_{50}/mL on 21 dpi for animal A. No antibodies were detected in the control animals. ELISA gave a positive result for only one sample (animal B on 17 dpi), corresponding to a neutralizing antibody concentration of $5.1x10^3$ DN_{50}/ml.

Post mortem examination

The results of post mortem examinations are summarized in Table II. Upon necropsy, animal A presented signs of abundant yellowish diarrhea. Multifocal petechial hemorrhages were present in the serosa and mucosa of the digestive tract, mainly on the abomasum and small intestine, as well as the epicardium and pericardium. Lungs were highly affected with a sizeable hemorrhage on the right lung apical lobe and petechias on the pleura. All lymph nodes had increased in volume. Chamois B was emaciated and presented severe enteritis in the small intestine with signs of brown diarrhea, severe verminous tracheitis and verminous bronchopneumonia. Animal C presented chronic pulmonary abscesses unrelated to the viral infection and verminous bronchopneumonia. No macroscopic lesions were observed in any of the fetuses, either after infection of the mother (B-fetus and C-fetus) nor in control animals (D, D-fetus and the ewe).

Histopathologic examinations of all inoculated animals revealed mild lymphohistiocytic encephalitis associated with moderate (B and C) or moderately severe (A) lymphoid depletion. Concurrent infections reported macroscopically were confirmed and completed. Animal A presented severe bacterial necrotizing pneumonia, animal B enteric coccidiosis and animal C suppurative bacterial placentitis. The fetus of B had severe autolytic changes precluding a precise histologic evaluation. Nevertheless, it was possible to observe multifocal acute hemorrhages in the brain and a diffuse lymphoid depletion in the spleen. No histologic changes were noticed in control animals.

RTq-PCR was performed on the total RNA extracted from collected organs. Pestivirus RNA was detected in all the tissues of inoculated animals except one, the skin of animal A. Moreover, all the organs of B-fetus, C-fetus and A-fetus tested positive (table III). RTq-PCR was negative for all the organs of the control animals.

DISCUSSION

Three pregnant Pyrenean chamois were inoculated with a BDV-4 strain previously isolated from wild Pyrenean chamois. One died on 24 dpi with a mummified fetus, another died on 51 dpi and the third aborted on 46 dpi before being euthanized on 51 dpi. Viral RNA was detected in all fetal organs of inoculated females with high loads of viruses per mg.

This experiment was performed in a strictly ethical observance. We had to choose the manipulations that could be excluded to avoid excessive stress and those necessarily to have the best follow up of the experimental infection. Temperature and weight measurements were therefore excluded from the clinical examination. However, for practical reasons, we could not acclimatize animals to the experimental conditions for more than 10 days.

Within five days of inoculation, the total leukocyte count decreased for all chamois, including the control. However, after 5 dpi, the total leukocyte count decreased only for inoculated animals and remained stable for the control chamois. The stress induced by capture and transport affects hematologic, serum chemicals and clinical parameters (López-Olvera et al., 2007). Duncan and collaborators (Duncan et al., 1994) showed that the leukocyte response to stress is biphasic: a preliminary lymphocytic leukocytosis is followed by lymphopenia. In this experiment, after capture and transport, an acclimatization period of 10 days preceded inoculation. During this period, animals were sampled only once, five days before inoculation. After inoculation, they were sampled daily for three days and every two days for one week. Both inoculation and sampling procedures may have stressed animals, which can explain the general leukocytosis followed by leukopenia observed between 0 and 5 dpi. After 5 dpi, leukopenia was more severe for inoculated animals than for the control chamois, and lasted until the end of the experiment. Leukopenia is commonly associated with pestivirus infection in both domestic (Thabti et al., 2002) and wild species. In Pyrenean chamois,

leukopenia has been associated with BDV infection in both natural conditions (Fernandez-Sirera et al., 2011) and experimental conditions (Cabezón et al., 2011).

This experiment confirms that a specific course of infection occurs in chamois. Abortion and animal death were observed. Pestivirus was detected in the cotyledons of inoculated animals, which would indicate that the virus caused their abortion. Infections in livestock are usually shorter and milder. In lambs (Cabezón et al., 2010c) and pigs (Cabezón et al., 2010a) inoculated with the same strain, no clinical signs were reported and the associated viremia was short-lived. In non-pregnant Pyrenean chamois inoculated in the same conditions as in this study, two animals were found dead on 18 and 21 dpi with signs of hemorrhagic diarrhea and one died on 24 dpi after a coughing attack. The last two inoculated animals were euthanized on 34 dpi, without presenting any clinical signs other than weight loss and high body temperature (Cabezón et al., 2011). In the two experiments, secondary infections were the rule, which means that all inoculated animals had low defense level.

All inoculated chamois showed positive viremia until at least 17 or 51 dpi. Such long-lasting viremia associated with pestivirus infection has only been described in chamois (Cabezón et al., 2011). In contrast, during transient infection in sheep, viremia is short-lived, lasting no more than 12-15 days (Thabti et al., 2002). In other wild species such as elks (*Cervus elaphus*), viremia was only detected between 3 and 7 dpi (Tessaro et al., 1999).

Chamois A eliminated the virus on 17 dpi. It did not survive despite high VN titers. Chamois B (the oldest one, aged 14 years) had a very long-lasting and severe viremia until its death on 51 dpi. Despite a seroconversion on 12 dpi, VN titers remained very low and were negative before death. The third animal (C) seroconverted, with VN titers higher than chamois B but still lower than others described in the field (Marco et al., 2011; Martin et al., 2011). The ELISA test for antibody detection gave positive results only when VN titers reached $5.1x10^3$ DN_{50}/ml. These results provide important information for the epidemiologic study of pestivirus infection in natural conditions. Indeed, the usual methods used in the field are a preliminary screening of antibody prevalence with an ELISA test, followed by RT-PCR for viral detection in ELISA-negative samples (Martin et al., 2011). In livestock, to differentiate a PI animal from an animal in transient infection, two PCRs with a three-week interval have to be positive. However, the current study results show that chamois in transient infection have

362 this profile if the ELISA test is used for antibody detection. Great care must therefore be taken when
363 interpreting infection status for wildlife studies in the field.
364 These results also have important implications concerning disease epidemiology. Of three inoculated
365 pregnant females, one had a mummified fetus, one died before parturition and one aborted. As none of
366 the pregnancies reached their term, the usual method of determining a PI animal (serial virus
367 detections) could not be used. However, a multisystemic and systematic distribution of viruses in all
368 organs of infected fetuses was present, with high virus loads per mg. The virulence of the strain used
369 may have killed fetuses. The use of a hypovirulent strain would have been interesting to test, in order
370 to generate PI animals. In chamois, pestivirus RNA has already been isolated in a fetus (Cabezón et al,
371 2010b). An experimental infection of one pregnant Pyrenean chamois had already been carried out in
372 2008 (Vautrain and Gibert, 2008). The two RT-PCRs effected at birth and death (on 92 days) were
373 both positive. Generally speaking, the multisystemic distribution of the virus, its excretion by several
374 routes, the long-lasting viremia and possible birth of PI offspring revealed in this study all suggest that
375 infected chamois may efficiently transmit infection in natural populations.
376 Our results mostly confirm virologic and histologic observations made in the field. In all inoculated
377 animals, the virus had a multisystemic distribution. Histologic lesions were consistent with those
378 observed in animals infected in both natural and experimental conditions. In chamois naturally
379 infected by BDV type 4, the main lesions were observed in the brain with diffuse moderate spongiosis,
380 occasional glial nodules, neuronal degeneration and death, and occasionally, a non-purulent
381 perivascular inflammatory infiltrate (Marco et al., 2007). Besides, major lymphoid depletion was
382 present in all infected animals, which is usually observed with pestivirus infection. In an
383 experimentally-infected pregnant doe with BVDV, the most significant histologic abnormality was
384 diffuse depletion of B-lymphocytes in fawns (Duncan et al., 2008). Although weight loss was not
385 quantified, two out of three animals were seen to have lost weight during the experimentation, which
386 is in accordance with the cachexia observed in 15 of 23 virus-positive chamois (Marco et al., 2009).
387 However, clinical signs did not exactly correspond to observations of infections in natural conditions
388 (cutaneous signs or neurological alterations were not observed). In both experiments (Cabezon et al.,
389 2011 and the present study), infections lasted at least 18 days and were progressive: no individual

390 stayed without any sign or lesion but duration, signs and lesions differed. In experimental infections,
391 signs were expected to differ from natural infections, because individuals were stressed and had to
392 adapt to an unknown environment. The immunodepression caused by the virus, associated with the
393 stress induced by captivity favored the expression of secondary infections. Their precise timing and
394 signs were expected to be variable, as they are agent and individual dependant.
395 In the Pyrenean mountains, pestivirus infections have been shown to be associated with a marked
396 decline in Pyrenean chamois populations (Marco et al., 2011). On the other hand, in populations of
397 Alpine chamois (*Rupicapra rupicapra*) in the French Alps, infection was shown to be associated with
398 a decrease in fertility (number of kids/number of females per yr) (Martin et al., 2011). The results of
399 this experiment confirm both observations in natural populations: BDV-4 infection in Pyrenean
400 chamois led to abortion, fetus mummification and secondarily to animal death. Chamois fetuses were
401 shown to be infected by the virus. Their role in the epidemiology of pestivirus in chamois populations
402 needs to be assessed.
403

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562 **LIST OF FIGURES AND CAPTIONS**

563

564 **Figure 1**: Evolution of total leukocyte count after inoculation in three inoculated chamois (A, B and
565 C), one control chamois (D) and one control ewe.

566 Legend for figure 1

567 In order to compare individual variations, the mean of values obtained on -10, -5 and 0 dpi was
568 calculated and scaled to 100 for each animal, constituting the value at the time of inoculation.

569

570 **Figure 2**: Kinetics of viremia and viral RNAemia in the blood of the three inoculated animals (A, B
571 and C).

572

573 **Figure 3**: Evolution of pestivirus excretion by (3a) oral route, (3b) nasal route, (3c) vaginal route and
574 (3d) rectal route in the three inoculated chamois (A, B and C)

575

576 **Figure 4**: Kinetics of pestivirus-neutralizing antibodies in the three inoculated animals (A, B and C)

577

578

579

580 Table I. Clinical signs and associated scores.

581

System	Clinical sign		Score
General	Healthy		0-1
	Weakness		0-1
	Apathy		0-1
	Prostration		0-1
	Decubitus		0-1
	Lymph node hypertrophy		0-1
	Weight loss		0-1
	Death		0-10
Dermatology	Alopecia		0-1
	Ulcer	Mouth	0-1
		Lips	0-1
		Nostril	0-1
		Vulva	0-1
		Skin	0-1
	Hyperkeratosis		0-1
	Conjunctivitis		0-1
Reproductive system	Premature birth		0-1
	Blood discharge		0-1
	Stillbirth	Normal	0-1
		Mummified	0-1
		Generalized congestion	0-1
		Undeveloped	0-1
	Newborn	Malformations	0-1
		Weakness	0-1
Digestive system	Anorexia		0-1
	Excessive salivation		0-1
	Diarrhea		0-1
	Hemorrhagic diarrhea		0-1
Respiratory system	Cough		0-1
	Nasal discharge		0-1
	Lachrymal discharge		0-1
	Dyspnea		0-1
Locomotive system	Limping		0-1
Nervous system	Tremor		0-1
	Ataxia		0-1
	Paresis		0-1
	Paralysis		0-1

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Table II. General characteristics of the experimental animal and post mortem results.

General information				Post mortem results			
Identification of the animals	Status	Age (years)	Sum of clinical scores	Date of death (dpi)	Gross lesions	Histologic findings	Presence of RNA in organs
A	Inoculated	4	10	24	Diahhrea Multifocal petechial hemorrhages in digestive tract, epicardium and pericardium Pulmonary hemorrhages Generalized lymphadenomegaly	Mild lymphohistiocytic encephalitis Marked lymphoid depletion	All organs tested positive except skin
B	Inoculated	14	63	51	Emaciated Enteritis with yellowish diarrhea Thinness	Mild lymphohistiocytic encephalitis Moderate lymphoid depletion	All organs tested positive
C	Inoculated	7	7	51	Thinness Pulmonary abcess (older lesions)	Mild lymphohistiocytic encephalitis Moderate lymphoid depletion	All organs tested positive
A fetus (A-fetus)	Infected	-	-	Undetermined	Fetal mummification	N.D.	All organs tested positive
B fetus (B-fetus)	Infected	-	-	51	Normal	Multifocal acute hemorrhages in brain	All organs tested positive
C fetus (C-fetus)	Infected	-	-	46 (abortion date)	Normal	N.D.	All organs tested positive
D	Control	9	0	63	Normal	No histologic lesions	All organs tested negative
Ewe	Control	1	0	63	Normal	No histologic lesions	All organs tested negative
D fetus (D-fetus)	Control	-	-	63	Normal	No histologic lesions	All organs tested negative

N.D.: Not Done, dpi: days post inoculation

Table III. Quantitative PCR in organs (expressed in number of RNA copies/ g of organ).

Animal	Cotyledon	Brain	Kidney	Liver	Lung	Mesenteric node	Myocardium	Skin	Spleen
A	$1.5x10^{6}$	$3.4x10^{4}$	$7.5x10^{4}$	$7.5x10^{2}$	$3.4x10^{5}$	$2.3x10^{4}$	$9.4x10^{1}$	0	$2.3x10^{5}$
B	$1.3x10^{7}$	$3.4x10^{6}$	$5.9x10^{6}$	$3.4x10^{6}$	$8.7x10^{6}$	$5.0x10^{3}$	$8.4x10^{5}$	$9.7x10^{4}$	$5.6x10^{6}$
C	$1.0x10^{8}$	$1.2x10^{6}$	$4.0x10^{5}$	$1.9x10^{6}$	$3.7x10^{7}$	$2.6x10^{6}$	$1.0x10^{6}$	$1.9x10^{4}$	$3.7x10^{6}$
B-fetus	-	$1.5x10^{6}$	$2.3x10^{6}$	$2.1x10^{6}$	$1.1x10^{7}$	-	$6.5x10^{5}$	$8.7x10^{6}$	$5.9x10^{6}$
C-fetus	-	$4.1x10^{6}$	$2.2x10^{6}$	$3.4x10^{5}$	$8.7x10^{5}$	-	$2.6x10^{5}$	$2.2x10^{6}$	$4.7x10^{5}$
Ewe	-	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0
D-fetus	-	0	0	0	0	0	0	0	0

Figure 1.

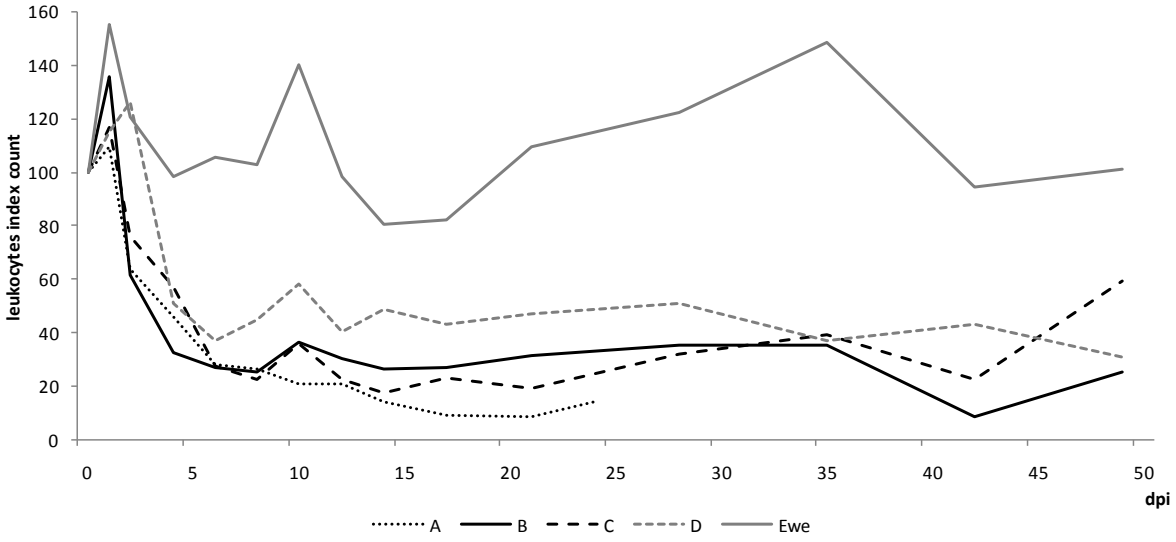
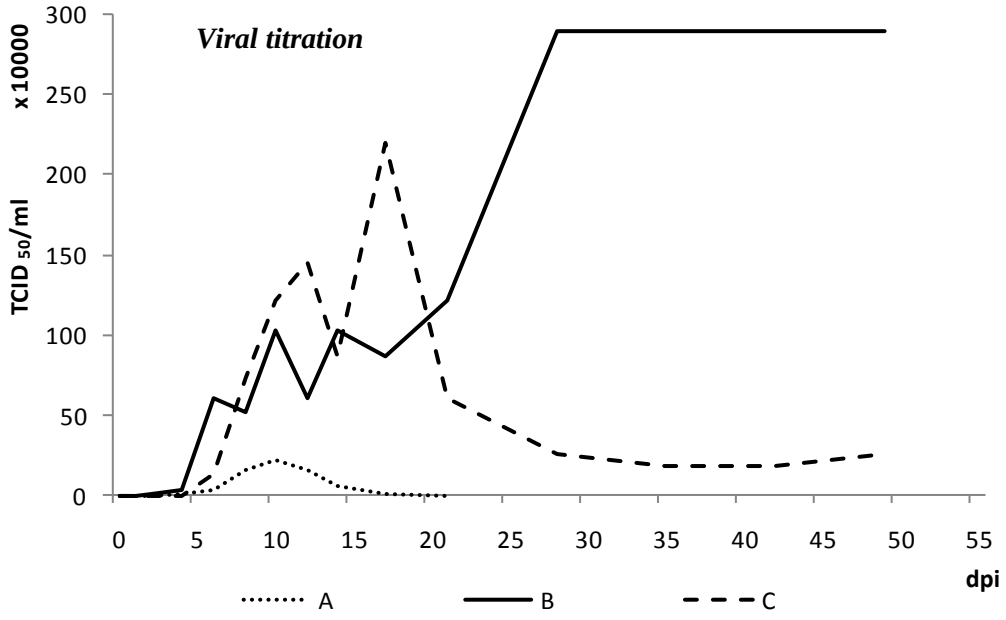

leukocytes index count
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
dpi
A
B
C
D
Ewe

Figure 2.


Viral titration
TCID 50/ml x 10000
300
250
200
150
100
50
0
0 5 10 15 20 25 30 35 40 45 50 55
dpi
A
B
C

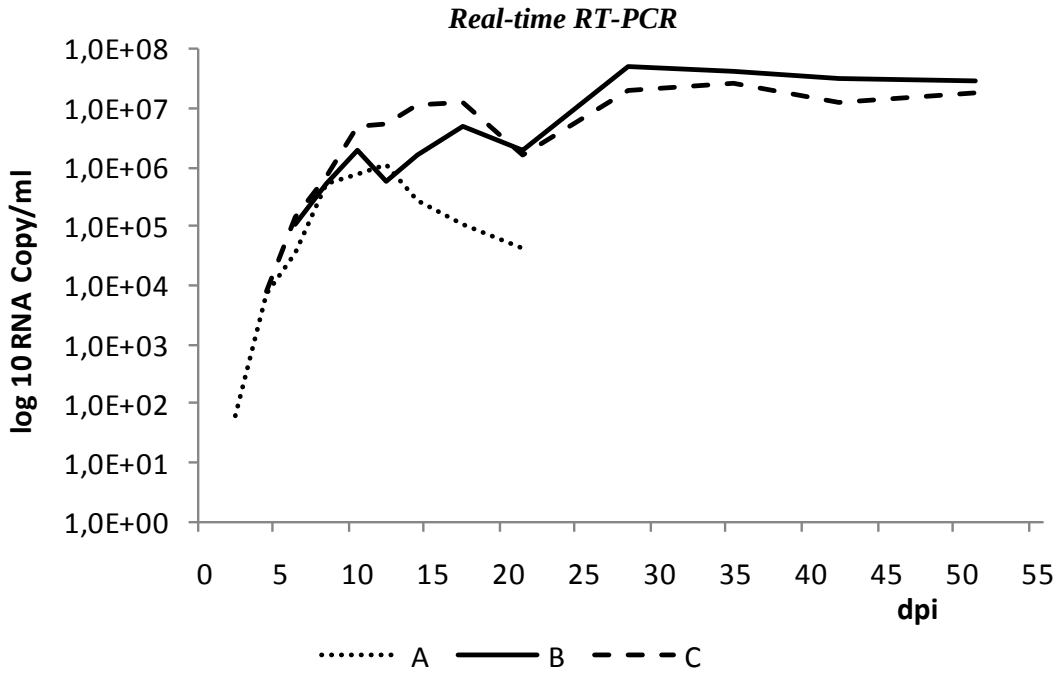

Real-time RT-PCR
log 10 RNA Copy/ml
1,0E+08
1,0E+07
1,0E+06
1,0E+05
1,0E+04
1,0E+03
1,0E+02
1,0E+01
1,0E+00
0 5 10 15 20 25 30 35 40 45 50 55
dpi
A
B
C

Figure 3.

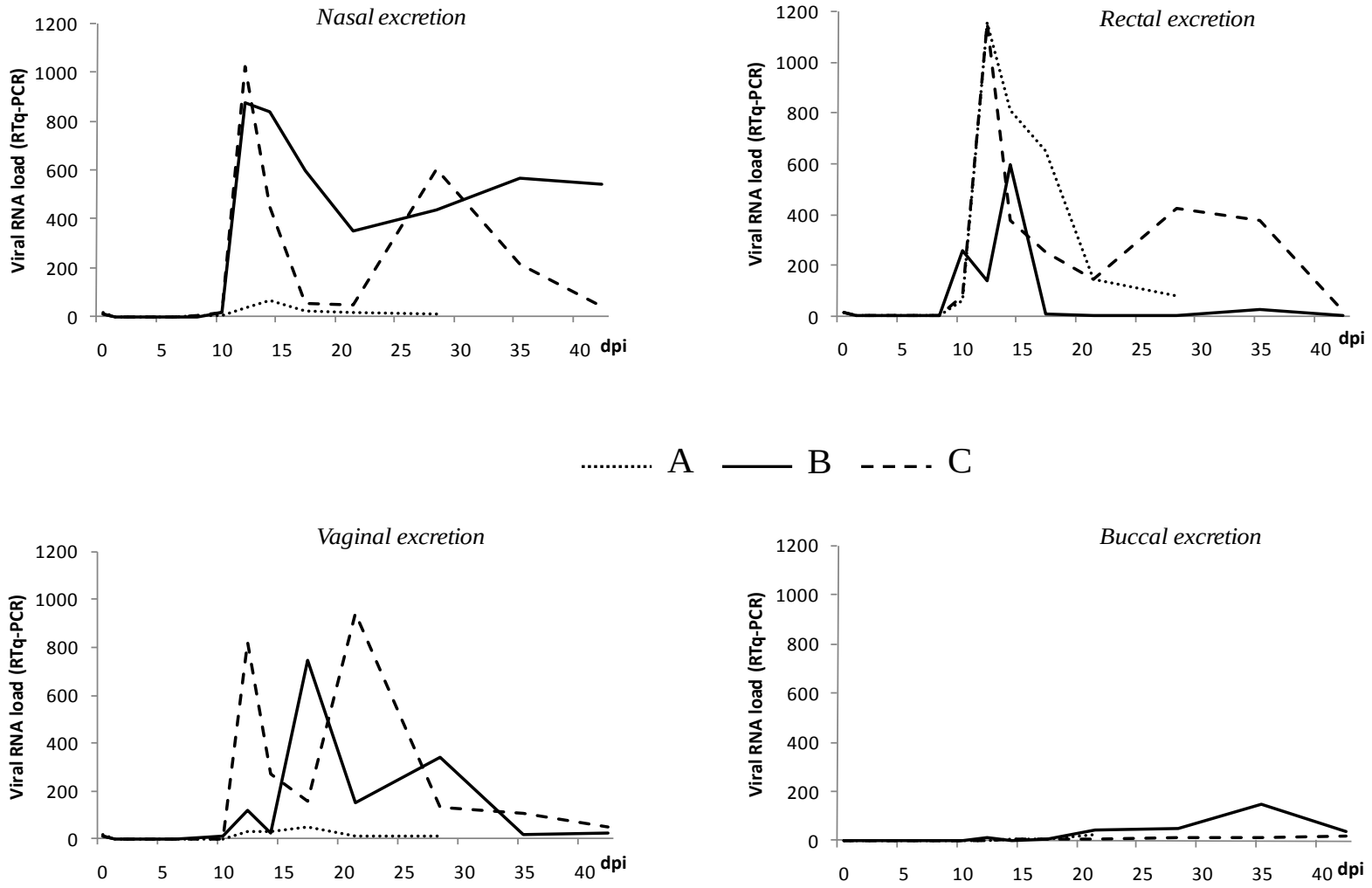
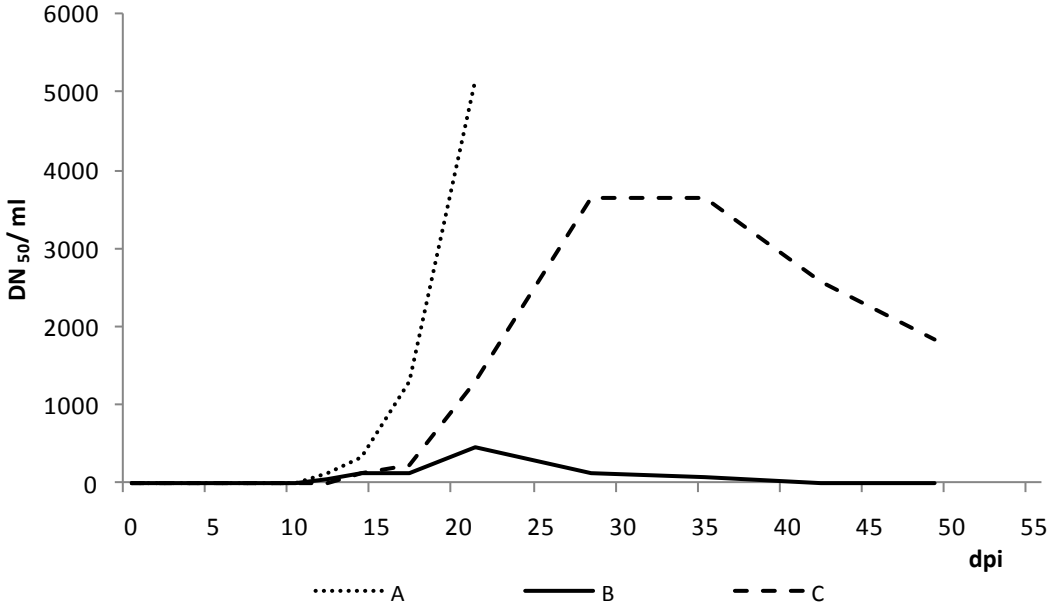

Nasal excretion
Rectal excretion
Vaginal excretion
Buccal excretion
Viral RNA load (RTq-PCR)
1200
1000
800
600
400
200
0
0 5 10 15 20 25 30 35 40 dpi
A B C

Figure 4.


6000
5000
4000
3000
2000
1000
0
DN50/ ml
0
5
10
15
20
25
30
35
40
45
50
55
dpi
A
B
C

PARTIE II :

ETUDE EPIDEMIOLOGIQUE

Cette seconde partie du travail se décompose en trois études présentées successivement.

La première est une étude bibliographique (acceptée dans *Veterinary Research*) qui a eu pour objectif de lister les infections ou maladies infectieuses déjà décrites chez les ruminants sauvages en Europe dans trois tableaux (ou « additionnal files ») : un tableau pour les infections ou maladies bactériennes, un pour les affections virales, et un pour les maladies ou infections parasitaires. Pour chacune des infections présentées, des exemples sont donnés et le rôle épidémiologique des espèces animales est décrit tel qu'énoncé par les auteurs des publications. De plus, les facteurs de risque associés aux interactions entre les animaux sauvages et domestiques ainsi que les mesures de contrôles déjà mises en place dans la faune sauvage européenne sont décrits et discutés.

Les deux dernières études présentent le travail de recherche mis en place dans la région PACA.

Une première étude épidémiologique longitudinale (acceptée dans *Veterinary Microbiology*) a été réalisée sur des prélèvements de chamois, mouflons et chevreuils obtenus de 2003 à 2007 dans le département des Hautes-Alpes. Cette étude a eu pour objectif de déterminer la prévalence des pestivirus au sein des ongulés sauvages dans ce département, et d'identifier les facteurs de risques associés à la séroprévalence trouvée. Des séroneutralisations croisées ont été mises en place dans le but de caractériser les réponses sérologiques des chamois.

Par la suite, une deuxième étude a été mise en place dans différentes zones de la région PACA chez les ruminants sauvages (chamois, chevreuils et mouflons) et domestiques partageant les mêmes alpages. Cette étude a été mise en place au sein de quatre zones situées dans la région PACA. Initialement, neuf zones avaient été pointées comme pouvant faire partie des zones d'études. Le choix de ces sites d'étude a été fait à l'aide d'une étude comparative présentée préalablement au manuscrit du dernier article (en préparation pour soumission dans *Veterinary Microbiology*).
Les objectifs de cette étude épidémiologique transversale ont été de rechercher la prévalence en anticorps ainsi que d'identifier et de caractériser des souches circulant à la fois chez les espèces sauvages et domestiques ; afin d'étudier une possible contamination croisée entre les différentes espèces de ruminants.

ARTICLE 3. ARTICLE DE REVUE

A survey of the transmission of infectious diseases/infections between wild and domestic ungulates in Europe

Publié dans Veterinary Research ("highly accessed paper")
Veterinary Research 2011, 42:70.

ARTICLE 3. Resumé

Etude des transmissions des maladies infectieuses entre les ongulés sauvages et domestiques en Europe

L'interface entre la faune sauvage et la faune domestique est actuellement un thème de recherche suscitant un intérêt grandissant dans la communauté scientifique. Bien que le nombre d'études soit en expansion, le rôle épidémiologique des animaux sauvages dans la transmission de maladies infectieuses est mal compris la plupart du temps. De multiples maladies infectant les troupeaux domestiques ont déjà été décrites dans la faune sauvage, et plus particulièrement chez les ongulés sauvages. Le premier objectif de cet article a été d'établir une liste des infections déjà reportées dans des populations d'ongulés sauvages européennes. Un premier tableau développe les infections bactériennes, un deuxième les infections virales, et un troisième les infections parasitaires ; spécifiant pour chacune les rôles épidémiologiques des espèces animales, tel qu'indiqué par les auteurs des articles. Par ailleurs, les facteurs de risque associés aux interactions entre les ongulés sauvages et domestiques sont résumés. Enfin, les mesures de surveillance de la faune sauvage mises en place en Europe sont présentées. De nouvelles perspectives de recherche sont présentées, afin de proposer des outils efficaces pour la prévention des transmissions de maladies entre les ongulés sauvages et les troupeaux domestiques.

Martin et al. Veterinary Research 2011, 42:70
http://www.veterinaryresearch.org/content/42/1/70

VETERINARY RESEARCH

REVIEW Open Access

A survey of the transmission of infectious diseases/infections between wild and domestic ungulates in Europe

Claire Martin[1,4], Paul-Pierre Pastoret[2], Bernard Brochier[3], Marie-France Humblet[1] and Claude Saegerman[1*]

Abstract

The domestic animals/wildlife interface is becoming a global issue of growing interest. However, despite studies on wildlife diseases being in expansion, the epidemiological role of wild animals in the transmission of infectious diseases remains unclear most of the time. Multiple diseases affecting livestock have already been identified in wildlife, especially in wild ungulates. The first objective of this paper was to establish a list of infections already reported in European wild ungulates. For each disease/infection, three additional materials develop examples already published, specifying the epidemiological role of the species as assigned by the authors. Furthermore, risk factors associated with interactions between wild and domestic animals and regarding emerging infectious diseases are summarized. Finally, the wildlife surveillance measures implemented in different European countries are presented. New research areas are proposed in order to provide efficient tools to prevent the transmission of diseases between wild ungulates and livestock.

1. Introduction

1.1. General introduction

The transmission of infectious diseases between wild and domestic animals is becoming an issue of major interest [1]. Scientists still lack of knowledge concerning the means and ways a large majority of infectious agents are transmitted. Wildlife can be exposed to domestic animal diseases resulting in severe consequences on their populations. On the other hand, numerous emerging infectious diseases (EIDs), including zoonoses, were shown to originate from wildlife [2,3]. Multiple publications dealing with wildlife diseases focus on zoonoses, while the present review targets the wild ungulates present in Europe (focussing on *suinae* and ruminants [4]), considering their close ecological and phylogenic relationship with livestock. The main objectives of this review are (i) for the first time, to establish a list as complete as possible of infectious agents already reported in European wild ungulates, (ii) to evaluate the possible role of both wild and domestic ungulates in the transmission of infectious diseases and (iii) to emphasize the importance of considering wildlife when studying the epidemiology of infectious diseases. Indeed, wild species may be infected by livestock pathogens and, at the same time, be a risk for the re-infection of livestock [5]. Thus, their importance in global animal health and in farming economy must be taken into account. This review is the first to list so exhaustively infectious diseases/infections already reported in European wild ungulates and, above all, to address their potential epidemiological role (e.g. reservoir, spillover, dead-end host and asymptomatic excretory animal). Bacterial, viral and prion, parasitic diseases are listed in three additional files (additional file 1, additional file 2 and additional file 3). In order to better understand the epidemiology of diseases/infections at the domestic animals/wildlife interface, global risk factors associated with the transmission of infectious diseases are reviewed. Finally, the different measures implemented by European countries regarding wildlife diseases/infections are summarized and new areas of research are suggested.

1.2. Methodology of bibliographic research

A list of bacterial, viral and parasitic diseases known to affect wild ungulates or livestock in Europe was

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established. The starting point was the list of diseases reportable to the World Organization for Animal Health (OIE). A bibliographical research was performed, combining the [name of pathogens] or the [name of the disease associated] with [ungulate] or [wildlife] or [wild ungulate] on web medical servers and databases (Medline, PubMed, CAB abstracts and ISI Web of Knowledge). Researches on prevalence or seroprevalence studies were mostly carried out from October 2008 to March 2009. No time limits of publication were imposed. For each pathogen, the most recent publications covering a maximum of European countries were selected. Furthermore, for each risk factor or perspective considered, a bibliographic review was launched in both Pubmed and ISI Web of Knowledge databases to identify the most suitable publications (fitting with keywords introduced, and illustrating problematic of concerns).

2. Current situation/status of European wild ungulates

2.1. Species and countries of concerns

This review targets wild ungulates present in the European continent (not only the European Union). They are listed in Table 1 according to their phylogenic relationship. Data about the origin of populations (natural vs. introduced) as well as their geographical distribution are adapted from a recently edited book [6].

2.2. Definition of important concepts

2.2.1. Definition of an infectious disease/infection

The definition of an infectious disease/infection is the first step towards understanding the mechanisms involved in the transmission of a pathogen between animals. The first definition was given by Koch in four postulates at the end of the 19th century. However, they are stated in a "one disease-one agent" model and are almost exclusively based on laboratory considerations. Several characteristics such as carrier state, opportunistic agents or predisposing factors are not taken into account with this definition. A disease may be currently defined as "any perturbation, not balanced, of one or more body function(s)" [7], which includes responses to infectious as well as non infectious agents [8]. In wild animals, characterized by feeding, reproduction and movements mostly independent from human activities (in opposition to domestic animals) [9], disease is strongly associated with environmental factors. Ecological factors are of major importance in the dynamics of wild populations as their survival rate and fecundity may be influenced by diseases [8]. A new concept of disease ecology recently emerged. For a well defined target population, the study of a disease/infection should be related to the study of interactions between the environment, pathogens and human activities [1,10]. For practical reasons, in this review, the term disease will be used to design both disease and infection.

2.2.2. Definitions of epidemiological roles

Studying and controlling an infectious disease implies the knowledge of all actors involved in its transmission. A reservoir, or maintenance host, "is able to maintain an infection in a given area, in the absence of cross-contamination from other domestic or wild animals" [11]. Some authors distinguish different types of reservoirs (1) true reservoir (the species alone maintains the infection), (2) accessory reservoir (maintains the infection secondarily to the main reservoir), (3) opportunistic reservoir (accidentally infected, but without serious consequences) and (4) potential reservoir (can be a reservoir for biological or ecological reasons, but, to date, has not been identified as such under field conditions) [7]. For each category, the reservoir is related to a target population [12]. Spillover hosts can maintain the infection after recurrent contacts with an external source [11]. However, the categorisation of a species is not definite and may be a question of time: the integration in the maintenance or spillover categories of hosts is dynamic as a spillover species may become a reservoir as suspected in the French Brotonne forest: cervids were initially spillover hosts for *Mycobacterium bovis* but because of a high density of animals, the infection spread among them and they now act like maintenance hosts [13]. Wildlife pathogens can also spill back to domestic animals [3]. A dead-end host may be infected by a pathogen but does not allow its transmission in natural conditions; such status may be lost by a species under modified environmental conditions [7]. Finally, an infected animal can excrete a pathogen without showing obvious clinical signs. It is important to mention that the environmental survival of pathogens may also determine wether or not an asymptomatic excretory animal may be considered as reservoir.

Although definitions seem to be clearly delimited, it is not so easy to determine the particular role of a species. Indeed, out of 295 descriptions of wildlife infections reported in the additional files, their epidemiological role is only suggested by the authors in 34.2% of cases (N = 101). Authors often lack of data concerning species interactions as well as the infection status in other species. Besides, to determine the epidemiological role of a wild species towards domestic animals, it is required to assess the real status of livestock, which might not be always the case [14].

2.3. Review of some infectious diseases already reported in European wild ungulates

A global view of infectious diseases affecting domestic animals but already reported in European wild ungulates is presented in additional file 1 (bacteria), additional file 2 (viruses and prions) and additional file 3 (parasites). The

Table 1 Classification, origin of the populations and geographical distribution of ungulates presents in Europe (from [5])

Family	*Sub-family*	*Species*	Latin name	Natural/introduction	European location
Suidae		Wild boar	*Sus scrofa*	Natural populations Introductions in Great Britain	All European countries
Cervidae	Cervinae	Chital	*Axis axis*	Introductions	Croatia, Istrian peninsula
		Fallow deer	*Dama dama*	Introductions Almost all populations are farmed animals.	All European countries
		Red der	*Cervus elaphus*	Natural populations Introductions in Corsica Introduction in Sardaigna	All European countries
		Sika deer	*Cervus nippon*	Introductions in the XIX" century	Northern Europe
		Reeves' muntjac	*Muntiacus reevesi*	Introductions in beginning of XXe century (native from China)	Great Britain
	Hydropotinae	Chinese water deer	*Hydropotes inermis*	Introductions	Great Britain
	Capreolinae	European roe deer	*Capreolus capreolus*	Natural populations	All European countries
		Elk	*Alces alces*	Natural populations	Northern Europe
		White-tailed deer	*Odocoileus virginianus*	Introductions (native from North America)	Finland, Czech Republic, Serbia, Croatia
		Reindeer	*Rangifer tarandus*	Natural populations Introduction in Iceland	Scandinavia Iceland
Bovidae	Bovinae	European bison	*Bison bonasus*	Natural populations or reintroductions	Central Europe (Poland, Byelorussia, Lithuania, Ukraine)
	Caprinae	Barbary sheep	*Amnotragus lervia*	Introductions	Spain
		Muskox	*Ovibos moschatus*	Introductions	Norway, Greenland
		Mouflon	*Ovis gmelinii*	Natural populations and introductions	All central and South of Europe
		Alpine chamois	*Rupicapra rupicapra*	Natural populations	Alpine mountains
		Pyrenean chamois	*Rupicapra pyrenaica*	Natural populations	Pyrenean mountains (France and Spain) Cantabric mountains (Spain) Abruzzi (Italia)
		Wild goat	*Capra aegragrus*	Introductions	Mediterranean islands (Balearic Islands, Crete)
		Alpine ibex	*Capra ibex*	Natural populations and reintroductions	Alpine mountains (France, Switzerland, Italy)
		Spanish ibex	*Capra pyrenaica*	Natural populations and reintroductions	Mountains of Spain and Portugal

epidemiological role of each species with respect to the pathological agent is specified. Nevertheless, it is not an exhaustive list of all diseases affecting wild ungulates as these studies only focused on pathogens affecting domestic animals. Pathogens were generally characterized by laboratory tests developed for domestic livestock. Some results such as apparent prevalence may therefore be biased [14]. In addition, the achievement of studies will also largely depend on the geographical accessibility of the region [15].

3. Risks factors associated with the transmission of diseases

A wide range of factors related to the ecology of diseases, e.g. environmental and ecological parameters, are constantly changing and will subsequently induce modifications in the transmission of pathogens. According to the OIE *Terrestrial Animal Health Code*, an EID is "a new infection resulting from the evolution or change of an existing pathogenic agent, a known infection spreading to a new geographic area or population, or a previously unrecognised pathogenic agent or disease diagnosed for the first time and which has a significant impact on animal or public health" [16]. Approximately 75% of the pathogens having affected or affecting humans for the last 20 years originate from animals [17]. Moreover, 72% of human EIDs reported between 1940 and 2004 find their origin in wildlife [18]. The role of wild ungulates as a reservoir of infectious diseases,

for both humans and livestock, is now well established [19]. Over 250 species of human pathogens have been isolated from ungulates [20]. The main factors affecting the transmission of pathogens among populations of wild ungulates are listed hereafter. Factors related to the host, the pathogen and the environmental changes are considered separately [21]. Most environmental modifications are anthropogenic because directly or indirectly linked to human activities, thus, they are expected to change with time [3]. A spatial classification (local vs. global) of the main factors involved in the transmission of pathogens between wild and domestic ungulates are illustrated in Figure 1.

3.1. Global level (national or European level)

3.1.1. Environmental changes

3.1.1.1. Distribution of geographical spaces Different factors can explain the constantly increasing interactions between wild and domestic animals. A major parameter is the growing human population, which increased four times during the previous century to now reach 6.9 billion people [22]. Such human population involves a huge and diversified protein demand constantly increasing [23]. In most European countries, large populations of wild ungulates are concentrated in small delimited areas because of high human distribution and densities. Degradation and fragmentation of wild spaces are the main anthropogenic factors associated with the emergence of diseases in wildlife [10,24]. The Food and Agricultural Organization (FAO) website [25] provides surface areas of the different type of land cover (agricultural, forestry, crops, meadows, etc.) since 1961 for almost all European countries: their evolution rates in Europe are summarized in Table 2. Until the nineties, areas dedicated to permanent crops and permanent pastures were increasing, leading to a diminution of natural landscape available for wild animals. However, a recent increase in forests areas as well as a global reduction of agricultural areas are observed, reflecting a decreasing importance of agriculture in the economy and additional space for wild populations (positive for wildlife conservation). What will be the real impact on the transmission on infectious diseases between wild animals is still to be assessed.

3.1.1.2. Chemical pollution Chemical pollution may have a negative impact on wildlife demography or disease susceptibility. Direct impact on reproductive parameters and sex ration has been described [24].

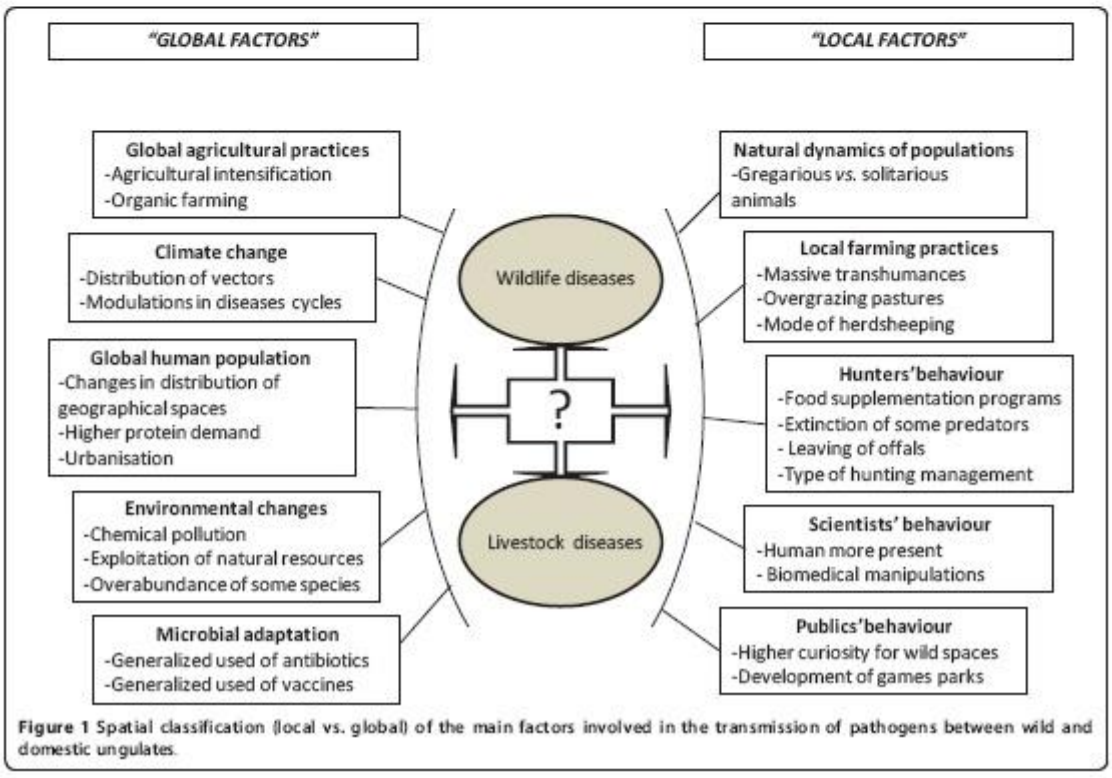

Figure 1 Spatial classification (local vs. global) of the main factors involved in the transmission of pathogens between wild and domestic ungulates.

Table 2 Evolution of European lands resources

	1990/1961	2008/2000
Country area	1.000	1.000
Agricultural area	0.993	0.967
Arable land	0.935	0.964
Arable land and Permanent crops	0.939	0.963
Fallow land	*	*
Forest area	*	**1.005**
Inland water	1.003	1.008
Land area	1.000	1.000
Other land	*	1.014
Permanent crops	**1.024**	0.948
Permanent meadows and pastures	**1.047**	0.973
Temporary crops	*	*

Ratios (i) equal 1 mean that the area stayed constant during the period considered (ii) lower than 1: diminution of the area (iii) higher than 1: augmentation of the area concerned.

These ratios were obtained dividing land areas (in 1000 Ha) of 2 years. We performed 2 ratios, [area in 1990]/[area in 1961] and [area in 2008]/[area in 2000], to have a constant total European countries area (which changed between 1990 and 2000).

*unavailable data

Data obtained from the fao website, consulted 19 December 2010 (updated on September 2010). http://faostat.fao.org/site/377/DesktopDefault.aspx?PageID=377#ancor. Request was effectuated with the selection: (i) Country: "Europe + (Total)" and "Europe > (List); (ii) Year: "1961, 1970, 1980, 1990, 2000, 2008"; (iii) Item: "Country area, Agricultural area, Arable land, Arable land and Permanent crops, Fallow land, Forest area, Inland water, Land area, Other land, Permanent crops, Permanent meadows and pastures, Temporary crops".

Immunodepression can directly result from a toxic accumulation of chemicals at subclinical levels and increase the susceptibility to infectious diseases [26]. Several studies targeting the consequences of chemical pollution on wildlife reported a direct negative impact on birds and rodents but only few studies focused on wild ungulates [27]. In France, wildlife intoxication reports are registered by the SAGIR Network, in charge of the wildlife health surveillance [28]. Twenty five percent of mammalian intoxication reports concerned ungulates, but only 2.1% of cases were confirmed by positive findings [27]. Scientists reported a biomagnification of chemical concentrations via a food-chain transfer: for instance, liver concentrations of chlordecone, a carcinogenic insecticide, were lower in herbivores (bottom of the food chain) than in carnivores, and concentrations in scavengers were still more elevated (top of the food chain) [29]. The season of sampling should be considered whenever using wildlife as an accumulative bioindicator of environmental pollution. Indeed, seasonal variability in metal levels measured in roe deer kidneys found its origin in the difference of nutrition, both quantitative and qualitative. Seasonal peaks for the majority of metals are observed in a very narrow period (summer-autumn). Some plant taxons, such as fungi, are an important pathway for heavy metal intake into the mammalian organism [30]. In addition, consequences and interactions of chemicals on the expression of a disease are not entirely elucidated yet.

3.1.2. Global agricultural practices

The last century was marked by an evolution of agricultural practices especially through industrialisation. Until the nineties, populations of "European classic livestock species" (cattle, sheep, goat, pig) were globally increasing (Table 3), along with an increase of areas dedicated to farming (Table 2). In such systems, domestic animals were genetically selected for a specific production, and as a result, they are less hardy and resistant to a high exposure rate of pathogens. However, since a few years, everywhere in Europe, public opinion is getting worried about the environment: people are in favour of an agriculture respectful of the environment. Development of organic farming is thus gaining much interest: areas dedicated to such farming were occupying more than 6% of the total agricultural areas in 2008 in Europe [25].

Table 3 Evolution of the number of living animals in Europe

	1970/1961	1980/1970	1990/1980	2000/1990	2009/2000	Global rate 2009/1961
Cattle	**1.13**	**1.15**	0.98	0.60	0.85	0.65
Goats	0.76	**1.01**	**1.28**	0.86	0.84	0.71
Pigs	**1.11**	**1.33**	**1.05**	0.77	0.94	**1.12**
Sheep	0.96	**1.04**	**1.11**	0.50	0.89	0.49
Donkeys	0.69	0.72	0.81	0.59	0.79	0.19
Buffaloes	0.89	0.85	**1.04**	0.40	**1.49**	0.47
Camels	0.86	0.97	**1.11**	0.04	0.70	0.02
Horses	0.70	0.72	0.92	0.69	0.90	0.29
Mules	0.57	0.52	0.63	0.70	0.85	0.11

Ratios (i) of 1 mean the numbers remained constant during the period of concern (ii) ratios < 1: decreased number (iii) and > 1: increased number. These ratios were obtained by dividing numbers of animals aged 2 years.

Data obtained from the fao website, consulted 19 December 2010 (updated on September 2010). http://faostat.fao.org/site/573/default.aspx#ancor. Request was effectuated with the selection: (i) Country: "Europe + (Total)" and "Europe > (List); (ii) Year: "1961, 1970, 1980, 1990, 2000, 2009"; (iii) Item: "Cattle, Goats, Pigs, Sheep, Asses, Buffaloes, Camels, Horses, Mules".

In opposite to the global intensification of agricultural practices, extensive farming systems regain interest, facilitating contacts between livestock and wildlife.

3.1.3. Microbial evolution and adaptation

Pathogens lacking intermediate stages such as viruses, bacteria or protozoans are the main recently emerged pathogens of wildlife [15]. Out of 31 pathogens identified as having a real impact on the dynamics of mammals, 41% are viruses [31]. Because of their high mutational rate, RNA viruses are perfect candidates for emergence. However, even if the evolution of pathogens plays a key role in the emergence of diseases, the ecological factors described below also favour their emergence [26].

3.1.4. Climate change

According to the last report of the Intergovernmental Panel on Climate Change (IPCC), the earth's surface and oceans temperatures are increasing by leading to the constant reduction of land snow cover and the melting of sea ice and glaciers [32]. The global mean surface air temperature increased of an average of 0.75 C since the mid-twentieth century and climate experts expect this increase to continue during the 21th century [33]. As a result, changes in ecosystems are occurring in many parts of the world: the distribution of species and timing of events in some seasonal cycles are affected [34]. In Europe, changes are less obvious than in other sensible parts of the world such as arctic or tropical ecosystems. However, epidemiological cycles are affected since the temperature threshold may modulate the cycle of vector-borne microorganisms [35]. Climate changes might favour the emergence of vector-borne diseases and be responsible of outbreaks of known diseases in regions where they were never reported before. The prevalence and distribution of well-known vector-borne diseases have already increased during the last decade [33]. In the Mediterranean region, bluetongue virus (BTV) recently emerged and became enzootic in livestock [36]. Wild ungulates were proved to be receptive to the virus in all European regions [37,38]. In southern Spain, BTV antibodies were detected in wild ruminants in areas where no outbreak had been reported in livestock, suggesting their potential role of reservoir for BTV, but this statement requires further confirmation [39]. The distribution of ticks is evolving along with climate changes. Indeed, during the last 20 years, the upper limit of tick distributions shifted from 700-800 m to 1200-1300 m above the sea level [40]. Consequences on wildlife infections were immediate: in 2005, tick-borne babesiosis was reported for the first time in chamois (*Rupicapra rupicapra*) in Switzerland [41].

3.1.5. Global increased mobility and trade

The last decades were marked by an increased human and animal mobility as well as a constantly evolving animal trade. The translocation of wild or domestic animals is one of the major factors responsible for the introduction of diseases. The trade of living animals was multiplied by a factor 10 between 1995 and 2005: global imports and exports were respectively 8.8 and 13.5 times more important in 2005 than in 1995 [42]. Transports are often carried out under very poor conditions because animals are piled up and stressed. Their susceptibility to infections increases. Even if it mainly concerns species other than ungulates, wildlife trade is one of the main problems in a potential cross-species transmission of infectious agents [43]. One should also consider (re)introduction of wild animals for hunting purpose when focusing on wildlife trade. The presence in Europe of most non-native species of ungulates may be explained by such practices. It is currently almost impossible to quantify the global wildlife trade as it is mostly illegal. However, the economic impact resulting from outbreaks caused by wildlife trade has globally reached hundreds of billions dollars to date [23]. Spatial mobility of humans was multiplied by more than 1000 since 1800. A 222% increase is expected for the number of passenger per km by 2035 [44]. As the incubation period of most infections exceeds the time necessary to transfer an animal from a country to another [45], the propagation of pathogens and vectors has reached an unprecedented rate.

3.2. Local level (regional or district)

3.2.1. Natural dynamics of populations

The social organisation of populations impacts the transmission rate of infections: the probability of contacts is higher for gregarious animals than for solitary species. Besides, the reproduction period is characterised by increased contacts between individuals [9]. Furthermore, the exposure to pathogens depends on the presence/absence of migratory flows [3]. European wild ungulates are not migratory animals as such, except reindeer (*Rangifer tarandus*). Nevertheless, once wild populations colonize and occupy a given area, some animals might later radially disperse to close areas and be at risk for contamination [5]. Natural and artificial barriers are likely to limit animal movements and may thus reduce the transmission of pathogens.

3.2.2. Human behaviours

Contacts between wildlife and livestock are also increasing because behaviours of farmers, hunters, scientists and the general public are changing.

3.2.2.1. Farmers Along with a global change of agricultural practices at the European scale, it is important to consider local agricultural practices. Changes of farmers' behaviours mostly impact contact rates between wild and domestic ungulates. Pastures are places where the transmission rate of infectious diseases is the highest

[46]. Farmers' management of pastures are thus of major importance. Some practices such as salt deposits in alpine pastures enhance the risk of indirect transmission of pathogens, like *Pasteurella* for example [47]. Mountain transhumance (summer moving of domestic flocks to alpine meadows) was initially performed at walking-distance. Nowadays, flocks are moved by cattle-trucks, allowing long-distance transportations of more animals; alpine meadows are overgrazed and the probability of contacts with wildlife increases. Besides, whereas initially created to protect biodiversity, national parks allow domestic flocks to graze inside their central part in some countries, which may have detrimental effects for both sides.

*3.2.2.2. **Hunters*** Hunting behaviours may play a major role in the transmission of diseases between or among wild populations. Food supplementation programs implemented to increase the number of hunting bags have drastically disturbed the natural regulation and spatial distribution of populations. Various wild populations, e.g. wild boar [48] or red deer [49], are constantly growing. For example, in Wallonia (Belgium), red deer and roe deer populations have increased twofold while wild boar populations have more than tripled between 1980 and 2005 [50]. In some other European areas, populations are overabundant. The hunting of predators led to their extinction and a subsequent imbalance of interactions between species. Offals of dead wild ungulates are generally left in the field, which may reach at the European scale thousands of tons of potentially infected materials in free access to other species. When an infectious disease is prevalent in wild populations, directed shots of sick animals are often applied. However, during a recent outbreak of infectious keratoconjonctivitis in Alpine wild ungulates, such measure seems to have prevented the natural immunisation of populations (Gauthier, personnal communication). A global reduction in hunting pressure may therefore be preferred, especially to protect reproductive adults.

*3.2.2.3. **General public*** For many city dwellers, contacts with nature are limited to controlled areas such as national parks or wildlife game parks. National/regional natural areas are government parks, of which the first objective is to protect natural lands (ecosystems). Wild ungulates may or may not be hunted in function of local legislation. In these opened parks, public frequentation is constantly increasing, as people are in search of a closer contact with nature under protected conditions. The frequency of contacts between wild species and humans increases as a consequence of natural tourism [51]. Wildlife game parks could be associated to 'game zoos': species belonging to the native European wild fauna are parked in closed areas. Densities of populations are often high and animals are frequently translocated between different parks. The high density rate can be implicated in the transmission of diseases [52]. Deer farming is promoted by several European governments like Switzerland [53]. In France, 400 deer farms are inventoried [54]. The proximity of several species (including humans) will subsequently play a key role in the contact rate.

*3.2.2.4. **Scientists*** More and more scientific studies focus on monitoring of wild populations. Even if carefully controlled, the intrusions of scientists may be a risk of disease transmission. Even if some introduction programs prevent animal transfers from one region to another, or between different countries, some wounded animals are brought to health cares and released after successful treatment. While it mainly concerns wild species other than ungulates, such practices can also increase the risk of diseases transmission.

4. Control measures of infectious diseases already implemented in European wildlife

The section below develops the measures already implemented or to be implemented by European countries to control the transmission of diseases between wild and domestic animals, at three different levels: (i) European; (ii) national, (iii) regional (local).

4.1. At European level

The continuity between all living beings involved in the transmission of infectious diseases must be treated from an international point of view.

4.1.1. Wildlife-livestock-human continuum

As previously described, the importance of contacts between wildlife, livestock and humans is such that some authors suggested a "wildlife-livestock-human continuum" [55]. In 2008, King suggested to use the term "interdependence" instead of "independence" of these three compartments [56]. As a consequence, a new concept of conservation medicine emerged for the protection of animal, human and ecosystem healths [57]. The main goals are to promote the development of scientific studies for problems occurring at the interface between environmental and health (human and animal) sciences [58]. In this context, studies of the community ecology should be performed, in order to better understand the epidemiological links between all actors of the wildlife-livestock-human-continuum [59].

4.1.2 Biodiversity and wild heritage

As already mentioned, infectious diseases affecting wildlife have several impacts such as depletion of populations and rare species (on their own or in concert with other factors) but management actions also have an environmental impact [60]. Nevertheless, if diseases are a risk for wildlife conservation, preserving biodiversity helps also avoiding their emergence. For example, the

prevalence of vector-borne diseases will decrease if the variety of food sources (native hosts) increases, as the infestation rate within each species will be reduced [61].

4.1.2.1. Wild mammals The first modern complete inventory of mammals was established in 1982, with a list of 4 170 species identified (cited in [62]). The 1993-inventory included 4 629 different species [63]. In 2005, the complete list of mammals indexed 5 416 species the total number being estimated at around 5 500: 99% of mammalian species are thus probably already known [64]. Such increasing number of identified species is due to the separate listing of newly discovered phenotypes and genotyping through molecular biology (taxonomic revision). Two hundred and forty species of *Artiodactyla* pertaining to 89 genera are described, most of them living in the biodiversity "hot spots" located in Sub-Saharan Africa. European species of *Artiodactyla* are by contrast less numerous (see Table 1).

4.1.2.2. Domestic species Through selection, man created numerous breeds of domestic animals, e.g. there are approximately 700 breeds of cattle identified worldwide [65]. Nevertheless, many of them are on the verge of extinction, decreasing the genetic variability of cattle.

4.1.2.3. Role of biodiversity in disease ecology The influence of human activities on endangered and unmanaged wild fauna is of major concern. Out of 31 cases of disease emergence in wildlife, only 6 were not influenced by humans [15]. Eighty-eight percent of mammals at risk for severe infections and listed by the International Union for Conservation of Nature (IUCN) Red List of Threatened and Endangered Species are carnivores or artiodactyls [31]. Most livestock and companion animals belong to these categories. The degradation of ecosystems, the loss of habitats and diminishing food resources force some species to use alternative alimentary sources [1]. Biodiversity acts as a primordial barrier against infectious pathogens. Besides, anthropogenic factors causing losses of biodiversity increase the risk of disease emergence [26] by modifying the abundance, the behaviour or the condition of hosts or vectors [66]. It is then crucial to preserve biodiversity in an integrated and sustainable manner [67].

4.1.3. OIE working group on wildlife diseases

In order to develop specific surveillance guidelines for wildlife diseases, the OIE recently created a Working Group on Wildlife Diseases [68]. It provides information on the wild animal health status, either in the wild or in captivity. Its most important missions are: (i) the elaboration of recommendations and the reviewing process of scientific publications on wildlife diseases; (ii) the implementation of surveillance systems of the wildlife-domestic animals-human continuum and (iii) the control of emerging and re-emerging zoonoses.

4.1.4. Prioritization of wildlife diseases

Based on an OIE imported framework, a method of "rapid risk analysis" was developed in New Zealand with the aim to prioritize pathogens for the wildlife disease surveillance strategy [69]. Authors first listed all wildlife pathogens likely to interfere with animal or human health. They selected the pathogens likely to have a serious impact on wildlife, livestock and/or humans, after consulting experts of each sector. The risk estimate for each pathogen was scored on a semi-quantitative scale (from 1 to 4). The likelihood and consequences of spread were assessed for free-living and captive wildlife, livestock (distinction between consequences on productivity, welfare and trade), humans and companion animals. The risk of introduction in New Zealand was also assessed (scores: 0 or 1). Finally, pathogens were ranked and authors listed the top exotic and endemic dangerous wildlife pathogens for each population of interest (wildlife, domestic animals or human). Summing the risk estimate for each population gave a "total risk estimate" [69]. In Europe, the French agency for food, environmental and occupational health safety (Anses) multidisciplinary working group also elaborated a two-phase risk prioritization method [35]: (i) identification of diseases of which the incidence or geographical distribution could be affected by climate change, (ii) the risk assessment for each disease. Twenty diseases likely to be influenced by climate changes were selected. The authors qualitatively assessed the risk of each disease for its impact on human and animal health and on economy, considering the likelihood of disease evolution and the impact level. Three diseases affecting ungulates were selected for which some measures needed to be implemented (BTV, Rift Valley Fever and African horse sickness).

The prioritisation of diseases is useful to (re)-direct and target funds allocated to diseases surveillance and research. Organisms involved in wildlife conservation will be more inclined to financially support the control of wildlife diseases [69]. However, several current EIDs should in fact be considered as re-emerging [70]. To focus wildlife surveillance on prioritized agents could lead to a reduced vigilance/surveillance of "old" diseases. Their implementation in a global surveillance of wildlife diseases should be conducted carefully.

4.2. At country level

Some decisions will depend on the organization of national governments and bodies in charge of sanitary surveillance.

4.2.1. Surveillance programs

Disease surveillance is defined by the World Health Organization (WHO) as "the ongoing systematic collection, analysis and interpretation of data but also the

dissemination of information to the different actors involved in wildlife management" [71]. For the OIE, surveillance is "aimed at demonstrating the absence of disease/infection, determining the occurrence or distribution of disease/infection, while also detecting as early as possible exotic or emerging diseases" [72]. Several European Member States (MSs) have already implemented a health monitoring of their main wild populations. Surveillance systems of wildlife diseases are usually declined in passive surveillance, which consists in reports and necropsies of all animals found dead, and active surveillance, declined as the sampling of some populations in order to assess the (sero)-prevalence of infections. Such systems are now well developed in Belgium [37], Spain (Gortazar, personal communication), France (SAGIR Network) [73] and Switzerland (Ryser-Degiorgis, personal communication). A National Health Surveillance Program for cervids (HOP) was implemented in Norway in 2001 [74]. In Sweden, a monitoring of wildlife health exists since 1945 and became an integrated part of the National Environmental Monitoring Programs [75].

Such systems should be developed at a larger scale. Each State should be able to provide relevant information on the health status of its wild populations. To help other countries developing surveillance systems, it may be interesting to provide guidelines with different modalities in function of the specific epidemiological situation. Standardization of protocols between the different countries would permit a better global and harmonized evaluation of diseases status, and would allow the implementation of an efficient surveillance system. Moreover, the implementation of epidemiological surveillance should be based on both epidemiological (regular collection and analysis of epidemiological information and early warning systems for animal diseases) and ecological monitoring (surveillance of vectors and wild reservoirs) [35].

4.2.2. Vaccination programs

Several reasons may justify the implementation of vaccination programs in wild animals: (i) conservation of endangered species, (ii) reduction of disease impacts, (iii) protection of human health (zoonotic agents) and (iv) prevention of transmission to domestic animals (and subsequent economic losses) [58]. Besides, vaccination is an alternative to global culling of wild reservoirs. However, it is important to keep in mind the goals of a vaccination programme. Indeed, a safe and effective vaccine can be used in restricted threatened populations and provide expected results. To eliminate a pathogen in a large area or in large populations, vaccination programs may be used in a multiple-hosts system or at a too-large scale and be unsuccessful. The majority of available vaccines have been developed for domestic animals, and their efficacy and safety are in most cases unknown for wildlife. An ideal vaccine for wildlife should be (i) administered *per os*, (ii) mono-dose (iii) safe for target and non-target species and, if possible, (iv) inexpensive to produce [76]. For example, in Europe, vaccination programs have been implemented in wild boar for classical swine fever (CSF). In France, a quantitative and retrospective study showed that a preventive vaccination (using oral baits) in a determined region improved the control of CSF, but did not eradicate the disease [77]. For multi-hosts pathogens such as *Mycobacterium bovis*, vaccination programs may be more difficult to implement [78], the previous identification of reservoir(s) being essential. Vaccination programs against *M. bovis* were recently started in the UK for badgers [76] or in Spain for wild boar [79]. In conclusion, vaccination programs can be used in wildlife under specific conditions, especially for small populations or in restricted areas [58].

4.2.3. Sentinel animals

A sentinel species is an animal/species different from the target animal/species. The use of sentinel animals may be applied in three main situations: when adequate sampling of the target species is difficult (e.g. rare or endangered species), when the sentinel species is more abundant (e.g. use of sentinel chickens instead of wild birds for West Nile virus monitoring) and finally, when the species provides useful information on lower trophic level (e.g. the study of scavengers or carnivores) [8,80]. The place a species occupies in the food chain determines its probability of contamination [81]. The target and the sentinel population must be epidemiologically linked, at least spatially and the response of sentinel animals against a particular pathogen must be demonstrable [82]. For example, red deer are used as a sentinel species for the surveillance of BTV in Spain [38].

4.3. At local level (district or region)

(Inter)-national regulations must be implemented at local levels also, involving the participation of local structures, such as farmers groups or hunter organisations.

4.3.1. Adaptation of livestock farming

Wild animals are often considered as reservoir of infectious diseases [19]. However, in many cases, infections originate from domestic animals. For instance, bovine herpesvirus 1 (BoHV-1) can induce a moderate infection in deer, whereas cattle is not at risk for the cervid herpesvirus 1 [83]. Thus, contacts should be limited but, at best, avoided between wild fauna and livestock [67]. In some regions of North America, brucellosis became endemic among wapitis (*Cervus elaphus*) and bisons (*Bison bison*). Bisons were infected by cattle around 1900, and the disease became endemic in those wild

populations after their release. Although this example concerns non-European wild populations, the measures implemented are interesting to develop in this review. Despite the implementation of feedgrounds and vaccination, habitat improvement and prevention of commingling, livestock still remains infected. Other management options were then proposed: (i) removing cattle from public lands, (ii) developing and implementing brucellosis vaccines more effective for elks and bisons, (iii) managing cattle through vaccination and physical separation from elks and bisons and (iv) using contraceptives in elks to reduce pregnancies and abortions [84]. In the U.S. Sierra Nevada, a model assessing the impact of different management strategies of domestic sheep (grazing allotment closure, grazing time reductions and reduced probability of contact with stray domestic animals) on the transmission of respiratory diseases from domestic herds to endangered bighorn sheep was built [85]. In order to reduce the risk of disease transmission, the best solution was to avoid an overlapping between domestic sheep and bighorn sheep grazing areas.

Such epidemiologic studies show the importance of identifying and assessing the risks in order to implement preventive measures. Efforts should be devoted towards avoiding contacts between wild and domestic animals. Compartmentalisation and zoning are biosecurity measures advised by the OIE *Terrestrial animal health code* to avoid contacts between domestic and wild animals. However, such measures are often impossible to achieve in field conditions. The total surface area of the European continent occupied by national parks, protected zones where grazing is forbidden, is in fact very limited [83]. Efforts should be devoted to improve biosecurity in farms. In the UK, cattle often contract *Mycobacterium bovis* tuberculosis in pasture contaminated by badger excreta [86]. In order to reduce the risk of contamination in pasture, different practices such as the presence of ungrazed wildlife strips, and the greater availability, width and continuity of hedgerow may be proposed. The management of grazing has shown to reduce the risk of contamination. Here are other examples of efficient measures: rotational grazing system, off-fencing of setts and latrines, the avoidance of grazing pasture too short, the non-introduction of cattle to recently cut fields, the moving of cattle to fresh pasture in the afternoon and the absence of supplementary feeding on pasture [87].

4.3.2. Specific hunting measures

While hunters may play an important role in the transmission of diseases, they can also be important for their control. Indeed, most scientific studies dealing with infectious pathogens in wildlife require an effective collaboration with hunters, as sampling is facilitated on carcasses of hunted animals. Such collaborations should be promoted at a larger scale. Besides, the establishment of controlled management plans for different known diseases should be promoted.

5. Perspectives

Interdisciplinary collaboration is a requisite to the success of management programs. Studies involving biologists, ecologists, veterinarians, epidemiologists and medical doctors should then be promoted. Nevertheless, further research is needed to clearly assess all consequences of the diseases transmitted between wildlife, livestock and humans. A better knowledge of wild populations (size and distribution) of each species should be promoted by applying harmonized methods among the different regions and/or countries. Besides, more studies could be performed in order to understand and analyse the infectious strains circulating among wild animals, but, above all, to compare them to strains circulating among domestic livestock. In most cases, researchers ignore if strains circulating among domestic and wild populations are similar. The epidemiological cycles of infectious diseases in all populations of concern are not well assessed to date. Then, it would be interesting to study methods of space sharing between wild and domestic animals. Costs associated as well as benefits for biodiversity and economical incentives for livestock farming should be evaluated. Because of numerous factors such as globalisation or climate changes, the threat of EIDs is clearly present. The impact of EIDs on economy and public health is not always easily predictable, and should receive more attention, through prioritization procedures for example. Awareness campaigns of politics via a direct estimation of costs generated by EIDs would allow funding research projects for wildlife health surveillance. Ecology and protection of the environment should also be integrated in research programmes without neglecting the surveillance of already known 'old' diseases.

To focus wildlife surveillance on prioritized agents could lead to a reduced vigilance/surveillance of "old" diseases. Their implementation in a global surveillance of wildlife diseases should be conducted carefully. The implementation of surveillance programs and research studies is not achievable without the involvement of local partners. However, the latter often complain about significant discordances between research (most of the time carried out at the European Union level) and field conditions (regional level). Awareness campaigns and a better communication between all sectors would ensure a better involvement of all surveillance actors and thus benefit to the global system. For example, the attribution of definite roles at the different levels would provide a more efficient distribution of work. Furthermore,

information provided by the surveillance of wildlife should be available for the whole scientific community, in order to facilitate the development of spatio-temporal epidemiological methodologies to improve and refine it. Such approach would encourage interdisciplinary collaborations by involving all partners. Surveillance programs have already been implemented in wildlife such as the PREDICT project [88] developed by the Davis University of California: it uses a risk-based approach focused in areas where zoonotic diseases are most likely to emerge and where host species are likely to have significant interaction with domestic animals and high density human populations [88]. This proactive novel approach should be adapted to the specific EU situation. For some domestic species, epidemiologic networks are already in place, such as the RESPE network (Epidemiosurveillance Network of Equine diseases) in France [89]. This network is based on the existence of different specialized networks. It involves owners/farmers, veterinarians and laboratories. The role of each member is well definite, which comes out onto a well-working network. Besides, decisional trees may be suggested to local partners in order to adapt their management of wild populations and surveillance of diseases. These trees may propose different approaches for the populations' management in function of diseases or clinical signs reported. Such trees may simplify the decision making for local partners, when, for example, an epizooty starts in wildlife populations. Management plans will then be adapted more easily and more quickly.

A preliminary stage would be to categorise the diseases according to different parameters such as its mode of transmission, its pathogeny or the type of clinical signs it generates. Demographic specificities of the populations of interest (gregarious vs. solitary) must be taken into account also. According to the category of disease and the type of populations, management plans may be well adapted or not.

6. Conclusion

In 2004, King [45] reminded that knowledge and strategy were still missing for the prevention and control of wild animal diseases. Nowadays, governments and scientists become aware of the necessity to provide means for research on wildlife; scientific studies focusing on wildlife ecology as well as surveillance programs are indeed in expansion [1]. Nevertheless, numerous factors influencing the transmission and ecology of diseases reached a threshold without precedent, and are of major concern for the control of wildlife diseases, such as increasing pressure of humans on natural ecosystems and rising interactions between the different species. A better surveillance of wildlife diseases implemented in an integrated system involving international, national and local actors would be of major relevance to understand the origin of diseases and subsequently to control them. Efforts are required to reduce disagreements and misunderstandings between all actors involved in sanitary surveillance of wildlife. The preservation of biodiversity is crucial for diminishing the risk of disease transmission, as well as the improvement of farm biosafety.

Additional material

Additional file 1: [90-137]. Selected bacterial diseases reported in wild ungulates in Europe. This file is a table presenting a list of bacterial diseases already reported in wild ungulates in Europe.

Additional file 2: [138-171]. Selected viral diseases reported in wild ungulates in Europe. This file is a table presenting a list of viral diseases already reported in wild ungulates in Europe.

Additional file 3: [172-202]. Selected parasitic diseases reported in wild ungulates in Europe. This file is a table presenting a list of parasitic diseases already reported in wild ungulates in Europe.

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Authors' contributions
CM and CS participated in the conception and the design of the survey. CM carried out the majority of the bibliographic search and the redaction of the manuscript. PPP and BB revised the manuscript. MFH participated in the English improvement and in the revision of the manuscript. CS participated in the coordination as well as in the revision and commenting of the manuscript. All authors read and approved the final manuscript.

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Additional file 1. Selected bacterial diseases reported in wild ungulates in Europe

Pathogen	***Ungulate specie (Latin name)***	**n**	**N**	**Prevalence**	**Sero-prevalence**	**Diagnostic method**	**Epidemiological role from author's opinion**	**Years**	**Country**	**Reference**
Anaplasma spp. (Anaplamosis)	*Capreolus capreolus*	14	17		X	ELISA	Reservoir	2005	Spain	[90,90]
Anaplasma ovis (Anaplasmosis)	*Capreolus capreolus*	9	17	X		PCR	Reservoir	2005	Spain	[90]
Anaplasma phagocytophilum (Anaplasmosis)	*Capreolus capreolus*	23	72	X		ELISA	Reservoir	2003	Poland	[91]
		18	132	X		PCR	Unspecified	2004	Italy	[92]
		?	56		94%	IFA	Unspecified	1998	Slovenia	[93]
		19	96	X		PCR	Reservoir	2001	Italy	[94]
		52	121	X		PCR	Reservoir	Unspecified	Austria	[95]
		217	227		X	IFA	Reservoir	2002-2003	Denmark	[96]
		101	237	X		PCR	Reservoir	2002-2003	Denmark	[96]
		1	-	(case report)		PCR	Unspecified	2004	Norway	[97]
	Cervus elaphus		32		35%	IFA	Unspecified	1998	Slovenia	[93]
		15	150		X	ELISA	Reservoir	2000-2003	Spain	[98]
		2	7	X		PCR	Unspecified	Unspecified	Austria	[95]
	Bison bonasus	5	8	X		Nested PCR	Reservoir	2003	Poland	[99]
	Dama dama	31	70		X	IFA	Unspecified	2004-2005	Italy	[100]
		21	29	X(μ)		PCR	Unspecified	2004-2005	Italy	[100]
Borrelia burgdorferi (Lyme disease)	*Capreolus capreolus*	83	227		X	IFA	Unspecified	2002-2003	Denmark	[96]
	Rangifer tanrandus(&)	1	13		X	ELISA	Unspecified	1969-1998	Germany	[101]
	Alces alces (&)	2	13		X	ELISA	Unspecified	1969-1998	Germany	[101]
	Ovis amnon (&)	3	18		X	ELISA	Unspecified	1969-1998	Germany	[101]
Brucella suis (Brucellosis)	*Sus scrofa*	168	763		X	ELISA	Zoonotic reservoir	1995-1996	Germany	[102]
		98	424		X	RBT+CFT ELISA	Possible reservoir	2003-2004	Croatia	[103]
Brucella suis biovar 2 (Brucellosis)	*Sus scrofa*	6	93	X		Isolation	Possible reservoir	2003-2004	Croatia	[103]
	Sus scrofa	198	1841	X		Bacteriology test	Unspecified	2001-2007	Italy	[104]
Brucella suis biovar 3	*Sus scrofa*	1	93	X		Isolation	Possible reservoir	2003-2004	Croatia	[103]
Brucella melitensis biotype 2	*Capra ibex*	1	7	(case report)		Isolation	High seroprevalence (4%) present among domestic animal in the area	1996	Italy	[105]
Brucella melitensis	*Rupicapra rupicapra*	1		(case report)		Isolation	Sporadic case	1988	France	[106]

biotype 3										
Brucella spp. (Brucellosis)	*Cervus elaphus*	2	54		X		Unspecified	Unspecified	France	[107]
			5821		0.4 [0.3-0.6]	ELISA	Not reservoir	1999-2009	Spain	[108]
	Rupicapra pyrenaica		1410		0.8 [0.4-1.4]	ELISA	Not reservoir	1999-2009	Spain	[108]
	Capra pyrenaica		1086		0.1 [0-0.6]	ELISA	Not reservoir	1999-2009	Spain	[108]
	Capreolus capreolus	0	696		X	Unspecified	Unspecified	Unspecified	France	[107]
	Sus scrofa	180	1821		X	ELISA	Unspecified	2001-2003	Switzerland	[109]
		15	342		X	ELISA	Unspecified	2005-2006	Italy	[110]
			4454		33 [31.6-34.4]	ELISA	Possible threat	1999-2009	Spain	[108]
		448	2267		X	RBT + CFT	Unspecified	2001-2007	Italy	[104]
		62	211		X	RBT + CFT	Unspecified	1996-2000	Croatia	[103]
Campylobacter jejuni	*Capreolus capreolus*	1	38	X		Culture	Unspecified	2002	Norway	[74]
Chlamydia spp.	*Capreolus capreolus*	5	155		X	Complement fixation	Unspecified	1979	France	[111]
	Bison bonasus	28	60		X	CFT	Unspecified	1980-1983	Poland	[112]
Chlamydia abortus	*Sus scrofa*	2	14	X		PCR + sequencing	Possible reservoir	2002	Germany	[113]
Chlamydia psittaci	*Sus scrofa*	4	14	X		PCR + sequencing	Possible reservoir	2002	Germany	[113]
Chlamydia suis	*Sus scrofa*	2	14	X		PCR + sequencing	Possible reservoir	2002	Germany	[113]
Chlamydophila pecorum	*Rupicapra rupicapra*	1	-	(case report)		Isolation	Unknown	Unspecified	Italy	[114]
Chlamydophila abortus	*Capra ibex*	3	306		X	ELISA	Unspecified	2006-2008	Switzerland	[115]
Coxiella burnetti (Q fever)	*Capreolus capreolus*	3	175		X	Complement fixation	Unspecified	1979	France	[111]
		4	78	X		PCR	Unspecified	2001-2006	Spain	[116]
		6	39		X	IFA	Possible reservoir	2004-2005	Spain	[117]
	Cervus elaphus	1	54		X	Unknown	Unspecified	1982-1985	France	[118]
		34	116		X	IFA	Possible reservoir	2004-2005	Spain	[117]
	Bison bonasus	7	60		X	CFT + MAT	Unspecified	1980-1983	Poland	[112]
		36	47		X	Unknown	Endemic disease	Unspecified	Poland	[119]
	Capra ibex	8	269		X	ELISA	Unspecified	2006-2008	Switzerland	[115]
	Sus scrofa	4	93	X		PCR	Unspecified	2001-2006	Spain	[116]
Escherichia coli	*Cervus elaphus*	3	206	X		PCR	Reservoir	2005-2006	Spain	[120]
Francisella tularensis (Yersiniosis)	*Sus scrofa*	24	763		X	ELISA	Zoonotic reservoir	1995-1996	Germany	[102]
Foot necrobacillosis complex (#)	*Rangifer tarandus tarandus*	100	3000	X		bacteriological examination + PCR	Independent cases	2007	Norway	[121]

Leptospira interrogans (Leptospirosis)	*Capra ibex*	2	153		X	MAT	Unspecified	2006-2008	Switzerland	[115]
	Sus scrofa	9	342		X	MAT	Unspecified	2005-2006	Italy	[110]
	Bison bonasus	35	60		X	MAT	Cross reaction	1980-1983	Poland	[112]
Mycobacterium bovis (Tuberculosis)	*Cervus elaphus*	9	72	X		Culture	Reservoir	2001-2002	France	[13]
		33	138	X		Culture	Reservoir	2005-2006	France	[13]
		86	543	X		Gross lesions	Spill over	1999-2004	Spain	[122]
		1		(case report)		Isolation	Unspecified	1991	Czech republic	[123]
		26	95	X		Culture	Unspecified	2006-2007	Spain	[124]
		33	121*	X		Culture	Unspecified	1996-2002	Spain	[125]
	Capreolus capreolus	1	53	X		Culture	Spillover		France	[13]
	Sus scrofa	25	85	X		Culture	(£)	2001-2002	France	[13]
		65	155	X		Culture	(£)	2005-2006	France	[13]
		269	474	X		Gross lesions	Spill over	1999-2004	Spain	[122]
		51	96*	X		Culture	Unspecified	1996-2002	Spain	[125]
		65	126	X		Culture	Reservoir	2006-2007	Spain	[124]
		3		(case report)		Isolation	Unspecified	1992	Slovakia	[123]
	Dama dama	60	89*	X		Culture	Unspecified	1996-2002	Spain	[125]
		18	97	X		Culture	Reservoir	2006-2007	Spain	[124]
	Bison bonasus	12		(case report)		Isolation	Unspecified	1997-1999	Poland	[123]
	*Capra aegragus**	1		(case report)		Isolation	Unspecified	1991	Czech republic	[123]
	Ammotragus lervia	33	67		X	ELISA	Possible reservoir	1999	Spain	[126]
Mycobacterium avium subsp paratuberculosis (Paratuberculosis)	*Alces alces*	10	537		X	ELISA	Unspecified	1992-1999	Norway	[127]
	Cervus elaphus	106	709*	X		RFLP	Unspecified	1999-2001	Czech Republic	[128]
			95	42.6 [95% CI : 32.6-52.6]		PCR	Unspecified	2006-2007	Spain	[129]
		257	852		X	ELISA	Unspecified		Spain	[130]
		14	371		X	ELISA	Unspecified	1998	Norway	[127]
	Ammotragus lervia	13	67		X	ELISA	Possible reservoir	1999	Spain	[126]
	Dama dama	4	385*	X		RFLP	Unspecified	1999-2001	Czech Republic	[128]
			101	65.1 [95% CI : 55.1-75.1]		PCR	Unspecified	2006-2007	Spain	[131]
		1	94	X		PCR	Unspecified	2001-2003	Spain	[131]
		2	5	(case report)		PCR	Unspecified	1997-1998	Spain	[132]
	Ovis musimon	16	416*	X		RFLP	Unspecified	1999-2001	Czech Republic	[128]
	Capreolus capreolus	2	858	X		RFLP	Unspecified	1999-2001	Czech Republic	[128]

		6	49		X	ELISA	Unspecified	1997	Norway	[127]
	Rangifer tarandus	0	91		X	ELISA	Unspecified	1996	Norway	[127]
		11	325		X	ELISA	Unspecified	1994	Norway	[127]
	Sus scrofa	1	2	X		RFLP	Accidental host	1999-2001	Czech Republic	[128]
			127	25.7 [95% CI : 17.6-33.8]		ELISA	Unspecified	2006-2007	Spain	[129]
		1	65	X		PCR	Unspecified	2001-2003	Spain	[131]
Mycoplasma conjunctivae	*Capra ibex*	16	136	X		PCR	Possible carrier	2006-2007	Switzerland	[133]
Mycoplasma agalactiae	*Capra pyrenaica*	46	422	X		PCR	Unspecified	1996-2003	Spain	[134]
Mycoplasma suis (Porcine infectious anemia)	*Sus scrofa*	36	359	X		PCR	Possible reservoir	2007-2008	Germany	[135]
Mycoplasma hyopneumoniae	*Sus scrofa*	92	428		X	ELISA	Not a reservoir	2000-2008	Spain	[136]
Mannheimia sp (Pneumonia)	*Ovibos moschatus*	71	276	X		Isolation for some cases	Unspecified	2006	Norway	[137]
Salmonella spp.	*Ammotragus lervia*	9	67		X	Agglutination test	Unspecified	1999	Spain	[126]
	Sus scrofa	66	342		X	ELISA	Unspecified	2005-2006	Italy	[110]

Legend: n=number of positive animals; N=number of animals tested; CFT = Complement Fixation Test; IFA = Indirect immunofluorescence Assay; ELISA: Enzyme Linked Immuno Sorbent Assay; MAT = Microscopic Agglutination Test; PCR = Polymerase Chain Reaction; RBT = Rose Bengal Test; RFLP = restriction fragment length polymorphism method; * = animal from game park (isolated from the wild) or extensives farms; (&) = zoo animals; (£) = no epidemiological conclusion possible because of the sampling was not done randomly; (µ): 29 PCR were done on sera with positive serology; *(#):* foot necrobacillosis complex= *Fusobacterium necrophorum, Arcanobacter pyogenes, Streptococcus agalactiae, Staphylococcus aureus.*

Additional file 2. Selected viral diseases reported in wild ungulates in Europe.

Pathogen	Ungulate specie (latin name)	n	N	Prevalence	Sero-prevalence	Diagnostic method	Epidemiological role from author's opinion	Year	Country	Reference
Aujezsky's disease virus (Pseudo-rabies)	*Sus scrofa*	294	3143		X	ELISA	Endemic	1991-1994	Germany	[138]
		9	16		X	IFAT	Unspecified	2000	Spain	[139]
		101	338		X	ELISA	Reservoir	2004-2005	Czech Republic	[140]
		92	929		X	ELISA	Enzootic disease	1993-2000	Germany	[141]
			192	30,6 ± 6,7%		PCR	Widespread infection	2004-2005	Spain	[142]
			185		45.9 ± 7.8%	ELISA	Widespread infection	2004-2005	Spain	[142]
		111	427		X	ELISA	Reservoir	2003-2004	Slovenia	[143]
		63	1857		X	ELISA	Sporadic cases	2001-2003	Switzerland	[109]
		62	152	X		PCR	Reservoir	2002-2003	Italy	[144]
		306	693		X	ELISA	Unspecified	2000-2003	Spain	[145]
		0	24		X	ELISA	Unspecified	2004	Lithuania	[146]
		423	12025 *		X	ELISA	Reservoir	1991-1998	France	[147]
		24	44		X	ELISA	Reservoir	1999	Croatia	[148]
		105	342		X	ELISA	Unspecified	2005-2006	Italy	[110]
African swine fever virus (African swine fever)	*Sus scrofa*	14	147		X	ELISA	Not a reservoir	1991-1993	Spain	[149]
Border disease virus (Border disease)	*Sus scrofa*	240	12025 *		X	ELISA	Unspecified	1991-1998	France	[147]
	Rupicapra pyrenaica	227	323		X	ELISA	Unknown	1994-2005	France	[150]
		17	167	X		ELISA+RT-PCR	Unknown	1994-2005	France	[150]
		82	114		X	ELISA	Emerging disease	2002-2006	Spain	[151]
		10	10 (£)	X		RT-PCR	Emerging disease	2005-2006	Spain	[151]
Bovine herpes virus -1 (Infectious bovine rhinotracheitis)	*Rangifer tarandus*	237	831		X	VNT	Endemic disease	1993-2000	Norway	[152]
	Cervus elaphus	3	589		X	VNT	Unspecified	1993-2000	Norway	[152]
		17	73		X	VNT	Spill over	2000-2002	Germany	[153]
	Capreolus capreolus	18	602		X	VNT	Unspecified	1993-2000	Norway	[152]
		4	38		X	VNT	Spill over	2000-2002	Germany	[153]
	Dama dama	1	46		X	VNT	Unspecified	2000-2002	Germany	[153]
	Bison bonasus	5	60		X	ELISA	Unspecified	1980-1983	Poland	[112]
	Rupicapra pyrenaica ornata	7	27		X	Microseroneutralisation	Unspecified	1990-1993	Italy	[154]
Bluetongue virus (Blue Tongue)	*Cervus elaphus*		513		40.4%	ID Screen Bluetongue Competition assay	Unspecified	2007	Belgium	[37]
		309	1409		X	ELISA	Unspecified	2005-2007	Spain	[38]

	Capreolus capreolus	2	39		X	ELISA	Unspecified	2005-2007	Spain	[38]
	Dama dama	34	96		X	ELISA	Unspecified	2005-2007	Spain	[38]
	Ovis aries	9	68		X	ELISA	Unspecified	2005-2007	Spain	[38]
		4	6	X (BTV-1)		RT-PCR	Unspecified	2007	Spain	[155]
	Ammotragus lervia	1	4		X	ELISA	Unspecified	2005-2007	Spain	[38]
	*Lama pacos**	1		(Case report)		PCR	Unspecified	2007	Germany	[156]
Bovine viral diarrhea virus (Bovine viral diarrhea)	*Capra ibex*	13	273		X	ELISA	Unspecified	2006-2008	Switzerland	[115]
	Sus scrofa	2	352		X	ELISA	Rarely exposed	2004-2005	Czech Republic	[140]
		2	44		X	ELISA	Reservoir	1999	Croatia	[148]
	Rangifer tarandus	34	810		X	VNT	Endemic disease	1993-2000	Norway	[152]
	Capreolus capreolus	78	635		X	VNT	Endemic disease	1993-2000	Norway	[152]
		12	123		X	VNT	Unspecified	1990-1992	Germany	[157]
	Cervus elaphus	7	658		X	VNT	Unspecified	1993-2000	Norway	[152]
		2	20		X	VNT	Unspecified	1995-1996	Denmark	[158]
	Alces alces	35	1794		X	VNT	Unspecified	1994-1999	Norway	[152]
Caprine herpes virus -1 (Caprine herpesvirosis)	*Cervus elaphus*	10	75		X	VNT	Unspecified	2000-2002	Germany	[153]
	Capreolus capreolus	1	38		X	VNT	Unspecified	2000-2002	Germany	[153]
Classic swine fever virus (Classic swine fever)	*Sus scrofa*	0	6471		X	ELISA	Unspecified	1999-2005	Czech Republic	[140]
		28	1767		X	ELISA	Sporadic cases	2001-2003	Switzerland	[109]
		0	591		X	ELISA	Unspecified	2004	Lithuania	[144]
		585	5286		X	ELISA	Reservoir	2002-2004	France	[159]
		128	301		X	VNT	Reservoir	2002-2004	France	[159]
		96	2767	X		PCR	Reservoir	2002-2004	France	[159]
		80	12025 *		X	ELISA	Unspecified	1991-1998	France	[147]
		17	44		X	ELISA	Reservoir	1999	Croatia	[137]
Chronic wasting disease agent (Chronic wasting disease)	*Cervus elaphus*	0	739	X		ELISA	Unspecified	-	Italy	[160]
		0	674	X		ELISA	Unspecified	2001-2003	Belgium	[161]
	Capreolus capreolus	0	192	X		ELISA	Unspecified	2001-2003	Belgium	[161]
Encephalomyocarditis virus (Encephalomyocarditis)	*Sus scrofa*	13	20		X	VNT	Unspecified	1994-2006	Greece	[162]
	Rupicapra pyrenaica ornata	5	27		X	Microseroneutralization	Unspecified	1990-1993	Italy	[154]
Foot and mouth disease virus (Foot and mouth disease)	*Sus scrofa*	0	504		X	ELISA	Unspecified	2004	Lithuania	[146]
		0	208		X	ELISA	Unspecified	2001	The Netherlands	[163]

Cervid herpes virus-1	*Cervus elaphus*	15	73		X	VNT	Unspecified	2000-2002	Germany	[153]
	Capreolus capreolus	2	38		X	VNT	Unspecified	2000-2002	Germany	[153]
Hepatite E virus (Hepatitis E)	*Sus scrofa*	143	676		X	ELISA	Possible reservoir	2001-2008	Spain	[164]
		9	74	X		RT-PCR	Possible reservoir	2001-2006	Hungary	[165]
		165	1039		X	ELISA	Unspecified	2005-2008	The Netherlands	[166]
		8	106	X		RT-PCR	Unspecified	2005-2008	The Netherlands	[166]
	Cervus elaphus	3	38		X	ELISA	Unspecified	2005-2008	The Netherlands	[166]
		6	39	X		RT-PCR	Unspecified	2005-2008	The Netherlands	[166]
	Capreolus capreolus	11	32	X		RT-PCR	Possible reservoir	2001-2006	Hungary	[165]
Orf virus	*Ovibos moschatus*	19	170	X		Characterization	Spill-over	2004	Norway	[167]
Parapox virus (Contagious echtyma)	*Rangifer tarandus**	48	6		X	Characterization	Unspecified	2000	Norway	[127]
Porcine circo virus -2 (Postweaning multisystemic wasting syndrome)	*Sus scrofa*	57	134		X	IFAT	Unspecified	2005	Czech Republic	[140]
		335	531	X		Nested PCR	Unspecified	2004-2007	Germany	[168]
		314	656		X	IPMA	Unspecified	2000-2003	Spain	[169]
Pestiviruses (unprecised) (Pestivirus infections)	*Rupicapra pyrenaica ornata*	6	35 *		X	ELISA	Unspecified	1990-1993	Italy	[154]
	Rupicapra rupicapra	28	110		X	ELISA	Unspecified	1999	Italy	[170]
		145	343		X	ELISA	Unspecified	2004-2007	France	[14]
	Cervus elaphus	8	136		X	ELISA	Unspecified	1999	Italy	[170]
	Ovis amon	11	18		X	ELISA	Unspecified	2006-2007	France	[14]
	Sus scrofa	7	56		X	ELISA	Unspecified	1999	Italy	[170]
Porcine parvovirus (Porcine parvovirus infection)	*Sus scrofa*	187	254		X	HIT	Unspecified	2004	Lithuania	[146]
		27	342		X	ELISA	Unspecified	2005-2006	Italy	[110]
Porcine reproductive and respiratory syndrome virus (porcine reproductive and respiratory syndrome)	*Sus scrofa*	33	909 *		X	ELISA	Reservoir	1991-1998	France	[147]
		129	342		X	ELISA	Unspecified	2005-2006	Italy	[110]
Small ruminant lentivirus	*Capra ibex*	3		(case report)		PCR	Independant cases	2006	France	[171]

Swine vesicular disease virus (Swine vesicular disease)	*Sus scrofa*	0	12	X	ELISA	Unspecified	2004	Lithuania	[146]
Transmissible gastroenteritis virus(Transmissible Gastroenteritis)	*Sus scrofa*	1	134	X	IFAT	Sporadic case	2004-2005	Czech Republic	[140]

Legend: n: number of positive animals; N: number of animals tested; *: semi-domesticated animal or farmed animals; £: Only 10 animal tested, because were found sick or already dead in the field. Suspicion of the disease was present before doing the test; IFAT: Indirect Fluorescence Antibodies Test; ELISA: Enzyme Linked Immuno Sorbent Assay; HIT: Haemagglutination Inhibition Test; PCR: Polymerase Chain Reaction; RT-PCR: Reverse Transcriptase Polymerase Chain Reaction; VNT: Virus Neutralization Test.

Additional file 3. Selected parasitic diseases reported in wild ungulates in Europe

Pathogen	*Ungulate specie (latin name)*	n	N	Prevalence	Serology	Diagnostic method	Epidemiological role from author's opinion	Year	Country	Reference
PROTOZOAN										
Babesia capreoli	*Rupicapra rupicapra*	6	7	(case report)		PCR	Emerging disease	2005	Switzerland	[41]
		1	48	X		PCR	Unspecified	2006-2007	Switzerland	[172]
	Cervus elaphus	1	9	X		PCR	Unspecified	2006-2007	Switzerland	[172]
	Capreolus capreolus	12	46	X		PCR	Reservoir	2006-2007	Switzerland	[172]
Babesia divergens	*Rupicapra pyrenaica*			15.79 [4.2-27.38]			Reservoir		Spain	[173]
	Capreolus capreolus	40	75		X	Indirect IF	Unspecified	1979	France	[111]
			51	54.9 %		PCR	Zoonotic reservoir	1996-2000	Slovenia	[174]
	Cervus elaphus		30	16.7%		PCR	Zoonotic reservoir	1996-2000	Slovenia	[174]
Babesia ovis	*Ovis musimon*	6	50		X	IFAT	Reservoir	1991-1996	Spain	[175]
	Capra pyrenaica	155	475		X	IFAT	Unspecified	1992-1995	Spain	[176]
Babesia spp	*Capra pyrenaica*	1	1	(case report)		Microscopic examination	Unspecified	1995	Spain	[177]
	Capreolus capreolus			53.3 [42.04-64.62]			Unspecified		France	[111]
		83	202	X		PCR	Unspecified	2004-2008	France	[164]
Babesia EU1	*Capreolus capreolus*		51	21.6%		PCR	Zoonotic reservoir	1996-2000	Slovenia	[174]
Cryptosporidium spp.	*Cervus elaphus*		118*	14,4		IFA+PCR	Unspecified	2003-2005	Poland	[178]
		1	289	X		Fecal examination	Reservoir	2001-2003	Norway	[179]
	Capreolus capreolus		22	9.1		IFA+PCR	Unspecified	2003	Poland	[178]
		18	291	X		Fecal examination	Reservoir	2001-2003	Norway	[179]
	Bison bonasus		55	29.1		IFA+PCR	Unspecified	2003-2005	Poland	[178]
	Alces alces	15	455	X		Fecal examination	Reservoir	2001-2003	Norway	[179]
	Sus scrofa		5	0		IFA+PCR	Unspecified	2003	Poland	[178]
Cryptosporidium parvum	*Dama dama*	1	16	X		Fecal examination	Possible reservoir	1995-1998	England	[180]
	Muntiacus reevsi	4	42	X		Fecal examination	Possible reservoir	1995-1998	England	[180]
Dicrocoelium dendriticum	*Capreolus capreolus*	1	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Cervus elaphus	4	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Giardia spp.	*Cervus elaphus*		118*	1,7		IFA	Unspecified	2003-2005	Poland	[178]
			285	1		Fecal examination	Unspecified		Croatia	[182]
		5	289	X		Fecal examination	Reservoir	2001-2003	Norway	[179]
	Capreolus capreolus		22	4.5		IFA	Unspecified	2003	Poland	[178]
			14	27			Unspecified		Croatia	[183]
		45	291	X		Fecal examination	Reservoir	2001-2003	Norway	[179]
	Bison bonasus		55	7.5		IFA	Unspecified	2003-2005	Poland	[178]
	Alces alces	56	455	X		Fecal examination	Reservoir	2001-2003	Norway	[179]
		1	1	X		Fecal examination	Unspecified	2002-2008	Sweden	[184]

	Rangifer tarendus	11	155	X		Fecal examination	Reservoir	2001-2003	Norway	[179]
	Sus scrofa		144	1.7		Fecal examination	Unspecified		Croatia	[182]
Neospora caninum	*Ammotragus lervia*	1	13	X		ELISA + IFAT	Unspecified	1993-2005	Spain	[185]
	Capreolus capreolus	2	33	X		ELISA + IFAT	Unspecified	1993-2005	Spain	[185]
	Cervus elaphus	28	237	X		ELISA + IFAT	Unspecified	1993-2005	Spain	[185]
	Sus scrofa	1	298	X		ELISA + IFAT	Unspecified	1993-2005	Spain	[185]
		102	565	X		ELISA	Unspecified	1999-2005	Czech Republic	[186]
Toxoplasma gondii	*Alces alces*	270	2142	X		Cdat	Unspecified	1992, 1994-2000	Norway	[187]
	Cervus elaphus	1	67		X	ELISA	Possible reservoir	1999	Spain	[126]
		1	10	X		MAT	Unspecified	1993-2005	Spain	[188]
	Capra pyrenaica	1	3	X		MAT	Unspecified	1993-2005	Spain	[188]
	Dama dama *Ovis ammon*	12	32	X		Isolation	Possible reservoir	2003-2008	France	[116]
		258	760	X		cDAT	Unspecified	1994, 1999-2000	Norway	[187]
		7	33	X		MAT	Unspecified	1993-2005	Spain	[188]
	Cervus elaphus	1	4	X		Isolation	Possible reservoir	2003-2008	France	[116]
		69	441	X		MAT	Possible source of zoonosis	1993-2005	Spain	[188]
		44	571	X		cDAT	Unspecified	1993-1999	Norway	[187]
	Dama dama	18	79	X		MAT	Unspecified	1993-2005	Spain	[188]
	Ovis ammon	4	27	X		MAT	Unspecified	1993-2005	Spain	[188]
	Ovis gmelini	1	7	X		Isolation	Possible reservoir	2003-2008	France	[116]
	Ovis orientalis musimon	17	77		X	ELISA	Unspecified	-	Italy	[189]
	Rangifer tarandus	9	866	X		cDAT	Unspecified	1999-2000	Norway	[187]
	Rupicapra rupicapra	2	10	X		MAT	Unspecified	1993-2005	Spain	[188]
	Sus scrofa	26	148	X		MAT	Reservoir	2002-2008	France	[190]
		148	565	X		IFAT	Unspecified	1999-2005	Czech Republic	[186]
Theileria sp. OT3	*Cervus elaphus*			85.7			Reservoir		Spain	[173]
	Capreolus capreolus			46.4			Reservoir		Spain	[173]
	Rupicapra pyrenaica			26.3			Reservoir		Spain	[173]
Theileria sp. 3185/02	*Cervus elaphus*			53.6			Reservoir		Spain	[173]
	Capreolus capreolus			10.1			Reservoir		Spain	[173]
Sarcoptes scabei	*Rupicapra rupicapra*	1696	10000	X			Unspecified	1995-2004	Italy	[191]
	Rupicapra pyrenaica parva		1600	12,9%		Observation	Unspecified	1994-1995	Spain	[192]
	Cervus elaphus	1		(case report)		M.E.	Unspecified	1995-2004	Italy	[191]
	Capreolus capreolus	1		(case report)		M.E.	Unspecified	1995-2004	Italy	[191]
	Ovis gmelini musimon	1		(case report)		M.E.	Unspecified	1995-2004	Italy	[191]
	Capra pyrenaica		2096	49.2 ± 7.9		M.E.	Unspecified	1995-2006	Spain	[193]
	Rupicapra pyrenaica	43	63	X		M.E.	Unspecified	1988	Spain	[194]

	Capra ibex	157		(case report)		M.E.	Unspecified	1995-2006	Italy	[195]
	Spanish ibex			100 (epizoology)	-	Histopathology	New infection of a naive population		Spain	[194]
TREMATODA										
Dicrocoelium dendriticum	*Capreolus capreolus*	1	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Cervus elaphus	4	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Fasciola hepatica	*Capra pyrenaica*	10	2096$	X		Necropsy	Unspecified	1995-2006	Spain	[193]
		5	380$	X		Coprology	Unspecified	1995-2006	Spain	[193]
	Cervus elaphus	5	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Capreolus capreolus	1	1	Case report		Necropsy	Unspecified	2006	France	[196]
		1	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Alces alces	1	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Fascioloides magna	*Cervus elaphus*			Case report		Necropsy	Unspecified		Croatia	[197]
Liorchis scotiae	*Alces alces*	4	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Parafasciolopsis fasciolaemorpha	*Alces alces*	8	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Capreolus capreolus	2	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Paramphistomum cervi	*Cervus elaphus*	3	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Paramphistomum ichikawai	*Alces alces*	6	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Capreolus capreolus	2	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
CESTODA										
Echinococcus granulosus	*Alces alces*	3	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Cervus elaphus	3	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Moniezia benedeni	*Alces alces*	5	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Taenia hydatigena	*Alces alces*	8	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Capreolus capreolus	1	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Cervus elaphus	2	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Taenia krabbei	*Cervus elaphus*	2	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
NEMATODA										
Bunostomum trigonocephalum	*Alces alces*	5	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Chabertia ovina	*Capreolus capreolus*	8	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Dictyocaulus eckerti	*Alces alces*	4	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Capreolus capreolus	2	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Cervus elaphus	9	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Nematodirus oiratianus	*Cervus elaphus*	4	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Oesophagostomum venulosum	*Alces alces*	3	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Capreolus capreolus	5	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Cervus elaphus	5	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]

Onchocerca flexuosa	*Cervus elaphus*	10	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Setaria cervi	*Capreolus capreolus*	4	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Trichuris ovis	*Alces alces*	6	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Capreolus capreolus	6	18	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
	Cervus elaphus	5	16	X		H.E.	Unspecified	1981-1998	Belorussian Polesie	[181]
Trichinella spp.	*Sus scrofa*	13	1035		X	ELISA	Unspecified	2003-2004	Slovak republic	[198]
		30	1492	X		ELISA	Unspecified	2006-2008	France	[199]
Trichinella britovi	*Sus scrofa*	1	1		Case report	Artificial digestion + PCR	Unspecified	2004	Belgium	[200]
		3	3		Cases report	Artificial digestion + PCR	Unspecified	Unspecified	Roumania	[201]
Trichinella spiralis	*Sus scrofa*	2	2		Case report	Artificial digestion + PCR	Unspecified	Unspecified	Roumania	[201]
		-	458		6.8%	ELISA	Unpecified	Unspecified	The Netherlands	[202]
Toxocara spp.	*Sus scrofa*	85	1173		X	ELISA	Unspecified	2003-2004	Slovak republic	[198]
Ascaris suum	*Sus scrofa*	45	411		X	ELISA	Unspecified	2003-2004	Slovak republic	[198]

Legend: *: farmed animals; $: same study total of 2096 ibexes: all were analysed by necropsy and 380 of them were additionally analysed by coprology; cDAT: commercial Direct Agglutination Test; ELISA: Enzyme Linked Immuno Sorbent Assay; IF: immunofluorescence; IFAT: Indirect Fluorescent Antibody Test; H.E.: Helminthological Examination: dissection and organ compression; MAT: Modified Agglutination Test; M.E.: Microscopic Examination; PCR: Polymerase Chain Reaction.

ARTICLE 4. ARTICLE ORIGINAL

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

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ARTICLE 4. Resumé

Epidémiologie des infections avec des Pestivirus chez les ongulés sauvages dans les Alpes du Sud françaises

Les transmissions inter-espèces sont régulièrement incriminées dans l'épidémiologie des pestiviroses. Le but de cette étude était de rechercher la prévalence des pestivirus au sein de certaines espèces d'ongulés sauvages de montagne, et de déterminer leur rôle dans la transmission des pestivirus. Entre 2003 et 2007, une étude épidémiologique longitudinale a été mise en place sur des ongulés sauvages dans le département des Hautes-Alpes. Des anticorps dirigés contre la protéine p80 des pestivirus commune à tous les pestivirus ont été trouvés dans 45,9% (IC95%: [40.5-51.3%]) des 343 chamois (*Rupicapra rupicapra*). De plus, une séroprévalence importante atteignant 61.1 %(IC95%: [38.6-83.6%]) a été mise en évidence sur des mouflons (*Ovis gmelinii musimon*). Ces résultats sérologiques obtenus par ELISA ont été confirmés par un test de séroneutralisation comparative, réalisé sur 7 souches de pestivirus en utilisant 15 sérums séropositifs. Les titres en anticorps les plus élevés étaient dirigés contre deux souches de BDV (souches 33s et Av), plutôt que contre une souche de BDV-4, responsable d'une précédente épizootie de pestivirose dans des populations d'isard. Les tests de séroneutralisation confirment une circulation de BDV dans les ongulés sauvages dans les Alpes du Sud françaises. Les animaux âgés et les femelles ont montré être deux facteurs de risque associés à la séropositivité. Cependant, aucune souche virale n'a pu être mise en évidence dans les sérums et rates. Des efforts doivent être réalisés pour améliorer le protocole afin de pouvoir isoler et caractériser les souches virales locales.

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Research article

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

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ABSTRACT

Inter-species transmission is often incriminated in the 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 (also named 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 seven 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.

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 camelopardis*) 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 three main criteria for species discrimination. The subdivision between all species is also antigenically

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supported by poor serological cross-reactivity (Avalos-Ramirez et al., 2001).

Pestiviruses are enveloped spherical viruses, 40–60 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 two subgroups (Vilček et al., 2005), CSFV into three subgroups (Paton et al., 2000) and BDV into seven subgroups (Valdazo-Gonzalez et al., 2007).

Ruminant *Pestiviruses* 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 (Frölich 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 the sharing of 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 (it) 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 h after shooting. Blood samples were centrifugated and sera were frozen at −20 °C within 12 h after shooting. A total of 53 spleens originating from chamois only were frozen at −20 °C within 12 h 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) (Fig. 1). Altitudes range between 1300 m and 3000 m. 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 two 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 mixture of pine, foliage trees, larch forests and alpine pasture. Contacts may occur between chamois, mouflon, roe deer, alpine ibex (*Capra ibex ibex*), red deer (*Cervus elaphus*), and wild boar (*Sus scrofa*). Domestic livestock (small and large ruminants) share the pastures with wild animals during the grazing season from June to September (Fig. 2).

2.3. Serological tests

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

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

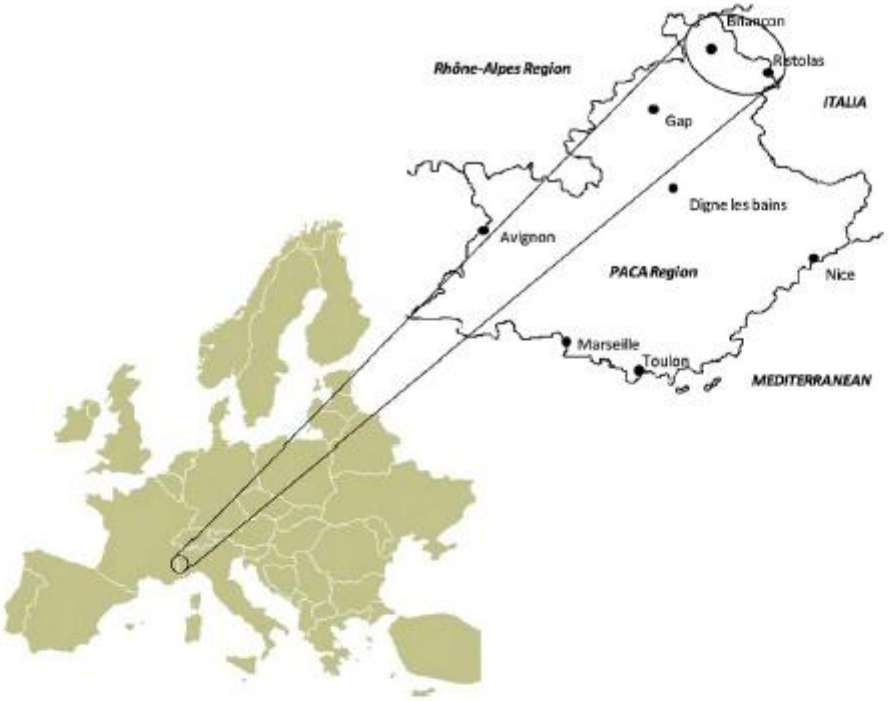

Fig. 1. Location of the study area (French South Alps).

number AY738080) both in the 5′UTR and the Npro regions.

A fixed virus dose (fixed amount between 30 and 200 CCID50) was incubated for 2 h at 37 °C with twofold serum serial dilutions in an antibiotics enriched growth medium (i.e. penicillin, gentamicin and amphotericin B). MDBK cells (ATCC Number CCL-22) were added and the cultures were grown for 72 h at 37 °C in a CO_2 incubator. All sera were tested in duplicate, using a 1:2 or a 1:10 as starting dilution. Viruses were titrated in all assays. Titers were expressed as the reciprocal of the highest serum dilution yielding virus growth neutralization.

2.4. Virus detection

2.4.1. ELISA

Antibody negative serum samples collected between 2003 and 2006 were screened for *Pestivirus*-specific

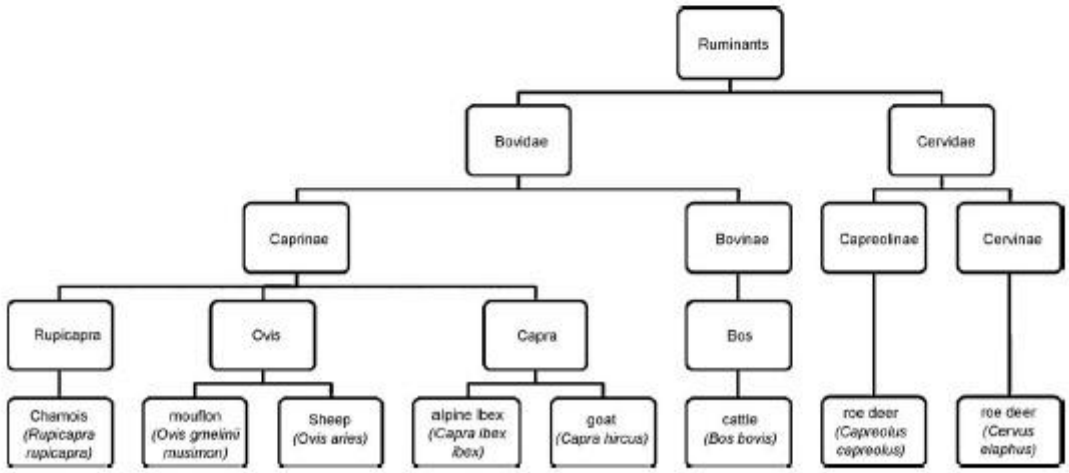

Fig. 2. Phylogeny of ruminants presents in the study area. Scientific names were taken from the NCBI Taxonomy Database (http://www.ncbi.nlm.nih.gov/taxonomy; consulted on November, 8th of 2009).

antigens (p80) using a sandwich ELISA antigen test (Synbiotics, Lyon, France). Sera collected in 2007 were analyzed by an Erns-capture ELISA (Idexx, Liebefeld-Bern, Switzerland).

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

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

The RNA was extracted using QIAamp RNeasy® Mini kit (QIAGEN) and was resuspended in 40 μL DEPC-treated water. The reverse transcription was carried out in a volume of 20 μL containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP, 150 pmol of the reverse primer B2, 200 U MMLV reverse transcriptase (GIBCO BRL) and 10 μL RNA. The cDNA was synthesized at 37 °C for 15 min and the enzyme was inactivated for 5 min at 95 °C. For conventional PCR, the 5′UTR region was amplified using primers BE 5′ CATGCCCTTAGTAGGACTAGC 3′ and B2 5′ TCAACTC-CATGTGCCATGTAC 3′ to amplify a 287 base pair fragment. In vitro amplification was realised in a thermocycler in a 50 μL-solution containing 20 mM Tris–HCl (pH 8.4), 3 mM $MgCl_2$, 0.5 mM dNTP, 75 pmol of each primer, 2.5 u Taq DNA polymerase (Invitrogen®) and 2 μL cDNA. Conditions of amplification were a first enzymatic activation for 5 min at 95 °C followed by 35 cycles of amplification (each cycle 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C). Amplified products were separated by electrophoresis in 1.5% agarose gel in Tris–borate EDTA buffer (Letellier et al., 1999).

For real-time RT-PCR, the primer pair F2: 5′ CTCGA-GATGCCATGTGGAC 3′ (position 224–242 of the NADL sequence) and PESTR: 5′ CTCCATGTGCCATGTACAGCA 3′ (position 391–371 of the NADL sequence) and the 5′ FAM CAGCCTGATAGGGTGCTGCAGAGGC TAMRA 3′ and the 5′ VIC CACAGCCTGATAGGGTGTAGCAGAGACCTG TAMRA 3′ probes were used. The Applied Biosystems ABI-PRISM 7900HT sequence detection system was used. The PCR conditions were as follows: 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 2× Universal Master Mix (Applied Biosystems), 300 nM of both primers and 200 nM 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, 200 μL of each serum were inoculated onto duplicate wells on 24-well microtiter plates containing MDBK cells, After 1 h 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 the presence of virus plaques.

2.5. Statistical analysis

The relationship between two 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 six 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 1: 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 it increases later (Table 2).

Table 1
Estimations of population size of the chamois population in the Game and Wildlife National Reserve of Ristolas.

Year	Population size
1986	415
1989	469
1992	510
1995	–[a]
1998	729
2001	–[a]
2004	344
2006	268

[a] Not realised for meteorological reasons.

Table 2
Reproduction rate in the chamois population in the Game and Wildlife National Reserve of Ristolas.

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

Reproduction rate = kid/female rate.

3.1.2. Serological results

Table 3 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 none was found positive, either in 2003 ($n = 3$) or in 2007 ($n = 3$). OD values are significantly higher for mouflon than for chamois and roe deer (Wilcoxon signed rank test, $P < 0.0001$ in both cases; 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 two were antibodies positive among 20 tested, in 2003 (Table 3). 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 large amount of the samples allows to standardize apparent prevalence on age distribution and to study related risk factors. Among 338 samples (age is unknown in five 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 in Fig. 3. The apparent prevalence is steadily going down. Interestingly, the prevalence drops 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

Table 3
Annual and species repartition of all blood samples.

	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)

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

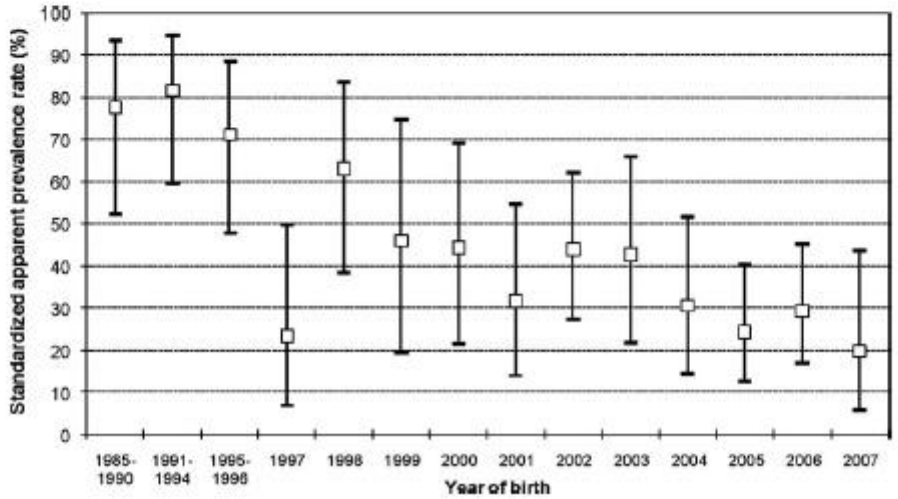

Fig. 3. Annual evolution of the standardized apparent prevalence rate (□) in chamois (*Rupicapra rupicapra*) originating from the French South Alps with 95% confidence interval (exact 95% binomial confidence intervals) of the *Pestivirus* p80 antibodies. The standardization of the apparent prevalence has been calculated considering five age states of chamois: 0–2 years, 2–4 years, 4–6 years, 6–8 years and more than 8 years.

Table 4
Virus neutralization titers on 15 chamois (*Rupicapra rupicapra*) originating from the French South Alps for seven *Pestivirus* strains.

Serum	Age (year)	Sex	Strain						
			Av	33s	02/1517	Frijters	137/4	NADL	3534
3.N.5	2	F	128	13	20	0	40	1,024	0
3.N.25	14	M	1,024	5,120	640	2,560	2,560	2,048	160
5.R.13	2	M	20,480	10,240	1,280	2,560	640	4,096	320
5.R.27	10	M	512	320	80	160	320	512	80
5.R.30	6	M	256	320	160	1,280	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	1,024	1,280	640	640	640	256	80
6.B.7	0	M	256	320	40	160	80	3	6
7.V.40	n.d.	n.d.	20,480	10,240	10,240	5,120	1,280	5,120	160
7.V.42	10	F	20,480	20,480	10,240	10,240	5,120	20,480	320
7.V.46	0	M	20,480	5,120	10,240	2,560	1,280	5,120	320
7.N.4	n.d.	n.d.	4,096	5,120	640	640	1,280	320	80
7.N.13	n.d.	n.d.	20,480	10,240	1,280	5,120	320	2,560	80
7.N.59	18	F	1,024	640	320	320	320	80	0

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

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 ≤ 2 years = 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 two 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 seven *Pestivirus* strains and are reported in Table 4.

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

Table 5
Arithmetic mean and standard error of titers obtained for each virus strain and *P*-value obtained with the Wilcoxon signed rank test.

Strain 1	Mean ± S.E.	*vs*	Strain 2	Mean ± S.E.	Wilcoxon signed rank test, *P*-value
Av	7,432.53 ± 2,125.47	–	NADL	2,785.8 ± 1,198.95	0.02
Av	7,432.53 ± 2,125.47	–	3534	108.4 ± 29.78	<0.001*
Av	7,432.53 ± 2,125.47	–	02/1517	2,412 ± 722.09	<0.001*
Av	7,432.53 ± 2,125.47	–	33s	4,683.53 ± 1,216.67	0.28
Av	7,432.53 ± 2,125.47	–	Frijters	2,138.67 ± 547.98	0.028
Av	7,432.53 ± 2,125.47	–	137/4	1,021.33 ± 322.90	0.027
NADL	2,785.8 ± 1,198.95	–	3534	108.4 ± 29.78	<0.001*
NADL	2,785.8 ± 1,198.95	–	02/1517	2,412 ± 722.09	<0.001*
NADL	2,785.8 ± 1,198.95	–	33s	4,683.53 ± 1,216.67	0.009
NADL	2,785.8 ± 1,198.95	–	Frijters	2,138.67 ± 547.98	0.86
NADL	2,785.8 ± 1,198.95	–	137/4	1,021.33 ± 322.90	0.31
3534	108.4 ± 29.78	–	02/1517	2,412 ± 722.09	<0.001*
3534	108.4 ± 29.78	–	33s	4,683.53 ± 1,216.67	<0.001*
3534	108.4 ± 29.78	–	Frijters	2,138.67 ± 547.98	<0.001*
3534	108.4 ± 29.78	–	137/4	1,021.33 ± 322.90	<0.001*
02/1517	2,412 ± 722.09	–	33s	4,683.53 ± 1,216.67	0.009
02/1517	2,412 ± 722.09	–	Frijters	2,138.67 ± 547.98	0.227
02/1517	2,412 ± 722.09	–	137/4	1,021.33 ± 322.90	0.819
33s	4,683.53 ± 1,216.67	–	Frijters	2,138.67 ± 547.98	0.003
33s	4,683.53 ± 1,216.67	–	137/4	1,021.33 ± 322.90	0.008
Frijters	2,138.67 ± 547.98	–	137/4	1,021.33 ± 322.90	0.135

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

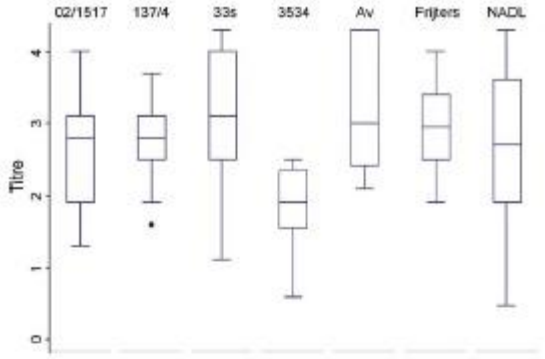

Fig. 4. Boxplot representation of the titer logarithm (*Y*-axis) obtained against each viral strain (*X*-axis).

obtained for each of the remaining strain (Table 5). 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 (Fig. 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 is the first published description of *Pestivirus* positive seroprevalence confirmed by VNT.

At the 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 of 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 other 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) was not confirmed by the RT-PCR carried out on the corresponding spleens, athough 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): four 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 h 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 four *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 was 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 Kingdom (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 Aveyron department in 1985 (Dubois et al., 2008). The 02/1517 strain was isolated in a Pyrenean chamois (Letellier, personal communication). 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, two 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 et al. (2008). They sequenced 23 of 32 strains isolated between 1985 and 2006 in four 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 two other ovine isolates, collected in 1996 in Vaucluse (one of the six PACA departments), clustered with the Tunisians isolates (Dubois et al., 2008). Thus, our results fit 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 two 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 et al. (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 Riekerink 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*. A 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 from 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. 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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Choix des sites d'étude pour l'étude épidémiologique transversale

Par la suite, une deuxième étude épidémiologique a été mise en place, visant à évaluer la présence des Pestivirus à l'interface ruminants sauvages/ ruminants domestiques. Le choix des zones d'étude s'est porté sur certaines zones, déterminées à l'aide d'un score d'aide à la décision. A cette fin, nous présentons dans un premier temps les informations initialement disponibles pour chaque zone potentielle d'étude. Dans un deuxième temps, une comparaison de ces zones a été réalisée, à l'aide de 8 critères notés de 0 à 5. Les zones ayant les notes les plus élevées ont été par la suite choisies. Pour certaines d'entre elles, un choix de secteurs a par la suite du être mis en place.

a. Données initiales disponibles par zone potentielle d'étude.

Pour chaque site potentiel d'étude, un tableau standardisé reprend les informations disponibles lors de la mise en place du protocole de suivi des *Pestivirus*. Des éléments généraux (département de la zone, gestionnaires de ces zones, altitude et superficie) caractérisent tout d'abord les zones. Les effectifs globaux (ou approximation des effectifs) d'ongulés sauvages et domestiques sont ensuite repris. Enfin, les principaux résultats d'éventuelles études scientifiques pertinentes pour ce sujet sont décrits dans les dernières lignes de ces tableaux.

i. Réserves de chasse et de faune sauvage gérées par l'ONCFS

Les réserves de chasse et de faune sauvage gérées par l'ONCFS proposées comme site d'étude sont la réserve du Caroux, la réserve des Bauges, la réserve d'Orlu.

- Réserve des Bauges

Département	73 – 74
Gestion	ONCFS ONF Parc Naturel Régional des Bauges
Altitude	800-2200 m
Superficie	5205 Ha
Espèce sauvages présentes	Chamois (2000) Mouflons (200) Chevreuil, sanglier
Espèces domestiques	Bovins (1200) Caprins (550) Ovins (130)
Dynamique de population	Évaluation des taux de survie selon les classes d'âges
Gestion faune sauvage / suivi sanitaire	4 zones de capture Données de capture depuis 1986 (≅ 700 chamois)
Prélèvements déjà réalisés	650 sérums depuis 1986
Données antérieures concernant les Pestivirus pour la faune domestique	Enquête séroprévalence des maladies abortives - résultats pour les pestivirus (E. Jourdain) Bv : 50% (594 ind - 20/24 troupeaux) Cp : 14,7 % (95 ind. - 2/3 troupeaux) Mt : 5,9 % (17 ind. – 1/1 troupeau) Estimation de la cohabitation faune sauvage/faune domestique à l'aide d'une occupation de l'alpage pendant 2 estives
Données antérieures concernant les Pestivirus pour la faune sauvage	chamois : 9/461 séropositifs entre 1986 et 2006 2003 : isolation d'un pestivirus, mais pas de typage possible (problème lors du transfert de laboratoire) 2004 : séroprévalence 42% 2005 : séroprévalence 20% depuis : 0%
Données futures pour la faune domestique	Pas de réseau actuellement disponible. Nécessité de passer du temps au contact direct des éleveurs
Bibliographie Pestivirus	Jourdain E, 2004. Thèse vétérinaire

Table I. Données initiales disponibles pour la réserve des Bauges

- Réserve du Caroux

Département	34
Gestion	ONCFS
Altitude	800-2200 m
Superficie	1700 Ha
Espèce sauvages présentes	Mouflons (>1500) Chevreuil, sanglier
Espèces domestiques	Ovins (100) Bovins (50) Caprins (qq)
Dynamique de population	Pas de données
Gestion faune sauvage / suivi sanitaire	Pas de données
Prélèvements déjà réalisés	Pas de données
Données antérieures concernant les Pestivirus pour la faune domestique	Pas de données
Données antérieures concernant les Pestivirus pour la faune sauvage	Séroprévalence = 8% (145 animaux testés) (Dupraz, 2004)
Données futures pour les Pestivirus	Pas de réseau actuellement disponible. Nécessité de passer du temps au contact direct des éleveurs
Bibliographie Pestivirus	Dupraz, 2004. Thèse vétérinaire.

Table II. Données initiales disponibles pour la réserve du Caroux.

- Réserve d'Orlu

Département	09
Gestion	ONCFS
Altitude	900 – 2765 m
Superficie	4250 Ha
Espèce sauvages présentes	Isard (500) Chevreuil, sanglier
Espèces domestiques	Ovins (2000) Bovins (200)
Dynamique de population	Comptages tous les ans survie femelles adultes : 0,94
Gestion faune sauvage / suivi sanitaire	1 zone de capture Données de capture depuis 1984 (≅ 426 isards)
Prélèvements déjà réalisés	280 sérums depuis 1994
Données antérieures concernant les Pestivirus pour la faune domestique	Enquête séroprévalence des maladies abortives : résultats pour les Pestivirus (Reynal, 2004) Bv : 51% (118 ind - 2/2 troupeaux) Mt : 18 % (972 ind. – 6/6 troupeau)
Données antérieures concernant les Pestivirus pour la faune sauvage	Circulation importante d'un Pestivirus, isolement de la souche Orlu (BDV-4)
Données futures pour la faune domestique	Pas de réseau actuellement disponible. Nécessité de passer du temps au contact direct des éleveurs
Bibliographie Pestivirus	Pioz et al., 2007 : *Transmission of a pestivirus infection in a population of Pyrenean chamois* (Vet. Microbiol)

Table III. Données initiales disponibles pour la réserve d'Orlu.

- Société de chasse Ségure-Viso et Réserve de Chasse et de Faune Sauvage de Ristolas

Département	05
gestion	ONCFS Réserve de Chasse et de Faune Sauvage de Ristolas Parc Naturel Régional du Queyras
Altitude	900 – 2765 m
Superficie	1692 Ha
Espèce sauvages présentes	Chamois (≅ 250) Mouflon (34) Bouquetin (28) chevreuil (65) cerf, sanglier
Espèces domestiques	Ovins Bovins
Dynamique de population	Suivi ongulés sauvages réalisé par l'ONCFS : - Données IPS (Indice d'abondance pédestre) - Données démographiques (tendance des effectifs, indices de reproduction, indice de survie hivernal) - condition physique des animaux, poids moyens - veille sanitaire.
Gestion faune sauvage / suivi sanitaire	Gestion des populations réalisée par la société de chasse et FdC05. Suivi sanitaire réalisé depuis 2004
Prélèvements déjà réalisés	Sérothèque depuis 2004
Données antérieures concernant les Pestivirus pour la faune domestique	Circulation importante d'un Pestivirus Pas encore d'isolement de souche
Données antérieures concernant les Pestivirus pour la faune sauvage	Néant
Données futures pour la faune domestique	possibilité d'obtenir les noms des éleveurs présents sur les alpages (GDS PACA) Collaboration possible avec les vétérinaires sanitaires Prélèvements peuvent être obtenus à partir du LVDHA 05 Centralisation possible des prélèvements au LDVHA 05
Bibliographie Pestivirus	Martin et al., *Epidemiology of Pestivirus infection in wild ungulates of the French South Alps*. Article soumis à *Veterinary Microbiology* au moment du choix des sites d'études.

Table IV. Données initiales disponibles pour la commune de Ristolas.

- Vallée de la Clarée

Département	05
Gestion	Communes de - Névache - Val des Près
Altitude	900 – 2765 m
Superficie	1692 Ha
Espèce sauvages présentes	Chamois Mouflon Bouquetin chevreuil cerf, sanglier
Espèces domestiques	Ovins Bovins
Dynamique de population	Comptages tous les 6 ans
Gestion faune sauvage / suivi sanitaire	Gestion des populations réalisée par les sociétés de chasse et FdC05.
Prélèvements déjà réalisés	Sérothèque depuis 2004
Données antérieures concernant les Pestivirus pour la faune domestique	Circulation importante d'un Pestivirus Pas encore d'isolement de souche
Données antérieures concernant les Pestivirus pour la faune sauvage	Néant
Données futures pour la faune domestique	possibilité d'obtenir les noms des éleveurs présents sur les alpages (GDS PACA) Collaboration possible avec les vétérinaires sanitaires Prélèvements peuvent être obtenus à partir du LVDHA 05 Centralisation possible des prélèvements au LDVHA 05
Bibliographie Pestivirus	Martin et al., *Epidemiology of Pestivirus infection in wild ungulates of the French South Alps*. Article soumis à *Veterinary Microbiology* au moment du choix des sites d'études.

Table V. Données initiales disponibles pour le Briançonnais.

- Vallée de l'Ubaye

Département	04 - 05
Gestion	Pas une zone protégée Fédération de chasseurs
Altitude	800-3412 m
Superficie	94000 Ha
Espèce sauvages présentes	Chamois Mouflon Bouquetin
Espèces domestiques	Ovins Bovins
Dynamique de population	Pas de données
Gestion faune sauvage / suivi sanitaire	Gestion des populations réalisée par les sociétés de chasse Pas de suivi sanitaire actuellement en place
Prélèvements déjà réalisés	Néant
Données antérieures concernant les Pestivirus pour la faune domestique	Néant
Données antérieures concernant les Pestivirus pour la faune sauvage	Néant
Données futures pour la faune domestique	Collaboration importante possible avec le GDS PACA Contact avec le Dr Eric Belleau : vétérinaire en contact avec les éleveurs locaux. Centralisation possible des prélèvements au LDVHA 05
Bibliographie Pestivirus	Néant

Table VI. Données initiales disponibles pour la vallée de l'Ubaye.

- Vallée de la Tinée

Département	06
Gestion	Parc National du Mercantour Fédération de chasseurs Territoire d'étude du programme Prédateur-Proie (PPP)
Altitude	1200-2800 m
Superficie	72000 Ha
Espèce sauvages présentes	Chamois Mouflons Cerf Chevreuil
Espèces domestiques	ISOLA : Ov - 3932 Cp - 82 Bv - 159 St DELMAS Ov - 6068 Cp - 88 Bv - 0 St ETIENNE Ov - 8360 Cp - 69 Bv - 0
Dynamique de population	St Delmas : Population suivie depuis 2006 dans le cadre PPP : connaissance des paramètres démographiques Saint Etienne : suivi réalisé par la FdC 06
Gestion faune sauvage / suivi sanitaire	St Delmas : PNM Saint Etienne : FdC 06
Prélèvements déjà réalisés	Zone de capture du PPP : environ 60-80 prélèvements par an depuis 2006
Données antérieures concernant les Pestivirus pour la faune sauvage	Données sérologiques obtenues lors des captures disponibles depuis 2006 au niveau du LVDHA 05 à exploiter (prévalence apparente en anticorps fluctuant entre 8 et 53% des animaux testés) Rates recueillies dans le cadre du suivi hivernal 2008 sur des carcasses également disponibles
Données antérieures concernant les Pestivirus pour la faune domestique	Néant
Données futures faune domestique	Contact avec le Dr Coulibaly (DSV 06)
Bibliographie Pestivirus	Néant

Table VII. Données initiales disponibles pour la vallée de la Tinée.

b. Comparaison des sites d'études entre eux

i. Critères comparatifs

Le tableau suivant reprend une série de 8 critères qui ont permis la comparaison des zones potentielles concernées par l'étude épidémiologique de terrain.
Pour chacun de ces critères, chaque zone d'étude est notée de 1 à 5. Les zones présentant les notes les plus élevées seront retenues.

- *Données préalables existantes* : zones dans lesquelles des travaux antérieurs ont déjà été réalisés sur la faune domestique et/ou sauvage et ont permis de générer des données utilisables dans des travaux futurs (notamment données portant sur les dynamiques de populations, données sanitaires)
    - 0 : Aucune donnée n'existe encore
    - 1 : Il existe très peu de données
    - 2 : Il existe quelques données
    - 3 : Il existe des données
    - 4 : Il existe beaucoup de données
    - 5 : Il existe un grand nombre de données

- *Pertinence des données* : les travaux antérieurs ont permis de montrer des résultats intéressants pour la thématique de ce doctorat.
    - 0 : Les données existantes n'ont aucun rapport avec la thématique de ce doctorat
    - 1 : Les données existantes sont très peu pertinentes pour la thématique de ce doctorat
    - 2 : Les données existantes sont peu pertinentes pour la thématique de ce doctorat
    - 3 : Les données existantes sont moyennement pertinentes pour la thématique de ce doctorat
    - 4 : Les données existantes sont pertinentes pour la thématique de ce doctorat
    - 5 : Les données existantes sont très pertinentes pour la thématique de ce doctorat

- *Proximité régionale* : des zones situées le moins loin possible du laboratoire généreront des frais de transports inférieurs à des zones plus éloignées. De plus, ce projet de doctorat est financé à 50% par la région Provence Alpes Côtes d'Azur. Les résultats obtenus pour des territoires de la région PACA permettront de justifier l'importance de tels partenariats pour des collaborations futures.
    - 0 : La zone proposée ne fait pas partie de la région PACA
    - 5 : La zone étudiée se trouve dans la région PACA

- *Réseau de collaboration pour la faune domestique* : il existe dans la zone d'étude un réseau de personnes déjà formé et prêt à opérer permettant de mettre en place un protocole de suivi des populations d'ongulés domestiques.

- 0 : ce réseau n'existe pas du tout ou aucun contact n'est encore mis en place
- 2.5 : un réseau existe, mais les contacts sont encore à établir de façon formelle
- 5 : le réseau existe, fonctionne et les contacts avec l'Anses sont déjà établis

- *Réseau de collaboration pour la faune sauvage* : il existe ou pas dans la zone d'étude un réseau de personnes déjà formé et prêt à opérer permettant d'aider à la mise en place un protocole de suivi des populations d'ongulés sauvages.

- 0 : ce réseau n'existe pas du tout
- 2.5 : les contacts avec des personnes locales sont déjà établis, le réseau reste à mettre en place de façon formelle
- 5 : il existe déjà un réseau pour le suivi sanitaire qui est très performant

- *Originalité du travail* : le travail qui sera fourni présentera une originalité par rapport à la bibliographie existante dans le domaine.

- 0 : les travaux prévus lors de ce doctorat ne présenteront aucune originalité par rapport aux travaux antérieurs déjà réalisés dans la zone
- 2.5 : les travaux prévus lors de ce doctorat présenteront une originalité par rapport aux travaux antérieurs déjà réalisés dans la zone (soit au niveau de la faune sauvage, soit au niveau de la faune domestique)
- 5 : les travaux prévus lors de ce doctorat seront complètement originaux par rapport aux travaux antérieurs déjà réalisés dans la zone

- *Publication déjà réalisées* : certaines zones ont déjà fait l'objet d'études antécédentes qui ont été publiées dans des journaux internationaux.

- 0 : une publication a déjà été publiée portant à la fois sur la problématique en faune sauvage et en faune domestique
- 2.5 : une publication a déjà été publiée portant sur une des 2 thématiques (faune sauvage ou faune domestique exclusivement)
- 5 : Aucune publication n'a encore été publiée dans cette zone

- *« Zone de référence » pour la faune sauvage* : cette zone a déjà fait l'objet d'études antérieures, et a permis l'élaboration de résultats. Ces résultats peuvent servir d'outil de référence par rapport à un phénomène encore peu connu. Le fait de continuer une surveillance active permet de confirmer ou de réévaluer les résultats précédents.

- 0 : les résultats antérieurs n'ont pas pu être validés scientifiquement
- 2.5 : les résultats antérieurs ont été validés soit en virologie soit en sérologie
- 5 : les résultats antérieurs sont validés à la fois pour la partie virologie et sérologie

ii. Tableau comparatif

Zone d'étude	Bauges	Belledonne Sept-Laux	Orlu	Bazès	Caroux	Vallée de l'Ubaye	Vallée de la Tinée	Ristolas	Vallée de la Clarée
Données préalables existantes	5	4	5	1	1	0	5	5	4
Pertinence des données	0	0	5	0	0	0	5	5	2
Proximité régionale	0	0	0	0	0	5	5	5	5
Réseau de collaboration pour la faune domestique	0	0	0	0	0	5	2.5	2.5	2.5
Réseau de collaboration pour la faune sauvage	5	5	5	5	5	2.5	2.5	5	5
Originalité du travail	2.5	5	2.5	5	5	5	5	2.5	2.5
Publication déjà réalisées	5	5	2.5	5	5	5	5	2.5	2.5
« zone de référence » pour la faune sauvage	0	0	5	0	0	0	0	2.5	2.5
SOMME	17,5	19	25	16	16	**22,5**	**30**	**30**	**26**

Table VIII. Tableau comparatif des zones potentielles d'étude

c. Conclusion

Des moyens financiers et humains ont limité le nombre de zones d'études. Par conséquent, seules les zones d'études présentant une note supérieure à 20 ont été retenues.
Les 5 sites d'études retenus sont constitués par les 4 zones situées dans la région PACA, et une zone extérieure à la région :

1. **la vallée de la Tinée (06)**
2. **la réserve de chasse et de faune sauvage de Ristolas (05)**
3. **la vallée de l'Ubaye (04)**
4. **la vallée de la Clarée (05)**
5. **la réserve de chasse et de faune sauvage d'Orlu (09)**

Cependant, par la suite et pour des raisons pratiques, il a été décidé de se limiter aux zones présentes au sein de la région PACA. La réserve de Chasse et de Faune Sauvage d'Orlu a donc été finalement exclue du protocole.

Pour l'ensemble des zones sélectionnées sauf pour la vallée de la Tinée, une collaboration a été mise en place avec certaines sociétés de chasse locales, détaillées ci-après :

- RNCFS de Ristolas : Société de chasse Ségure-Viso, située à Ristolas
- Vallée de l'Ubaye : Sociétés de chasse de Méolans-Revel, Jausiers, La Condamine,

Dans chaque société de chasse concernée, l'ensemble des animaux présentés au plan de chasse ont été prélevés. Concernant les troupeaux domestiques, il était prévu de réaliser un test sérologique (ELISA) sur un dixième des animaux prélevés lors du plan national de prophylaxie. Des échantillons de placenta, de sang total étaient envoyés par les vétérinaires locaux lors de suspicion clinique d'animaux IPI.

ARTICLE 5. ARTICLE ORIGINAL

Comparison between pestivirus infections in wild and domestic ruminants in the French Southern Alps suggest that interspecies transmission may occur

En preparation pour publication dans Veterinary Microbiology

ARTICLE 5. Resumé

Possible transmission inter-spécifique des infections avec des pestivirus entre les ruminants sauvages et domestiques dans les Alpes du Sud françaises

Les transmissions inter-espèces sont régulièrement incriminées dans l'épidémiologie des pestiviroses dans les alpages. Le premier objectif de cette étude a été d'investiguer les infections avec des pestivirus chez les ruminants sauvages et domestiques présents sur les mêmes alpages dans les Alpes du Sud françaises. De plus, les relations génétiques et antigéniques entre les souches isolées ont été étudiées. Un kit ELISA commercial (Synbiotics, Lyon, France) a été utilisé pour rechercher les anticorps dirigés contre des pestivirus dans tous les sérums provenant d'espèces sauvages et dans 10% des sérums prélevés sur les troupeaux ovins. La totalité des 38 troupeaux ovins testés présentaient une séroprévalence positive, atteignant 76,5% (intervalle de confiance à 95% [IC95%]: [74.2 – 78.8%]) des 1383 sérums testés. Chez les ruminants sauvages, 38.7% (IC95%: [33.8 – 43.9%]) des 369 chamois, 28.7% (IC95%: [17.4 – 38.1%]) des 72 chevreuils et 22.2% (IC95%: [6.5 – 37.9%]) des 27 mouflons testés étaient séropositifs. Un screening viral a été réalisé sur des échantillons de rate collectés sur les animaux sauvages chassés (n=160) et sur 15 ovins (cliniquement suspectés d'être des animaux IPI) par une RT-PCR conventionnelle. Trois souches de pestivirus ont été isolées sur des moutons et ont été classés parmi les génotypes BDV-3, BDV-Tunisian et BDV-6. Pour la première fois, une souche (appelée RUPI-05) a été isolée d'un chamois et classée parmi les BDV-6, montrant 92% d'homologie (au niveau de la région 5'UTR) avec la souche ovine isolée dans la même vallée (également classée parmi les BDV-6). Des séroneutralisations comparatives ont montré que les moutons avaient des titres supérieurs aux chamois pour l'ensemble des souches domestiques utilisées mais inférieurs pour la souche RUPI-05. Une circulation active des pestivirus a été montrée dans les ongulés sauvages comme domestiques. Les résultats obtenus indiquent une continuité génétique entre les souches circulant localement. Par ailleurs, les résultats de séroneutralisation croisée semblent montrer qu'un cycle épidémiologique est présent pour chacune des espèces chamois et ovine, et que des transmissions ponctuelles peuvent exister entre les deux espèces.

Comparison between pestivirus infections in wild and domestic ruminants in the French Southern Alps suggest that interspecies transmission may occur

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Abstract (318 Words)

In alpine pasture, interspecies transmission has recently been incriminated in the epidemiology of pestivirus infection. The first aim of this study was to investigate pestivirus infections in wild and domestic ruminants sharing pastures in the French Southern Alps. In addition antigenic and genetic relationships between isolated strains were studied. Sera were screened for pestivirus antibodies against the pestivirus NS3 protein by a commercial blocking enzyme linked immunosorbent assay (ELISA) (Synbiotics, Lyon, France) in all wild animal samples and in 10% of sera collected from domestic herds. All 38 domestic herds tested were positive for pestivirus-specific antibodies. Individual sero-prevalence reached 76.5% (95% confidence interval [CI95%]: [74.2 – 78.8%]) of the 1383 sheep tested. For wild ruminants, 38.7% (CI95%: [33.8 – 43.9%]) of the 369 chamois tested, 28.7% (CI95%: [17.4 – 38.1%]) of the 72 roe deer and 22.2% (CI95%: [6.5 – 37.9%]) of the 27 mouflons were seropositive. Virus screening was carried out on spleen samples from hunted wild animals (n=160) and from 15 domestic ruminants (clinically suspected to be persistently infected animals), by a conventional reverse transcription-polymerase chain reaction (RT-PCR). Three pestivirus strains were isolated from sheep and were classified in the BDV-3, BDV-Tunisian and BDV-6 genotypes. For the first time, one strain (named RUPI-05) was isolated from an alpine chamois and clustered in the BDV-6 genotype, showing 92% of similarities with the ovine isolate of the same area in the 5'UTR region. Comparative virus neutralization tests (VNT) showed that sheep had higher titers than chamois for all domestic strains but lower for the RUPI-05 strain. These results indicate a very high and unexpected seroprevalence in domestic ruminants, and a high seroprevalence in wild ruminants. The viruses isolated from chamois and sheep in the same area clustered in the same genogroup, which may indicate a genetic continuity among the circulating strains. Active circulation of pestivirus was demonstrated in both wild and domestic ungulates. Nevertheless, an epidemiological study needs to be completed in order to establish the relationship between wild and domestic animals for the transmission of pestivirus infection.

1. Introduction

Pestiviruses (family *Flaviviridae*) are single-stranded, positive-sense RNA viruses, officially divided into 4 species. Bovine Viral Diarrhea Virus type 1 (BVDV-1) and Bovine Viral Diarrhea Virus type 2 (BVDV-2) are mainly found in cattle, Border Disease Virus (BDV) in sheep and Classical Swine Fever Virus (CSFV) in pig (ICTV, 2011). In ruminants, diseases associated with pestivirus infection mainly include reproductive failure such as abortion, stillbirth, birth of weak lambs, and birth of persistently infected animals (PI) (if infection occurred during the second third of gestation) (Pastoret et al., 1988). Reduced conception rate, abortions, congenital defects, growth retardation, immunodepression possibly associated with respiratory disorders and a possible reduced milk

production lead to important financial losses directly related to pestivirus infections (Houe, 1999). Because of these financial losses, many European countries recently adopted official programs to eradicate pestivirus in domestic herds (Presi et al., 2011). In France, the *Association pour la Certification en Santé Animale* (ACERSA) recently adopted measures for voluntary certification of "non-PI cattle" (ACERSA, 2010; Legifrance, 2011).

Pestivirus infections have also been described in a wide range of wild ruminant species (Vilcek and Nettleton, 2006). Interspecies transmission has often been incriminated in the epidemiology of abortive infections such as pestivirus (Pioz, 2006). Indeed, alpine pastures have been shown to be places with a high rate of contact between wild and domestic animals (Richomme et al., 2006), which may represent a risk of domestic animal or wildlife contamination. In Austria, a survey was performed to investigate the influence of alpine pasturing on the spread of pestivirus infections in sheep and goats. The main result was a significant increase in seroprevalence in the 481 tested sheep (from 67.6% to 83%) (Krametter-Froetscher et al., 2007). In that case, the presence of PI animals in herds may only partly explain the increase in seroprevalence. The role of wildlife in the transmission could not be assessed but was suspected to be significant (Krametter-Froetscher et al., 2007).

Previous studies in the Alps showed different pestivirus seroprevalence in wild ungulates. Olde Riekerink et al., (2005) reported 25.5% of positive chamois (*Rupicapra rupicapra*) in the High Valley of Susa (Italy). Only six chamois and three alpine ibexes (*Capra ibex ibex*) from 546 sera tested in Switzerland were found positive (Casaubon et al., 2011). In Alpine ibex, seroprevalence reached 13.9% of 553 sera tested between 2006 and 2008 in Switzerland (Marreros et al., 2011), and in 7.17% (16 out of 223) of Alpine ibexes (*Capra ibex*) from the Italian Alps (Fernandez-Sirera et al., 2011). In the French Northern Alps, the pestivirus seroprevalence in chamois was very low, below 2% between 1980 and 2001 (Jourdain, 2003). In the French Southern Alps, a recent survey conducted in wild ruminants showed that 45.9% of the 343 chamois tested and 61.1% of the 13 mouflons (*Ovis gmelinii musimon)* tested were seropositive (Martin et al., 2011). But, to the author's knowledge, no isolation and classification of pestiviral strains has been published so far in this area.

The objectives of this study were to investigate the prevalence of antibodies directed against pestiviruses among wild and domestic ruminants sharing the same pastures, to characterize circulating strains and to assess the possible cross-contamination between different species of ruminant.

2. Material and methods

2.1. Study area and samples

Samples were collected from ruminants sharing pastures in four areas in three Districts (*Départements*) of the French Southern Alps, Ubaye Valley in the Alpes de Haute-Provence (04), Clarée Valley and the Game and Wildlife National Reserve of Ristolas in the Hautes-Alpes (05) and Tinée Valley in the Alpes Maritimes (06) (Figure 1). These valleys were known by local hunters and veterinarians for their high contact rate among wild and domestic species (hunters, personal

communication). For practical reasons, in this paper, we will name the areas by their District numbers (04, 05 and 06). For wildlife, samples were collected from hunted animals in Districts 04 and 05 and from captured animals in District 06. Biological samples (blood and spleens for hunted animals or sera and whole blood for captured animals) from 369 chamois (*Rupicapra rupicapra)*, 27 mouflons (*Ovis gmelini musimon)* and 72 roe deer (*Capreolus capreolus*) were collected during the 2009 and 2010 hunting seasons according to previously described conditions (Martin et al., 2011). General information (species, sex, age, weight, location where shot and overall sanitary status) were given as complementary data with each sample. Domestic herds (mainly sheep) pasturing in the same areas were sampled: sera from one tenth of each herd were collected by the departmental laboratories of concern and frozen until use. A total of 1067 samples were collected in 2009 in District 04 and 310 in 2010 in Districts 05 and 06. Samples were collected from non-vaccinated herds. Numbers of samples by areas and species are shown in Table 1.

2.2. Serological analyses

The presence of pestivirus-specific antibodies against the NS3 protein in sera was investigated using a commercial blocking enzyme linked immunosorbent assay (ELISA) (Synbiotics, Lyon, France) according to the manufacturer's recommendations. This ELISA test was chosen for data harmonization with a previously published study performed in the same area (Martin et al., 2011).

In order to characterize serological responses, a total of 12 sera of chamois, 12 of sheep and 6 of roe deer randomly chosen among the ELISA antibody-positive sera collected in 2009 in District 04 were analyzed in a comparative virus neutralization test (VNT). Seven different pestivirus strains were used for VNTs: 2 reference strains, the BVDV-1 strain NADL (Collet et al., 1988, GENBANK accession number M31182), and the BDV-1 strain Moredun (Vilcek et al., 1997; GENBANK accession number U65023.1), one strain isolated from Pyrenean chamois, the BDV-4 strain CADI-6 (Marco et al., 2009, GENBANK accession number AM905923), three local strains isolated from sheep, the BDV-3 strain 06M0150 (Martin et al., 2011; GENBANK accession number), the BDV-Tunisian strain 10F03401 (Martin et al., 2011; GENBANK accession number), and the BDV-6 strain 10F03356 (Martin et al., GENBANK accession number), and one local strain isolated from chamois, the BDV-6 strain RUPI-05 (Martin et al., GENBANK accession number). VNTs were carried out following the same protocol as Martin and collaborators (2011).

2.3. Virus detection, isolation and characterization

2.3.1. Virus screening by RT-PCR

A total of 163 samples were analyzed: 30 whole blood samples (collected in District 06 on live animals) and 133 spleens. Out of these 133 spleens, 11 came from necropsy of dead animals found by local partners (6 chamois, 2 roe deer, 1 red deer and 2 ibexes *[Capra ibex]*) and the others were randomly chosen among all spleens collected in Districts 04 and 05 (for financial reasons, we could

not analyze all of them). Total RNA was extracted from 100 µL of whole EDTA blood and from 100 µL of 10%-homogenate (w/v) of tissue in PBS using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA was suspended in 30 µL of RNase-free water and kept at -80° C until use (Martin et al., 2011, submitted).

Conventional reverse transcription-polymerase chain reactions (RT-PCR) were performed on each whole blood sample and spleen. The 5'UTR region was amplified using primers 324 and 326 to amplify a 249 base pair fragment using the method already described in Dubois et al., 2008. On each positive sample, the 5'UTR/N^{pro}/Erns was amplified using the primers P324 and 1400RC (Vilček et al., 2005) as described by Dubois and collaborators (2008).

2.3.2. Viral isolation

Viral isolation was performed in all RT-PCR positive samples. Briefly, 100µL of 10%-homogenate (w/v) of tissue in PBS were inoculated into a 7.5 cm² plate containing ETM52 cells using a previously described method (Martin et al., submitted). An RT-PCR was performed on cell supernatant to confirm viral multiplication. The virus was then multiplied by three passages in cell culture in order to obtain a sufficient quantity for its use in VNT.

2.3.3. Sequence analysis

RT-PCR positive products were purified by using the QiaQuick1 PCR Purification kit (Qiagen) and direct sequencing of both strands of cDNA was performed by Millegen (France). Each sample was treated in duplicate. For both the 5'UTR and Npro regions of the genome, 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. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura, Dudley, Nei, and Kumar 2007). Descriptive boxplots were used to graphically depict optical densities obtained with the ELISA test (Tukey, 1977).

2.4. Statistical analysis

Risk factors were assessed using odds ratio (OR). The 95% confidence interval was estimated using a binomial exact method and the Wilcoxon signed rank test (Dagnelie, 1998), given that titers values were 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); this involves dividing the P-value obtained with any of the tests by the number of multiple comparisons performed (i.e. 21 comparisons in this study).

3. Results

176

177 *3.1. Serological results*

178 Antibodies against pestivirus were found by ELISA in 76.6% of sheep (95% confidence interval
179 [CI95%]: 74.2-78.8%), 38.7% of chamois (95% CI: 33.8-43.9%), 27.8% of roe deer (95% CI: 17.4-
180 38.1%) and 22.2% of mouflons (95% CI: 6.5-37.9%). Apparent seroprevalence is shown in Table 2 for
181 chamois and sheep for each District. In chamois, the seroprevalence was 24.5% (95% CI: 17.7-32.4%)
182 in District 04, 47.7% (95% CI: 33.8-43.9%) in District 05 and 48.5% (95% CI: 30.8 - 66.5%) in
183 District 06. In sheep, values are significantly higher for all Districts and reach 76.9 % (95% CI: 74.3-
184 79.4%) in District 04, 65.4% (95% CI: 57.4-72.8%) in District 05 and 85.0% (95% CI: 78.5-90.1%) in
185 District 06. No significant difference was observed between Districts. The populations of roe deer and
186 mouflons being very low, they were not included in the statistical analysis.

187 In chamois, female gender was confirmed to be a risk factor (OR♀=2.51, 95% CI: 1.62-3.69). Besides,
188 seroprevalence was significantly lower in young animals (OR<2 years=0.49; 95% CI: 0.30-0.79). Out
189 of 117 young chamois tested (less than 2 years old), 32 were seropositive, indicating a recent viral
190 circulation. In roe deer, no risk factors were found.

191 Distributions of optical density (OD) values for sheep and chamois by District are presented in Figure
192 3. Homogenous results were found for sheep in all three Districts. Median optical densities were
193 always higher in sheep than in chamois. In District 04, OD values obtained by ELISA were
194 significantly higher for sheep than for chamois (Figure 3, Welsh test, $p<0.05$; data not shown).

195 Virus neutralization titers obtained against the seven pestivirus strains are reported in Tables 2a and 2b
196 for chamois and sheep, respectively. Except for one analysis, all roe deer had negative results (data not
197 shown) for all strains. The only roe deer (male, adult) had a positive titer against the BVDV-1 strain
198 NADL (titer equaled 22.6 DN_{50}/50 µl). In chamois, titers obtained against the BDV6 strain RUPI-05
199 were significantly higher (Wilcoxon signed rank test, $p<0.05$) than all other strains except the BDV-3
200 strain 06M0150. For the latter strain, titers against the RUPI-05 strain were higher but not significantly
201 so (Wilcoxon signed rank test, $p>0.05$). Titers were also higher against the two other local strains
202 isolated from sheep (the BDV-3 strain 06M0150 and the BDV-6 strain 10F03356).

203 In sheep, significant higher titers were found against the local strains isolated from sheep: the BDV6
204 strain 10F03356, the BDV3strain 06M0150 and the BDV-Tunisian strain 10F03401 (Wilcoxon signed
205 rank test, $p>0.05$). The BVDV-1 strain NADL was significantly less neutralized by sera of the two
206 animal species (Wilcoxon signed rank test, $p<0.05$).

207 Besides, the comparison of sheep with chamois showed that significantly higher titers were obtained
208 by sheep for the strains NADL, CADI-6, BPII, 10F03356, 10F03401 (Wilcoxon signed rank test,
209 $p<0.05$). RUPI-05 was the only strain neutralized more by chamois than by sheep (Table 2).

210

211 *3.2. Virological results*

Three different strains of pestiviruses were isolated from sheep, and one from a chamois. GENBANK accession numbers and their characteristics are presented in Table 2. The RUPI-05 strain was isolated from a juvenile female chamois (under 1 year old), captured in the Vars Pass (District 04: 48°51'39"N, 02°20'43'' E) and transferred to a care center. On arrival, the animal was emaciated, and died after 2 days with low nasal discharge and weakness. At necropsy, general amyotrophy and hypertrophy of the prescapular lymph node were the only macroscopic changes reported. Bacterial cultures were negative in all of the organs tested (spleen, heart, liver, kidney, lung). Although the animal did not show any sign of diarrhea, coccidiosis was present (2900 coccidia/g of fecal material). Pestivirus RNA was detected in the ground spleen by RT-PCR. Analysis of the 5'UTR and Npro genes showed that the RUPI-05 strain clustered in the BDV-6 genogroup (Figure 2).

The three strains isolated in sheep clustered in 3 subgroups of BDV: BDV-3, BDV-6 and BDV-Tunisian, respectively (Figure 2).

The ovine strains 06M0150 (BDV-3) and 10F03401 (BDV-Tunisian) were isolated in District 06. The two strains isolated in the Ubaye Valley (District 04) from chamois and sheep clustered in the BDV-6 genogroup, showed 92% of homologies.

4. DISCUSSION

This study showed an obvious circulation of pestiviruses among wild and domestic ruminants sharing pastures in the French Region of Provence-Alpes-Côte d'Azur (PACA). To the author's knowledge, this is the first published isolation of pestivirus in chamois in the Alps. In District 04, strains isolated in the same valley from sheep and the positive chamois clustered among the same BDV-6 genotype. The other pestiviruses isolated from sheep in District 06 clustered in two different subgroups of BDV: BDV-3 and BDV-Tunisian. Classification of domestic isolates is in accordance with previous results showed by Dubois et al. (2008): in the PACA region, ovine isolates clustered in the BDV-3, BDV-Tunisian and BDV-6 genotypes.

To the author's knowledge, this is the first study to investigate seroprevalence directed against pestiviruses in ovine herds of the PACA region. Seroprevalence reached 76.6% in sheep and was significantly higher than in all the wild species tested. In the French Northern Alps (in the Game and Wildlife National Reserve of Les Bauges), a survey carried out in 2001-2002 showed that 50% of 592 non-vaccinated bovines, 5.8% of 17 sheep and 147 % of 95 goats were antibody-positive (Jourdain, 2003). The mode of management of domestic herds may explain these differences. Indeed, in the Northern Alps, local bovine dairy herds are the main domestic animals present in alpine pasture whereas sheep herds are the main domestic flocks present in the PACA region. Most of them are translocated to alpine pastures in summer seasons (from June to October) (Champion, personal communication). In alpine pastures, contact exposure between chamois and sheep was shown to be directly linked to pastoral practices (Gauthier & Durand, 1996). In other European countries,

pestivirus seroprevalence in sheep was dependant on the country, from 29.4% in Austria (Krametter-Froetscher et al., 2007) to 69% in the Spanish Pyrenees (Marco et al., 2008).

In alpine populations of chamois, seroprevalence depends on the region considered: in the Southern French Alps, seroprevalence reached 45.9% (95% CI: 40.5-51.3%) of the 343 chamois tested during the 2003 to 2007 hunting seasons (Martin et al., 2011) whereas only eight animals were seropositive among 423 in the Northern Alps between 1980 and 2001 (Jourdain, 2003). In chamois, ELISA serologies showed that females were significantly more seropositive than males, and that older animals (more than 8 years old) were more often positive than younger ones. This is in accordance with and confirms previous results found in District 05 (Martin et al., 2011) or in the Pyrenees (Pioz et al., 2006).

Concerning other wild ruminant species, seroprevalence in mouflons was lower than previous results in the same region: 22.2% (95% CI: 6.5-37.9%) of mouflons were seropositive in this survey whereas 61.1% (95% CI: 38.6-83.6%) of the 18 animals previously tested were positive (Martin et al., 2011). Although both samples were taken within the PACA region, two Districts were concerned: 20 of the 27 mouflons sampled in the present study originate from District 04 whereas the previous sample only concerned animals from District 05. In order to understand such differences in seroprevalence, further studies are needed to identify biological characteristics of both mouflon populations (animal densities, frequency of contact with other species). In roe deer, 27.8 % of the 72 sera tested were antibody-positive with the ELISA test. The seroprevalence described in this species depends on the region and the country but was always lower than results obtained in the present study. In Spain, two surveys showed that only 8 animals tested out of 519 (1.5%) (Boadella et al., 2010), and none of the 43 roe deer tested in the Pyrenees (Marco et al., 2011) were antibody-positive. In Denmark and in Austria, no antibody-positive roe deer were detected out of 361 and 77 roe deer tested, respectively (Nielsen et al., 2000: Krametter et al., 2004). In Norway, pestivirus was shown to be endemic in roe deer with a seroprevalence of 12.3% (from among 635 roe deer tested) (Lillehaug et al., 2003). However, in the present study, only one among six ELISA positives was confirmed by a positive result in VNT: the only positive titer obtained in VNT was directed against the BVDV-1 strain NADL (titer of 22.6 DN_{50}/50µl). Two hypotheses may explain this divergence of results. First, a lack of specificity of the ELISA test could generate such false positive results. However, Lillehaug and collaborators (2003) used the same commercial kit and showed that the 78 ELISA positive sera were all confirmed by VNT, using the same BVDV-1 strain NADL as in the present study. The absence of antigenic relationship between the circulating strain in roe deer and the strains used for the VNT may explain such a difference. To test this second hypothesis, it would be interesting to test roe deer sera in a VNT using local strains isolated from cattle or *cervidae*.

Comparative VNTs were performed in order to characterize and to compare serological responses of chamois and sheep. Chamois had higher titers for the RUPI-05 strain than for all other strains. Except for the BDV-3 strain 06M0150, the difference was significant. Titers of sheep were higher for ovine

local strains. Besides, for all strains but one (the chamois RUPI-05 strain), sheep had higher titers than chamois. These results indicate that viral cycle seems to be specific to the host species: chamois have higher titers against the chamois strain whereas sheep have higher titers directed against local ovine strains. Previous studies showed that antigenic relatedness is low among pestivirus species (Avalos-Ramirez et al., 2001) but may be high within a genus. In the present survey, despite significant cross reactivity between strains, 6 of the 7 strains used in VNT were BDV strains, and significant differences were found between animal species. These results are important when studying the epidemiology of the infection.

The pathogeny of pestiviruses in wild species has only been studied in some determined species, which were shown either to be highly affected or to play a role in the epidemiology of domestic livestock. Recently, outbreaks of pestivirus infection were reported in Spanish Pyrenean chamois and were associated with high mortality (42 to 86%) and clinical signs such as behavioral changes and alopecia, with skin hyperpigmentation (Marco et al., 2007, 2009). Experimental infections of Pyrenean chamois with a chamois-BDV (isolated during a previous outbreak in Pyrenean chamois) showed that BDV infections can induce severe disease leading to abortion for pregnant females and the death of 5 animals out of 9 inoculated (Cabezon et al., 2011; Martin et al., submitted). In the Alps, only decreases in population have been reported, and were not associated with specific clinical signs (Martin et al., 2011). In the present survey, no clinical sign was reported in any of the chamois populations studied. Nevertheless, the RUPI-05 strain was isolated on a yearling chamois presenting weakness, which is the only clinical sign that can be associated with pestivirus infection. At necropsy, no macroscopic lesions were found. Unfortunately, histological analyses could not be performed. Only a spleen sample was available for RT-PCR investigation, meaning that it was impossible to assess the systemic distribution of the virus. In deer, Passler et al. (2009) showed that virus could be isolated from four organs of PI fawns including lungs, spleen, thymus and lymph node. It is not possible to know whether this animal was infected *in utero* and may be a PI animal or if it was in transient infection. To investigate the pathogeny of the circulating strain in chamois and to compare this with the pathogenicity of the BDV-4 chamois strain, experimental infections of alpine chamois with the alpine isolate are needed. It might also be interesting to give more attention to visual inspections associated with virological investigations in order to determine the viral pathogenicity.

The two strains isolated in the Ubaye Valley from chamois and sheep clustered in the BDV-6 subgroup. Similar observations were reported in the Pyrenean Mountains: strains isolated in Pyrenean chamois and sheep clustered together in the BDV4 subgroup (Marco et al., 2009; Valdazo-Gonzalez et al., 2006). Thus, we can assess that pestiviral isolates are geographically distributed, as shown by a spatial continuum of pestiviral strains among ruminant species. Over the last few years, interspecies transmission has become an issue of major interest (Gortazar et al., 2007). Scientists often try to

address a specific epidemiological role to species involved in the transmission of infections or diseases (Martin et al., 2011, Vet Res). For pestiviruses, cross contamination has been widely described but interspecies transmission in natural conditions has not yet been demonstrated. During an experimental infection, two persistently infected cattle (with BVDV1) shared food and water in the same pen with seven pregnant white-tailed deer for 60 days. Four pregnancies resulted in PI offspring, indicating that BVDV may efficiently cross the species barrier (Passler et al., 2009). The principal objectives of this study were to describe the present situation in all species involved in pestivirus transmission in alpine mountains, and, if possible, to investigate possible interspecies transmission. However, with the results obtained, it is difficult to attribute a specific epidemiological role to the different species. To make such attibutions, further studies are needed to investigate the virological prevalence among the various ruminant species, to isolate and sequence more strains from both species. Besides, further studies are needed to investigate modes of herd management, and how these are related with seroprevalence in both wild and domestic ruminants.

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Figures and tables

Figure 1.

Title: Location of the study area

Legend :

District 04: Alpes de Haute-Provence; District 05: Hautes-Alpes; District 06: Alpes-Maritimes

Figure 2a.

Title: Neighbour-joining phylogenetic tree constructed using 212 nucleotides from the 5'UTR region of the pestivirus

Numbers indicate the percentage of bootstrap replicates that supported each phylogenetic branch. BVDV: bovine viral diarrhea virus, BDV: border disease virus

Figure 2b.

Title: Neighbour-joining phylogenetic tree constructed using 489 nucleotides from the Npro region of the pestivirus

Figure 3:

Title: Distribution of the optical densities obtained by chamois and sheep in Districts 04, 05 and 06

Legend: DO: *Densités Optiques* (Optical densities). The vertical mark represents the limit of the positive optical density (OD=20)

Table 1.

Title: Number of serum samples collected in sheep and wild ruminants in 2009 and 2010

Table 2.

Title: Apparent seroprevalence directed to pestiviruses in sheep and chamois by areas collected

Legend: Nt, Number of tested animals; Np, Number of positive animals; SP, Seroprevalence; CI, Confidence interval (Exact binomial estimation)

Table 3.

Title: Origin, species and classification of isolated strains in the Provence-Alpes-Côte d'Azur (PACA) region

Legend: BDV: border disease virus.

Table 4.

Title: Virus neutralization titers against 7 different pestivirus strains

Table 1. Number of serum samples collected in sheep and wild ruminants between 2009 and 2010

District	Chamois (*Rupicapra rupicapra*)	Roe deer (*Capreolus capreolus)*	Mouflon (*Ovis gmelinii musimon)*	Sheep (*Ovis aries*)
04	143	47	20	1067
05	193	23	6	156
06	33	2	1	160
Total	369	72	27	1383

542 **Table 2. Apparent seroprevalence directed against pestiviruses in sheep and chamois by areas collected**

543

	Sheep			Chamois			Mouflon		Roe deer	
District	Nt	Np	SP (95% CI)	Nt	Np	SP (95% CI)	Nt	Np	Nt	Np
04	1067	821	76.9 (74.3 - 79.4)	143	35	24.5 (17.7 - 32.4)	20	1	47	13
05	156	102	65.4 (57.4 - 72.8)	193	92	47.7 (40.4 - 55.0)	6	5	23	7
06	160	136	85.0 (78.5 - 90.1)	33	16	48.5 (30.8 - 66.5)	1	0	2	0
Total	1383	1059	76.6 (74.2 - 78.8)	369	143	38.8 (33.8 - 43.9)	27	6	72	20

544

545 Legend: Nt, Number of tested animals; Np, Number of positive animals; SP, Seroprevalence; CI, Confidence interval (Exact binomial estimation).

546 Seroprevalence and confidence interval were not evaluated for mouflon and roe deer because of their low populations.

Table 3. Origin, species and classification of pestivirus isolates in 2010 in the PACA region

Virus isolate	Animal species and characteristics	District of isolation	Genogroup	GENBANK Accession no.	
				5'UTR	N^{pro}
10F03401	Sheep (PI animal)	06	BDV-Tunisian		
06M0150	Sheep (PI animal)	06	BDV-3		
10F03356	Sheep (abortion)	04	BDV-6		
RUPI-05	Chamois (yearling found dead)	04	BDV-6		

Table 4. Virus neutralization titers against seven different pestivirus strains

Table 4a. With 12 sera from chamois (*Rupicapra rupicapra*)

	NADL	**BPII**	**06M0150**	**CADI-6**	**RUPI-05**	**10F03356**	**10F03401**
	BVDV-1	BDV-1	BDV-3	BDV-4	BDV-6	BDV-6	BDV-T
2009-04-009	40	28,3	56	80	160	113	80
2009-04-0062	40	160	160	160	905	80	80
2009-04-0163	<10	160	320	80	640	113	56
2009-04-0177	0	<10	226	<10	160	56	14
2009-04-0181	28	40	80	40	640	113	56
2009-04-0186	<10	0	320	226	320	226	14
2009-04-0198	113	226	902	320	1280	320	160
2009-04-0214	14	160	320	80	320	160	40
2009-04-216	14	56	56	160	80	113	28
2009-04-0290	0	<10	226	<10	80	0	28
2009-04-313	0	0	80	80	226	56	<10
2009-04-314	0	0	80	0	160	0	0
Mean values	24(#)	83(#)	235	122(#)	414(*)	112(#)	50(#)

Table 4b. With 12 sera from sheep (*Ovis aries*)

	NADL	**BPII**	**06M0150**	**CADI-6**	**RUPI-05**	**10F03356**	**10F03401**
	BVDV-1	BDV-1	BDV-3	BDV-4	BDV-6	BDV-6	BDV-T
A	56	113	113	160	160	452	452
B	56	160	226	226	226	226	226
C	<20	56	226	226	226	452	640
D	80	56	226	113	113	640	452
E	113	226	113	226	320	640	452
F	160	320	1810	905	1280	905	640
G	80	160	113	160	226	905	905
H	160	320	320	226	452	640	452
I	20	113	160	160	452	320	640
J	56	80	1280	113	226	113	113
K	160	320	905	113	226	452	320
L	56	113	160	160	452	320	113
Mean values	90(#)	169(#)	471	232(#)	363	505(#)	450(#)

Legend:

(*) Except for the BDV-3 strain 06M0150 (for which titers were higher but not significantly), the BDV-6 strain RUPI-05 was significantly more neutralized than the other strains by chamois sera.

(#) Significant difference between titers of chamois and sheep

Figure 1. Location of the study area

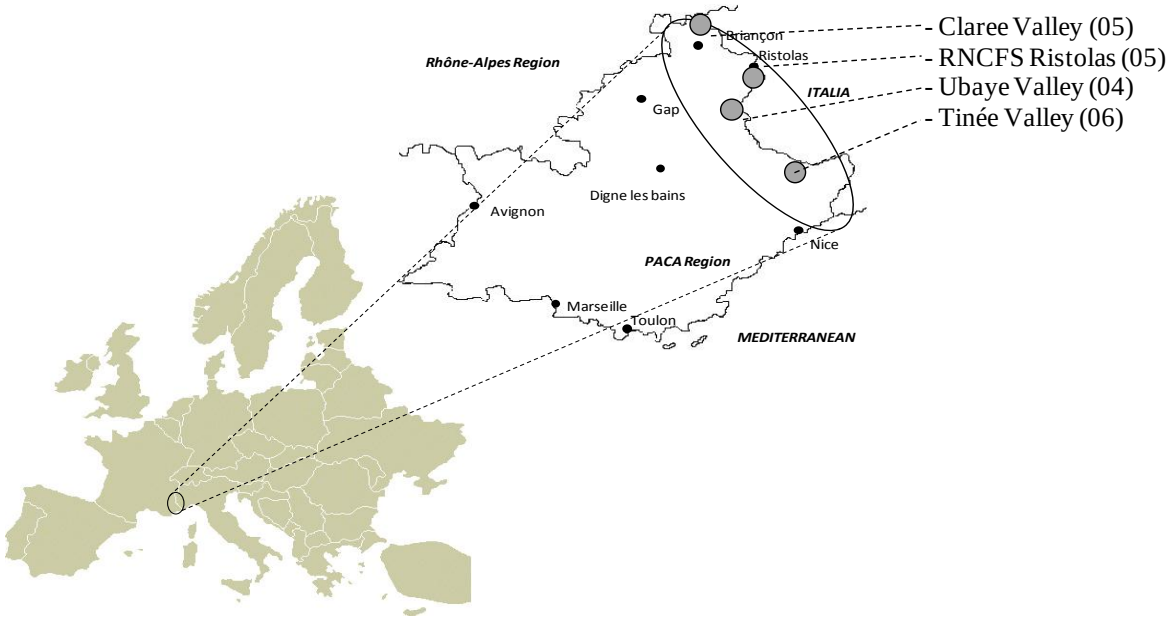


Figure 2a.

Neighbor-joining phylogenetic tree constructed using 212 nucleotides from the 5'UTR region of the pestivirus sequences found during this study and from the GENBANK database.

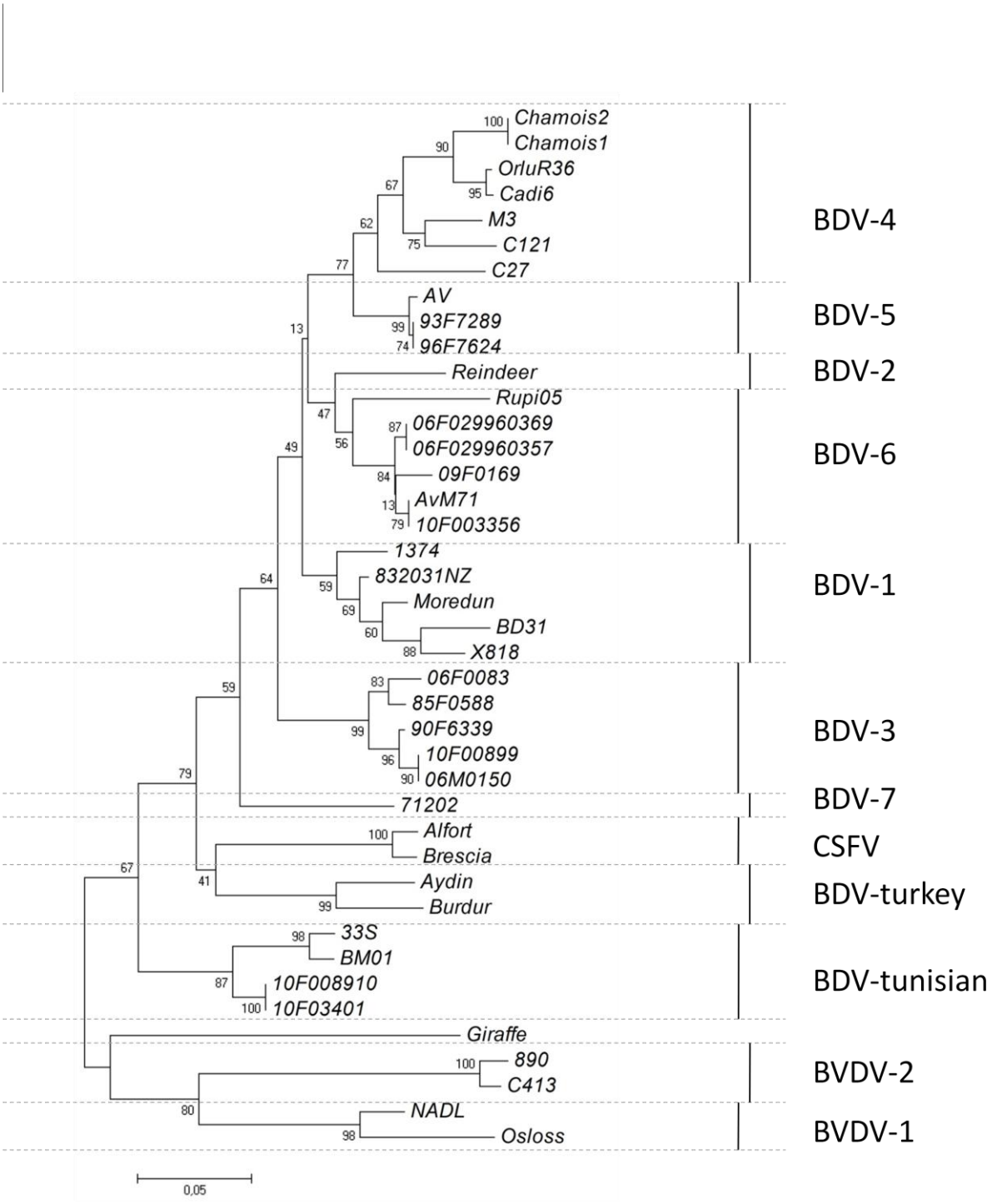


Figure 2b.

Neighbor-joining phylogenetic tree constructed using 489 nucleotides from the Npro region of the pestivirus sequences found during this study and from the GENBANK database.

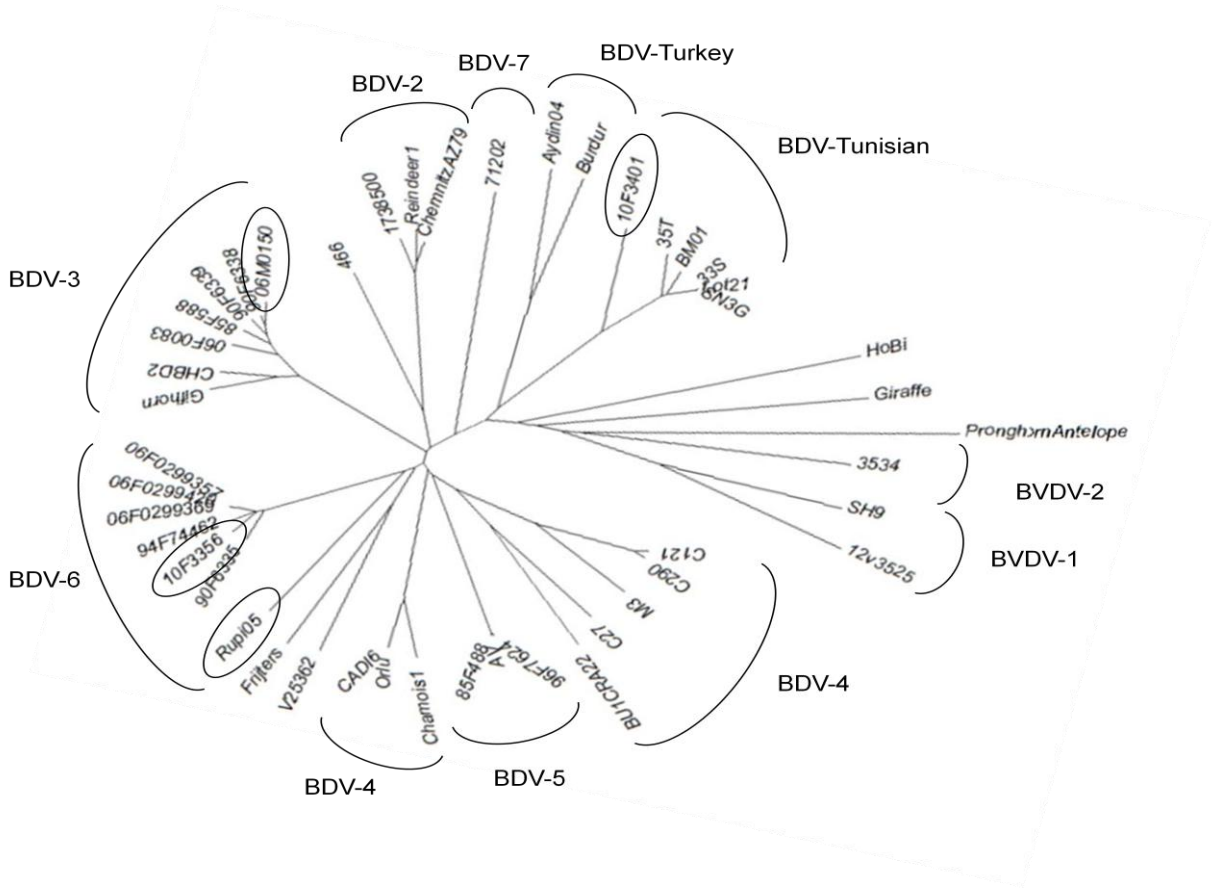
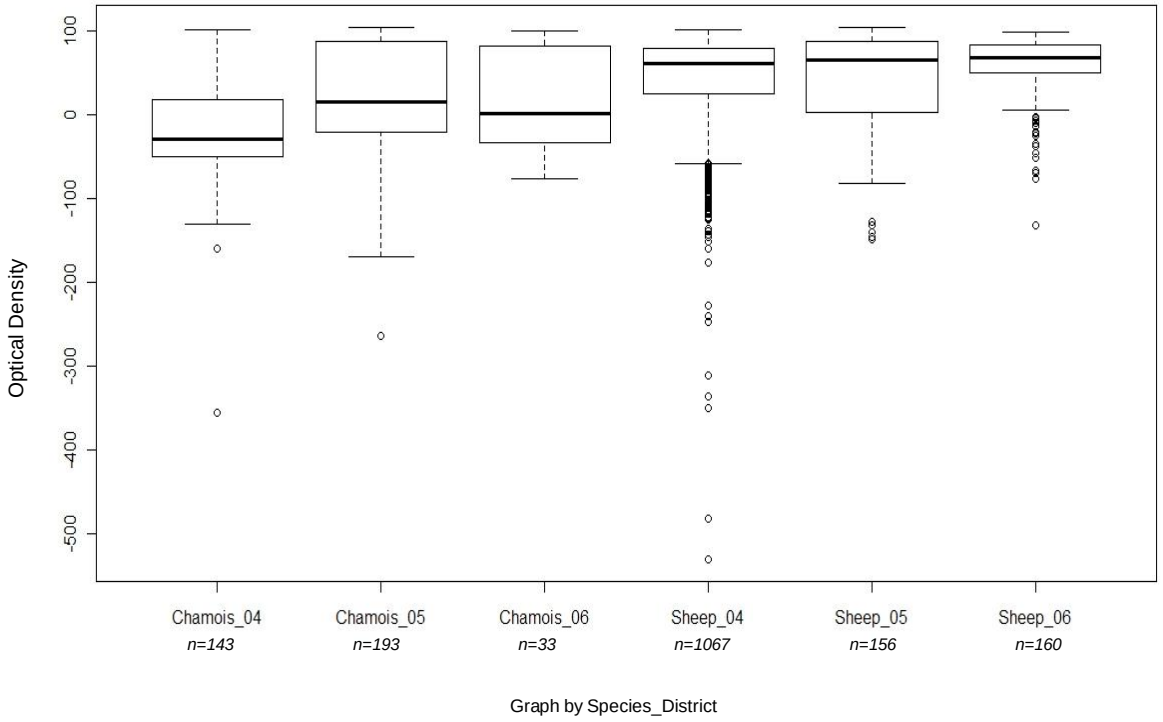


Figure 3.

Boxplot presentation of the distribution of the optical density (OD) values obtained by chamois and sheep in Districts 04, 05 and 06 with the antibody ELISA test.



CONCLUSION ET PERSPECTIVES

1. Rappel des principaux résultats

Au cours de ce travail, une première étude séro-épidémiologique, longitudinale sur des ruminants sauvages a été mise en place dans le département des Hautes-Alpes durant les campagnes de chasse de 2003 à 2007. Des anticorps dirigés contre les pestivirus étaient présents chez 45,9% (Intervalle de confiance à 95% [IC95%] : 40,5-51,3) des chamois et 61,1% (IC 95% : 38,6-83,6%) des mouflons (*Ovis gmelini musimon*). Une deuxième étude épidémiologique transversale, conduite à la fois chez les ruminants sauvages et domestiques lors des saisons de chasse 2009 et 2010, a montré des séroprévalences élevées, atteignant 38,8% (IC95% : 74,3 – 78,8 %) chez les chamois et 25,9% (IC95% : 9,4 – 42,4 %) chez les chevreuils (*Capreolus capreolus*). Tous les cheptels ovins testés (n=37) présentaient une séroprévalence positive, atteignant 76,6 % (IC95%: 74,3 – 78,8 %) pour l'ensemble des 1383 sérums analysés (prévalence individuelle). Dans le département des Alpes-Maritimes, deux souches ovines de pestivirus ont été isolées et classées respectivement dans le génogroupe BDV-3 (Border Disease Virus type 3) et dans le génogroupe BDV-Tunisien. Dans le département des Alpes de Haute-Provence, deux autres souches ont été isolées : l'une provenant d'une brebis avortée et, l'autre, pour la première fois, provenant d'un chamois (souche « Rupi-05 »). Les deux souches ont été classées parmi les virus du génogroupe BDV-6. Des séroneutralisations croisées ont montré que les chamois avaient des titres en anticorps supérieurs contre la souche « Rupi-05 », alors que les moutons réagissent de façon homogène envers les différentes souches ovines locales. De plus, les ovins ont des titres en anticorps neutralisants en moyenne plus élevés que les chamois pouvant laisser suspecter une circulation plus importante chez les moutons.
Une circulation active de pestivirus a donc été montrée dans la région PACA, chez les animaux sauvages comme domestiques. Dans les Alpes de Haute-Provence, les souches isolées des différentes espèces sont classées parmi le même génogroupe, montrant une continuité géographique dans la répartition des souches. Les résultats obtenus lors de l'infection expérimentale (réalisée avec une souche de BDV-4) montrent des effets importants sur la gestation, avec une possible présence d'animaux IPI chez les chamois comme chez les ovins. Bien que nos résultats ne permettent pas d'établir de façon précise un sens de transmission entre ces deux espèces de ruminants domestiques et sauvages, deux cycles épidémiologiques semblent être présents, caractérisés par une forte circulation intra-spécifique et connectés à des transmissions ponctuelles entre chamois et moutons.

Par ailleurs, lors de ce travail de thèse, deux études bibliographiques ont été conduites. Lors de la première, un outil permettant la comparaison d'expérimentations animales a été mis en place. Une approche originale basée sur une évaluation quantitative des méthodologies employées a été utilisée permettant de proposer des directives ou des conseils pour la réalisation de futures expérimentations. Au niveau temporel, ce travail bibliographique a été conduit essentiellement pendant les deux

dernières années de doctorat, soit après la période d'expérimentation conduite sur les isards. Nous avons donc pu inclure cette étude dans l'évaluation quantitative utilisée. Le score obtenu par notre expérimentation est de 26.33, ce qui nous permet d'être classés « en deuxième position ». De par le caractère particulier de cette expérimentation, nous avions en effet voulu avoir un suivi des animaux aussi complet que possible, ce qui a été validé par le système de scores, lui-même établi avec l'aide d'experts extérieurs. Cet outil peut être utilisé pour la comparaison d'autres types d'expérimentation animale, mais également être extrapolé pour une évaluation quantitative d'autres études scientifiques.
La seconde étude bibliographique présentée dans ce manuscrit a eu pour objectif de lister de façon la plus exhaustive possible des infections déjà décrites sur des ruminants sauvages en Europe, en donnant des exemples, et en essayant de montrer le rôle épidémiologique de chacune des espèces concernées. Après avoir montré les facteurs de risque pouvant influencer la transmission de pathogènes entre les espèces, nous avons montré qu'une meilleure surveillance de la faune sauvage devait être promue, notamment via la mise en place d'un système intégrant des acteurs locaux, nationaux et internationaux.

2. Difficultés rencontrées lors de ce travail

Lors de sa thèse présentée en 2006, Marylin Pioz (Pioz, 2006) avait défini les infections expérimentales comme une priorité dans l'étude de la pathogénie liée aux pestivirus dans la faune sauvage. Lors de ce travail, une infection expérimentale a pu être réalisée sur des femelles isards gestantes. Il faut tout d'abord souligner le caractère exceptionnel de cette étude. Le fait d'avoir pu avoir à disposition ces animaux, délivrés par Serge Mounard, a été une véritable oppotunité permettant l'étude de la pathogénie virale sur cette espèce.
Différentes difficultés inhérentes à l'utilisation d'animaux sauvages ont du être surmontées lors de la mise en place de l'infection expérimentale. Tout d'abord, il avait été initialement prévu de capturer six femelles. Cependant, sur ces six femelles, une n'était pas gestante, et une autre présentait des anticorps dirigés contre les pestivirus. Nous n'avons donc pu obtenir que quatre femelles gestantes, ce qui nous a incité à ajouter une agnelle dans le groupe des animaux témoins. Par ailleurs, des aménagements spécifiques (enrichissement du milieu de vie, adaptation des box) à l'utilisation d'animaux sauvages ont du être réalisés au sein de la station expérimentale afin de conduire l'expérimentation dans les meilleures conditions possibles.
De par son caractère rare, l'utilisation d'animaux sauvages limite également les possibilités de comparaison de résultats avec d'autres études. Dans notre cas, seule une étude similaire sur animaux non gestants avait été réalisée par l'équipe d'Ignasi Marco (Cabezon et al., 2011), nous permettant ainsi une comparaison des données. Peu de valeurs physiologiques de référence sont connues, ce qui

rend un peu plus difficile l'interprétation des suivis cliniques et hématologiques réalisés lors de l'expérimentation.

D'autre part, la mise en place d'une étude basée sur des prélèvements issus de la faune sauvage au sein de l'Anses Sophia-Antipolis a demandé la résolution d'un certain nombre de contraintes, liées notamment au fait que le laboratoire n'avait pas d'expérience préalable dans ce type de collecte de prélèvements. Bien qu'un réseau de professionnels existe déjà sur certaines zones d'étude (suivis sanitaires déjà en place dans les Hautes-Alpes, et présence du programme prédateur proie dans la vallée de la Tinée), la mise en place d'un réseau de collecte et son maintien a été nécessaire dans une autre zone (ex : Vallée de l'Ubaye). Un important travail de restitution des résultats est encore nécessaire.

L'échantillonnage réalisé au sein des espèces sauvages a été basé sur le recueil de prélèvements de chasse. Pour la faune sauvage, ce mode d'échantillonnage est généralement considéré comme un échantillonnage aléatoire au sein de la population cible (Gauthier, communication personnelle). La recherche de souches virales comme l'étude de la séroprévalence sont donc basées sur ce tirage aléatoire. Or, si celui-ci est la méthode de référence pour l'investigation de la séroprévalence, il n'est pas le plus adapté pour la recherche de souches virales. En effet, ce type d'investigation est plus aisé lorsque l'échantillonnage est directement ciblé vers les animaux infectés et virémiques. Actuellement, dans les Alpes, aucun signe clinique associé à une infection par des pestivirus n'a été rapporté sur des espèces sauvages. Même si, d'après les résultats obtenus dans cette étude, des avortements pourraient être présents, aucun signe n'est visible sur des animaux vivants. Il est dès lors difficile de cibler des animaux malades. De ce fait, malgré le nombre de prélèvements, nous n'avons pu isoler qu'une seule souche virale, ce qui reste une nouveauté dans cette région.

3. Perspectives

Les résultats obtenus lors de cette thèse laissent apparaître de nouvelles perspectives, tant au niveau méthodologique (outils terrain et outils de laboratoire) qu'au niveau de la compréhension de la dynamique de l'infection.

Tout d'abord, afin d'augmenter la pertinence des résultats des séroneutralisations croisées, il serait intéressant d'augmenter le nombre et la diversité des souches en culture afin de pouvoir avoir une meilleure comparaison possible des titres sérologiques. Localement, des efforts doivent être faits pour collecter plus de prélèvements issus d'avortements, ou de troupeaux ayant des problèmes de fertilité,

afin d'avoir un plus grand panel des souches circulantes, et de mieux évaluer les transmissions virales. Ces prélèvements doivent être réalisés régulièrement au sein des troupeaux ovins et bovins.
Cependant, l'utilisation de cette méthode pour déterminer la souche responsable de la séroconversion des animaux est longue et fastidieuse. Afin de s'en affranchir, des travaux sont actuellement menés au laboratoire pour mettre en place un test ELISA permettant la discrimination des génotypes sur base des réponses sérologiques dirigées vers la protéine E2. Cette protéine de surface est en effet la cible principale des anticorps neutralisants et varie selon les différents génotypes. Cet outil pourra être utilisé pour la mise en place d'études épidémiologiques rétrospectives, dans le but de caractériser les réponses sérologiques et de remplacer les seroneutralisations croisées.

Dans ce travail, nous avons utilisé une nested PCR pour la détection du virus dans les différents prélèvements provenant du terrain. Celle-ci a la propriété de détecter toutes les souches, y compris les plus exotiques comme la souche « HOBI ». Cette technique est assez longue, puisqu'elle nécessite deux cycles de PCR et un dépôt sur gel pour la visualisation des résultats, et peut potentiellement plus fréquemment conduire à la contamination des échantillons. Il serait donc intéressant de développer une RT-PCR pan-pestivirus en temps réel, permettant une quantification des acides nucléiques dans des échantillons provenant du terrain. Pour cela, il sera également important de recueillir ou d'isoler préalablement un panel représentatif de souches afin de garantir une bonne ubiquité de cet outil.

Nous avons pu observer, lors de l'infection expérimentale sur isards gestants, une grande variabilité des effets du virus. En effet même si les 3 animaux n'ont pas pu mener leur gestation à terme, une a avorté, et les deux autres sont mortes avant la mise-bas, dont l'une avec un fœtus momifié. L'implication du virus dans ces trois manifestations différentes est fortement suggérée par la détection de forte quantité de virus dans les différents organes, dont les cotylédons de la femelle ayant avorté. D'autre part, une virémie très longue a été mise en évidence sur deux femelles inoculées, de 4 jours post-inoculation à la mort des animaux. Ces résultats, surprenants, sont à approfondir. En effet, une telle durée de virémie positive n'a jamais été décrite chez les animaux domestiques. Dès lors, une infection expérimentale réalisée avec une souche moins virulente, ou avec un titre viral inférieur mais sur une durée longue, apporterait des informations importantes pour la compréhension de l'épidémiologie des pestiviroses chez les chamois. Cependant, ce type d'étude sur une longue durée est difficile à mettre en place, notamment pour les contraintes d'hébergements et de prise en compte du bien-être animal.

Par ailleurs, nous avons testé lors de cette expérimentation la pathogénie liée à une souche isolée sur des isards dans les Pyrénées. Les résultats trouvés sont pertinents en regard des observations faites en montagne (avortement, momification fœtale, et mort de l'animal), ce qui confirme la diminution de fertilité et les diminutions de taille de populations rapportées dans certaines zones. Cependant, il faut

rester vigilant quant à l'extrapolation de ces résultats pour les souches et espèces animales alpines. En effet, la pathogénie liée à la souche RUPI-05 isolée dans les Alpes n'est pas encore connue. L'autopsie et les analyses microbiologiques réalisées sur le chevreau positif semble indiquer que ce virus est bien la cause de la mort. L'étude de la pathogénie de cette nouvelle souche revêt dès lors une grande importance pour comprendre le rôle des pestivirus dans les problèmes démographiques rapportés cette fois dans les Alpes. De plus, avec les données disponibles, il nous est impossible de savoir si ce chevreau était un animal IPI ou s'il était en virémie transitoire. La cause de la mort de l'animal peut en effet être due à des effets délétères du virus lors d'une infection en phase aigüe, comme à une chute lymphocytaire importante, provoquant dès lors une forte immunodépression.

Les pestivirus ont un effet immunodépresseur important, tel que déjà connu chez les animaux domestiques et démontré ici par l'infection expérimentale. Dès lors, il serait intéressant d'évaluer leur rôle dans l'épidémiologie d'autres pathogènes pouvant infecter les animaux sauvages. Récemment, une vague épidémique de kérato-conjonctivite a atteint les chamois de la région PACA : elle a commencé par être décrite en 2005 dans le Queyras pour atteindre en 2006 les animaux situés dans la vallée de l'Ubaye puis se propager dans le Mercantour. Or, en 2004, dans la Société de Chasse de Ségure-Viso (située à Ristolas dans le Queyras) tous les animaux étaient soit séropositifs soit positifs en antigène avec des tests ELISA. L'éventuelle implication des pestivirus comme agent immunodépresseur pouvant faciliter la transmission d'autres agents serait intéressante à investiguer.

Parallèlement à ce que l'on observe chez les chamois, la population des mouflons semble être une espèce à étudier dans la dynamique des pestiviroses des alpages. En effet, sur 18 prélèvements réalisés en 2006-2007, 13 étaient séropositifs. Conjugués à la forte proximité phylogénétique existant entre les mouflons et les ovins, ces résultats méritent une attention particulière. Dans le cadre de ce travail, nous n'avons malheureusement pas pu étudier la pathogénie virale au sein de cette espèce ni leur rôle dans la transmission des pestivirus en alpage. Cependant, une étude génétique et antigénique des souches virales circulantes serait intéressante à mettre en place, via notamment l'utilisation de séroneutralisations croisées. En effet, récemment, des mortalités importantes de mouflons ont été rapportées, conduisant certaines sociétés de chasse à arrêter leurs prélèvements au sein de cette espèce. Différentes causes ont été localement évoquées pour expliquer ces diminutions de populations telles que le retour et la croissance des meutes de loups, ou la rigueur des récents hivers. Le rôle des pestivirus dans la dynamique des populations des mouflons doit être évalué.

Par ailleurs, les résultats obtenus sur l'espèce chevreuil ont montré une séroprévalence de 27.8 dans la vallée de l'Ubaye en 2009-2010. Par contre, ces résultats n'ont pas été confirmés par séroneutralisation (un seul animal sur les 20 séropositif a montré un titre séroneutralisant vis-à-vis

d'une souche de BVDV-1). Tel qu'exposé dans l'article 5, différentes hypothèses peuvent expliquer cette divergence de résultats, comme une mauvaise spécificité du test ELISA employé (non adapté à l'espèce chevreuil) ou une différence importante de la souche chevreuil par rapport aux souches testées. Dans un premier temps, à défaut d'isoler une souche spécifique de cette espèce, il serait par exemple intéressant de tester ces animaux vis à vis des souches circulant chez les bovins présents localement.

En Italie, des pestivirus ont été isolés sur des chèvres faisant partie d'un troupeau mixte ovins/caprins présentant des avortements (De Mia et al., 2005). Les souches ont été caractérisées mais n'ont pu être classées parmi un des génogroupes de BDV déjà existants. En effet, elles présentent 72 % d'homologies avec la souche « chamois » (numéro GENBANK AY738080) isolée dans les Pyrénées espagnoles sur un isard (Arnal et al., 2004), 72% avec une souche classée parmi la souche Bison-2 classée parmi les BDV-1 et 69 à 71 % d'homologies avec la souche SN3G classée parmi les BDV-Tunisian. Il serait dès lors intéressant d'investiguer les souches circulant au sein de l'espèce caprine, pour les comparer à celles circulant chez les chamois et moutons. De plus, dans certains massifs de la région PACA, des troupeaux de chèvres sont laissés en libre divagation pendant tout l'été, laissant une possibilité de contacts accrus entre les différentes espèces.

Une des priorités dégagée par ce travail est la mise en commun des données sanitaires et de dynamique des populations. En effet, pour certaines zones, des données existent (cas de la RNCFS de Ristolas, ou les populations sont suivies par le programme prédateur proie dans la vallée de la Tinée). Un important travail d'harmonisation des pratiques de suivi de dynamique de populations est à réaliser. En effet, chacune des études de démographie a été mise en place par des équipes différentes, ayant chacune un objectif propre. De telles pratiques d'harmonisation sont actuellement mises en place par les différents gestionnaires de la faune, mais un important travail de traitement de données reste à effectuer.

Une étude épidémiologique spatio-temporelle serait intéressante à mettre en place, en vue de comprendre si certains alpages sont plus à risque que d'autres. Si tel est le cas, une limitation éventuelle des mouvements d'animaux pourrait être envisagée, tout comme d'imposer dans certains cas des tests d'animaux domestiques montant en alpage (comme cela est réalisé en Suisse).

Un des défis de cette thèse a été d'unir au sein du même projet deux disciplines distinctes et pourtant indissociables pour l'étude d'une maladie, à savoir une approche virologique pure (étude de la pathogénie virale, ceci en passant par l'utilisation d'outils virologiques), à une approche épidémiologique. Or, nous avons vu que lors des perspectives dégagées, il est important d'associer l'ensemble des données présentées à une étude de dynamique de populations, en prenant en compte toutes les autres variables pouvant jouer sur la démographie d'une population d'animaux sauvages (climat, prédation, maladies, densité, disponibilité du milieu…) et entrer ainsi dans le domaine de l'écologie. Nous voyons donc l'importance d'aborder une telle problématique de façon multidisciplinaire et intégrée. Il est capital pour le futur de continuer les collaborations mises en place lors de ce projet, de développer, et de favoriser une bonne discussion entre les différents partenaires.

VALORISATIONS DE LA THESE

LISTE DES COMMUNICATIONS ASSOCIEES

Posters

- 3rd workshop of the student section of the European Wildlife Disease Association (EWDA, Veyrier du lac, 18-19th March 2009): Martin C., Letellier C, Gauthier D., Jean N., Shaffii A., Saegerman C., Epidemiology of pestivirosis infection in wild ungulates of the French South Alps (1)

- XII° Journées Francophones de Virologie (JFV, Paris, Mars 2010), Martin C., Duquesne V., Marco I., Gibert P., Gilot-Fromont E., Thiéry R., Dubois E. Infection expérimentale d'isards (*Rupicapra pyrenaica*) avec une souche spécifique de Border Disease Virus-4 isolée dans les Pyrénées espagnoles) (2)

- **European Wildlife Disease Association (EWDA, Vlieland, 13-16th September 2010) :** Martin C., Duquesne V., Adam G., Saegerman C., Thiéry R., Dubois E. Epidemiologic study of pestivirus infection in both wild and domestic ruminants: a survey in the Ubaye Valley (Alpine mountains, France) (3)

Communications orales

- Journées Scientifiques de l'Association pour l'Etude de l'Epidémiologie des Maladies Animales et de l'Association pour l'Etude de l'Epidémiologie des Maladies Animales (AEEMA, Paris, 4-5 Juin 2009) Martin Claire, Letellier Carine, Gauthier Dominique, Jean Nicolas, Saegerman Claude. Contribution à l'étude épidémiologique des Pestiviroses chez des ongulés sauvages dans les Alpes du Sud françaises (4)

- **Groupe d'Etude sur l'Ecopathologie de la Faune Sauvage de Montagne (GEEFSM, Marchairuz, 11-14 juin 2009) :** Martin C., Dubois E., Marco I., Gibert P., Gilot-Fromont E., Thiéry R. Reproduction expérimentale de la Border Disease chez l'isard gestant : premiers résultats (5)

- 4th workshop of the student section of the European Wildlife Disease Association (EWDA, Veyrier du lac, 14-17th April 2011): Martin C., Duquesne V., Adam G., Saegerman C., Thiéry R., Dubois E. Epidemiologic study of pestivirus infection in both wild and domestic ruminants: a survey in the Ubaye Valley (Alpine mountains, France) (6)

- XIII° Journées Francophones de Virologie (JFV, Paris, 28-29 Avril 2011), Martin C., Duquesne V., Adam G., Champions J.-L., Belleau E., Gauthier D., Thiéry R., Dubois E. Etude des transmissions

interspécifiques des pestivirus entre les ongulés sauvages et domestiques dans les Alpes de Haute-Provence (7)

- Journées des doctorants de l'Anses (Paris, 27-28 janvier 2011) : MARTIN Claire. Etude épidémiologique des pestiviroses en alpage (8)

- Journées des doctorants de l'Université de Nice Sophia Antipolis (JEDN, Nice, 8-9 Septembre 2011) MARTIN Claire, Richard Thiéry, Claude Saegerman, Eric Dubois. Les pestivirus chez les ruminants sauvages et domestiques en alpage - Etude épidémiologique dans la région PACA et pathogénie chez l'isard (9)

- Groupe d'Etude sur l'Ecopathologie de la Faune Sauvage de Montagne (GEEFSM, Nerja, 7-9 octobre 2011) : MARTIN Claire, DUQUESNE Véronique, GILOT-FROMONT Emmanuelle, GIBERT Philippe, VELARDE Roser, THIERY Richard, MARCO Ignasi, DUBOIS Eric. Etude expérimentale de la Border Disease chez l'isard (*Rupicapra pyrenaica*) gestant (10)

- Groupe d'Etude sur l'Ecopathologie de la Faune Sauvage de Montagne (GEEFSM, Nerja, 7-9 octobre 2011) : MARTIN Claire, DUQUESNE Véronique, SAEGERMAN Claude, GAUTHIER Dominique, BELLEAU Eric, CHAMPION Jean-Luc, THIERY Richard, DUBOIS Eric. Estudio epidemiológico de pestivirus en rumiantes salvajes y domésticos en los Alpes del Sur de Francia. Resumen de 4 años de estudio. *(Présentation orale présentée lors du concours d'étudiant du GEEFSM: 2° prix)* (11)

(1) Poster presented on the 3rd workshop of the student section of the European Wildlife Disease Association (March 2009)

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

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INTRODUCTION

In mountains area, common pasturing in summer seasons represents an important risk of contamination of both wild and domestic animals, either through contacts, or through succession in the same places, especially in locations such as salt deposits [1]. *Pestivirus* infections have been widely described in wildlife and has been associated with important mortalities of Pyrenean chamois [2].,

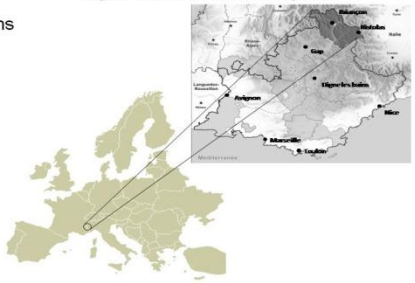
OBJECTIVES

The goal of this study was first to investigate the seroprevalence of *Pestiviruses* in chamois (*Rupicapra rupicapra*), mouflons (*Ovis amon musimon*) and roe deer (*Capreolus capreolus*) in the French South Alps and to identify the most relevant associated exploratory variables (risk factors). Moreover, cross virus neutralization tests were performed in order to determine the most probable *Pestivirus* specie circulating in the studied area.

MATERIAL AND METHODS

A total of 381 blood samples were collected during 2003-2007 hunting seasons and are reported and Table I. 53 spleens originating from chamois only were also collected. Species, sex, age, location of shot and assessment of health status of each hunted animal were given as complementary data.

Figure 1: Location of the study area



In the study area, contacts may occurs between chamois, mouflon, roe deer, alpine ibex (*Capra ibex ibex*), red deer (*Cervus elaphus*), and wild boar (*Sus scrofa*). Domestic livestock (small and large ruminants) share the pastures with wild animals during the grazing season from June to September.

RESULTS

	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)

Table I: Annual and specie repartition of blood samples (ELISA positive for *Pestivirus* antibodies)

Despite a very low number of animal tested, apparent prevalence of *Pestivirus* antibodies was very high in mouflons in 2006.
In chamois, the apparent prevalence is steadily going down. Risk factors associated were the age (oldness), the female gender, and the Briançonnais district.
No virus was isolated by RT-PCR.

Figure 2: Annual evolution of the standardized apparent prevalence rate () in chamois (*Rupicapra rupicapra*) originating from the French South Als with 95% confidence interval (-)* of the *Pestivirus* p80 antibodies.0

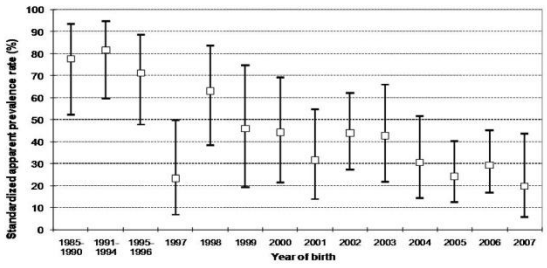
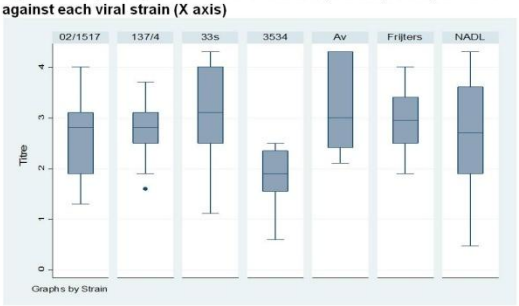


Figure 3: Boxplot representation of the titer logarithm (Y axis) obtained against each viral strain (X axis)



DISCUSSION

In order to classify the circulating strain, a comparative virus neutralisation test was performed. 2 strains of Border Disease titers obtained against Av and 33s strains (2 Border Disease Virus) were higher than those obtained all other strains. However this result cannot permit to exactly classify the circulating strain.

CONCLUSION

For the first time, a circulation of *Pestivirus* among wild chamois and mouflons in the South of French Alpine mountains was demonstrated. There is a wide probability that the circulating strain clusters within the BDV genotype. Further epidemiological (viral and serological) studies are needed to evaluate the importance of circulation among domestic animals, and to know the role of each species in the transmission of the pathogen.

This study was funded by the hunters' federation, the departmental veterinary laboratory of the Hautes-Alpes French department, the Veterinary and Agrochimical Research Center of Brussel and a research grant of the University of Liege.

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[1] Richomme, C., Gauthier, D., Fromont, E., 2006. Contact rates and exposure to inter-species disease transmission in mountain ungulates. Epidemiol. Infect. 134, 21-30.
[2] Marco, I., Rosell, R., Cabezon, O., Mentaberre, G., Casas, E., Velarde, R., Lopez-Olvera, J.R., Hurtado, A., Lavin, S., 2008. Epidemiological study of border disease virus infection in Southern chamois (*Rupicapra pyrenaica*) after an outbreak of disease in the Pyrenees (NE Spain). Vet. Microbiol. 127, 29-38.

(2) Poster présenté aux Journées Francophones de Virologie (Mars 2010)

Infection expérimentale d'isards (*Rupicapra pyrenaica*) avec une souche spécifique de Border Disease Virus-4 isolée dans les Pyrénées espagnoles

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Introduction

Des pestivirus classés dans le groupe Border Disease Virus génogroupe 4 (BDV-4) ont été associés à des tableaux cliniques sévères et à des diminutions de populations chez les isards dans les Pyrénées.
Cette étude a eu pour objectif de reproduire expérimentalement l'infection avec un BDV-4 chez des isards gestants, et notamment d'évaluer si cette infection pouvait aboutir à la naissance d'animaux infectés persistants immunotolérants (IPI).

Matériels et Méthodes

Groupe inoculé : 3 isards gestants
Groupe témoin : 1 isard gestant + 1 agnelle

Souche CADI-6 (BDV-4)
Voie intratrachéale

- Suivi clinique quotidien
- Quantification neutrophilique
- Suivi de la virémie : RT-PCR
- Suivi de la réponse immunitaire :
  - * ELISA (Synbiotics)
  - * Séroneutralisation
- Autopsie
- Histopathologie

Résultats

Animaux inoculés

❖Aucun animal inoculé n'a pu mener sa gestation à terme

❖ Neutropénie présente à partir du 5ème jour post-inoculation (JPI) et d'une durée variable en fonction des individus

❖Virémie décelée à partir de 4ème JPI et jusqu'à la mort des animaux (soit une durée de 20 à 49 jours)

❖Séroconversions détectées à partir de 10 à 12 JPI, dont les titres et les durées sont variables selon les individus (Fig.1).

Animaux témoins :

❖ Aucune virémie ni séroconversion n'ont pu être détectées

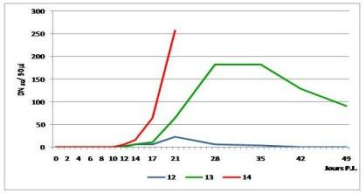

Figure 1. Evolution des titres en anticorps neutralisants (animaux inoculés)

Seul l'animal n°14 présentait une réponse positive avec le kit ELISA à 21 JPI.

	Animaux inoculés			Animaux témoins	
	12 (isard)	13 (isard)	14 (isard)	15 (isard)	03 (agnelle)
Signes cliniques	• Diarrhée profuse (à partir du 14ème JPI)	• Avortement à 46 JPI • Boiterie du postérieur droit (origine traumatique)	• Mort brutale	• Aucun signe clinique	• Aucun signe clinique
Mort	51 JPI	51 JPI (euthanasie)	24 JPI	63 JPI (euthanasie)	63 JPI (euthanasie)
Autopsie	• Bon état physiologique • Diarrhée profuse • Entérite • Pneumonie vermineuse grave • Fœtus viable	• Maigre • Pneumonie vermineuse modérée • Abcès pulmonaires (cicatriciels) • Abcédation de la plaie d'amputation • Arthrite du tarse	• Bon état physiologique • Diarrhée profuse • Lymphadénomégalie généralisée • Très nombreuses pétéchies sur l'ensemble du tube digestif • Hémorragies pulmonaires • Momification fœtale	• Bon état physiologique • Aucune lésion • Fœtus viable	• Bon état physiologique • Aucune lésion
Histo-pathologie	• Encéphale : spongiose légère, présence de quelques histiocytes	• Encéphale : spongiose modérée, histiocytes infiltrés • Placenta : nécrose diffuse	• Encéphale : spongiose modérée, infiltration périvasculaire • Poumons : pneumonie nécrosante bactérienne + pleurite fibrineuse	• n.d.	• n.d.

Discussion et Conclusion

❖Bien que l'effectif soit réduit, ces résultats préliminaires laissent supposer une forte sensibilité de l'isard à la souche virale CADI-6 et n'a pas abouti à la naissance de cabris IPI.

❖Certains signes cliniques observés en milieu naturel (amaigrissement et lésions cérébrales) associés à une longue virémie ont été retrouvés lors de cette infection expérimentale. Ces animaux sont issus d'un milieu semi-sauvage (Parc animalier), ce qui explique en partie la présence de pathologies concomitantes (pneumonies vermineuses notamment).

❖Ces premières conclusions devront être confirmées, notamment à l'aide de tests de RT-PCR en temps réel (quantification de la virémie sur les individus adultes et sur les fœtus ainsi que de l'excrétion virale).

XII^e Journées francophones de Virologie - 17-18 Mars 2010 – Paris, France

(3) Poster presented at the 9th confereence of the European Wildlife Disease Association (Octobre 2010)

Epidemiologic study of pestivirus infection in both wild and domestic ruminants
A survey in the Ubaye Valley (Alpine mountains, France)

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Background

Since several years, *Pestivirus* infections have been widely documented among wild ruminants[1,2]. Earlier epidemiologic studies often incriminated interspecies transmission between wild and domestic ruminants. In order to assess this statement, this study was carried out to investigate the apparent prevalence of pestivirus infection in both wild and domestic ruminants in the Ubaye valley.

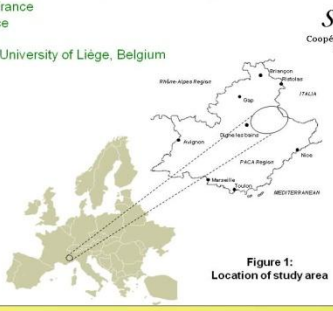


Figure 1: Location of study area

Material and Methods

❖ ***Geographic areas***: inside the Ubaye Valley (figure 1), sampling of animals was done in five areas identified for their high contact rates between wild and domestic ungulates.

❖ ***Samples, laboratories analysis:***

	Wild ruminants	Domestic ruminants
Sampling	Blood and spleens were collected by voluntaries wild game societies and by the Forest National Office	For serum: 1 out of 10 among sera collected for national prophylaxis of brucellosis For virologic study : samples based on local veterinatian clinical suspicion (swabs and spleens)
Virological analysis	RT-PCR directed on the 5'UTR sequence (on RNA extracted from spleens or swabs) followed by sequencing	
Serological analysis	Synbiotics SERELISA (on animal sera)	

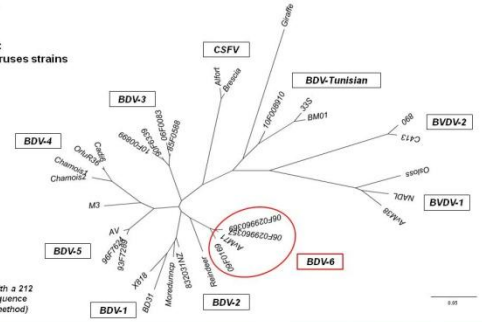
❖ ***Statistical analysis:*** Welch test was used to compare distributions of ELISA optical densities obtained between the different species.

Virological results

➢ Wild ungulates : no pestivirus was found in 77 samples tested.

➢ Domestic flocks : a strain was isolated and was clustered within the BDV-6 group[3] (figure 2).

Figure 2: BDV-6 among pestiviruses strains



(Phylogenetic tree prepared with a 212 bp-fragment of the 5'UTR sequence using the Neighbor-Joining method)

Serological results

➢ Apparent seroprevalence was calculated:

- **28.9%** (CI95%: [19.1-40.5%]) for chamois
- **25.9 %** (CI95%:[11.1-46.3%]) for roe deer
- **9.1 %** (CI95%: [0.2-41.3%]) for mouflons

and

- **76.5%** (CI95%: [74.2-79.4%]) for sheep

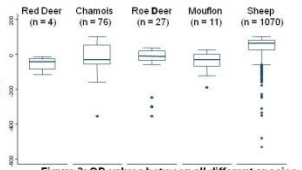


Figure 3: OD values between all different species

➢ OD values were significantly higher in sheep than in all other wild species (*Welch* test, figure 3).

➢ For chamois, apparent seroprevalence was significantly higher in females than in males (OR=3.15 [1.11-8.95]).

➢ Oldest animals (>8 years old) were significantly more seropositive (OR= 3.73 [1.09-12.84]).

➢ 6 out of 15 young animals (from 0.5 to 2 years old) were found seropositive.

Discussion and Perspectives

➢ These results do not allow us to clearly conclude about transmission direction between wild and domestic ruminants.

➢ An active circulation of pestiviruses has been demonstrated among wild and domestic ruminants in this area.

➢ To determine the epidemiological roles of both wild and domestic ruminants in pestivirus transmission, we need to :
  - ➢ Perform comparative virus neutralization test in order to:
    - determine the specificity of serological reactions
    - confirm ELISA results concerning differences between species .
  - ➢ Isolate and characterize circulating viral strain(s) from wild animals.

References: (1) Martin C., Letellier C., Caij B., Gauthier D., Jean N., Shaffii A., Saegerman C. Epidemiology of pestivirosis infection in wild ungulates of the French South Alps. *Vet Microbiol., 2010, doi:10.1016/j.vetmic.2010.07.010.*
(2) Marco I., Rosell R. Cabezón O., Beneria B., Mentaberre G., Casas E. , Hurtado A., López-Olvera J.R., Lavin S., Serologic and virologic investigations into pestivirus infection in wild and domestic ruminants in the Pyrenees (NE Spain), *Res. Vet. Science 87 (2009) 149–153.*
(3) Dubois E., Russo P., Prigent M., Thiéry R., Genetic characterization of ovine pestiviruses isolated in France, between 1985 and 2006. *Vet. Microbiol, 2008, 130: 69–79.*

9th Conference of the European Wildlife Disease Association, Vlieland, The Netherlands, 12-16 September 2010

(4) Résumé de la communication orale présentée aux journées de l'Association pour l'Etude de l'Epidémiologie des Maladies Animales (Juin 2009)

CONTRIBUTION A L'ETUDE EPIDEMIOLOGIQUE DES PESTIVIROSES CHEZ DES ONGULES SAUVAGES DANS LES ALPES DU SUD FRANÇAISES

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Résumé (maximum 200 mots) :

La transmission inter-spécifique est régulièrement incriminée dans l'épidémiologie des pestiviroses ; notamment dans les alpages où des ongulés sauvages et domestiques cohabitent. L'objectif de cette étude séro-épidémiologique longitudinale menée de 2003 à 2007 dans le département français des Hautes-Alpes a été de mettre en évidence une circulation de *Pestivirus* chez des ongulés sauvages pour évaluer leur implication dans la transmission de *Pestivirus*. Des taux de séroconversion atteignant 45,9% (intervalle de confiance à 95% : IC95% : [40,5-51,3%]) chez des chamois (*Rupicapra rupicapra*) et 61,1% (IC95% : [38,6-83,6]) chez des mouflons (*Ovis amon musimon*) ont été révélés et étaient associés à des densités optiques significativement supérieures chez les mouflons. Des séroneutralisations comparatives de 15 sérums positifs de chamois envers 7 souches de *Pestivirus* distinctes ont permis de montrer que 2 souches de Border Disease Virus (Av et 33s) étaient les plus neutralisées. Des titres significativement inférieurs ont été obtenus envers la souche 3534 (Bovine Viral Diarrhea Virus 2). Les résultats de séroconversion et de séroneutralisation indiquent une circulation d'un Border Disease Virus au sein de la zone d'étude. Une amélioration du protocole de prélèvement et de transport des échantillons a été envisagée afin de pouvoir isoler et caractériser la souche circulante.

(5) Résumé de la communication orale présentée aux journées du Groupe d'Etude pour l'Ecopathologie de la Faune Sauvage de Montagne (Juin 2009)

Reproduction expérimentale de la Border Disease chez l'isard gestant : premiers résultats

Claire Martin[a], Eric Dubois[a], Ignasi Marco[b], Philippe Gibert[c], Emmanuelle Gilot-Fromont[d], Richard Thiéry[a]

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De nombreuses études réalisées sur des isards (*Rupicapra pyrenaica*) dans les Pyrénées espagnoles et françaises ont montré que des virus classés dans le groupe BDV-4 (Border Disease Virus génogroupe 4) étaient associés à des tableaux cliniques sévères et à des diminutions de population. Toutefois, si la pathogénie de l'infection des virus de la maladie des frontières est désormais bien connue chez les espèces domestiques, les connaissances actuelles en demeurent à leurs prémices pour les espèces de ruminants sauvages, et en particulier chez l'isard. Cette étude a ainsi eu pour objectif de reproduire expérimentalement une infection à Border Disease chez l'isard gestant.Trois isards femelles gestantes, indemne de pestivirus et séronégatives, ont été inoculées par voie intra-trachéale avec 10^6 $TCID_{50}$ d'une souche de BDV-4 (souche CADI-6, Genbank AM905923) préalablement isolée sur des isards sauvages dans les Pyrénées espagnoles. Un groupe témoin, constitué d'un isard femelle et d'une agnelle, a permis de valider les résultats. Un animal inoculé a présenté de la diarrhée et 2 sur 3 ont présenté une momification fœtale ou un avortement. La période de virémie s'est étendue de 4 jours post-inoculation jusqu'à la mort des animaux, soit jusqu'à 24 et 51 jours post-inoculation. Une neutropénie a également été mise en évidence. D'après les résultats obtenus par test ELISA (Synbiotics SERELISA® BVD p80 Mono Blocking), un seul des animaux infectés a séroconverti à partir de 22 jours post inoculation. Bien que l'effectif soit réduit, ces premiers résultats semblent indiquer une forte susceptibilité de l'isard par rapport à cette souche virale. Des séroneutralisations sont prévues afin de mesurer avec précision l'évolution de la réponse immunitaire. La quantification de la virémie et de l'excrétion virale sera mesurée à l'aide de la mise en place d'une RT-PCR en temps réel.

(6) Résumé de la communication orale présentée aux 4th workshop of the student section of the European Wildlife Disease Association (April 2011)

Epidemiologic study of pestivirus infection in both wild and domestic ruminants in the French South Alps.

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Background: Since several years, *Pestivirus* infections have been widely documented among wild ruminants. Earlier epidemiologic studies often incriminated interspecies transmission between wild and domestic ruminants. In order to assess this statement, this study was carried out to investigate the apparent prevalence of pestivirus in both wild and domestic ruminants in a 3 alpine valleys.
Methods: In wild animals, samples (serum and spleen) were performed on hunted animals. Serum samples collected for brucellosis prophylaxis were used for the monitoring of domestic ruminants older than 2 years. Screening of pestivirus antibodies against p80 protein (named also NS3), common to all Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV), was achieved in all wild animal samples and in 10% of sera taken from domestic herds by blocking enzyme linked immunosorbent assay (ELISA) (Synbiotics, Lyon, France). Moreover, virus investigation was carried out in all samples collected from hunted animals and in association with local veterinarians for domestic ruminants (investigation based on clinical suspicion of persistently infected animals), using a conventional reverse transcription-polymerase chain reaction (RT-PCR).
Results: In a determined valley, for domestic ruminants, a total of 24 herds were screened. All herds were positive for pestivirus-specific antibodies. Individual sero-prevalence reached 76.5 % (95% confidence interval [CI95%]: [74.2 – 79.4 %]) of the 1039 animals tested. For wild ruminants, 29.7 % (CI95%: [19.6 – 39.8 %]) of the 79 chamois tested and 25.9% (CI95%: [9.4 – 42.4 %]) of the 27 roe deer were antibody positive.
Furthermore, in the same valley and for the first time, two pestivirus strains were isolated from both an ovine-herd and a chamois. The phylogenetic analysis of theses strains, based on the 5'untranslated region of the genome, permitted their classification among the BDV-genotype 6.
Discussion: These first results indicate a very high and unexpected sero-prevalence in domestic ruminants, and an important one in wild ruminants. The classication of the isolated strains in the same group may indicate a genetic continuity among the circulating strains.
Conclusions: An active circulation of pestivirus was demonstrated in both wild and domestic ungulates. Epidemiological study has to be completed in order to establish the relationship between wild and domestic animals for the transmission of pestivirus infection.

(7) Résumé de la communication orale présentée aux XIII° Journées Francophones de Virologie (Avril 2011)

Auteurs et coordonnées

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Résumé

Etude des transmissions interspécifiques des pestivirus entre les ongulés sauvages et domestiques dans les Alpes de Haute-Provence

Les transmissions inter-spécifiques sont régulièrement incriminées dans l'épidémiologie des pestiviroses ; particulièrement au niveau des alpages où des contacts sont régulièrement décrits entre ongulés sauvages et domestiques. L'objectif de cette étude a été de décrire l'épidémiologie des pestiviroses simultanément chez les ongulés sauvages et domestiques dans une zone alpine où des contacts inter-espèces sont connus comme importants.
Des échantillons de sang et de rate ont été prélevés sur les ruminants soumis à un plan de chasse (chamois, chevreuil, mouflon essentiellement) au sein de 6 sociétés de chasse situées dans la vallée de l'Ubaye (Alpes de Haute-Provence, France). Les prélèvements réalisés sur les troupeaux domestiques (ovins essentiellement) ont été réalisés sur les troupeaux (transhumant ou non) pâturant sur les territoires de chasse de ces 6 sociétés. Des prélèvements de sérum ont été réalisés en échantillonnant 10% des sérums prélevés lors de la prophylaxie pour la brucellose. De plus, des écouvillons vaginaux ou des échantillons de rate étaient envoyés par les vétérinaires locaux lors de suspicion clinique.
Une RT-PCR conventionnelle dirigée sur la région 5'UTR conduite sur l'ensemble des prélèvements a permis de caractériser et d'isoler deux souches virales, classées parmi les Border-Disease Virus (groupe BDV-6) issues chacune d'un élevage ovin et d'un chamois. L'étude de séroprévalence s'est effectuée à l'aide d'un kit ELISA (Synbiotics, Lyon, France).
En 2009, tous les troupeaux domestiques (n=24) présentaient une séroprévalence positive, située entre 61,5 et 95,3%. Sur la même période, 29.7 % (CI95%: [19.6 – 39.8 %]) des 79 chamois testés et 25.9% (CI95%: [9.4 – 42.4 %]) des 27 chevreuils testés étaient séropositifs. Ces résultats semblent montrer un continuum viral circulant de façon importante entre les animaux sauvages et domestiques. Afin de confirmer ces résultats, des séroneutralisations croisées seront réalisées dans le but de déterminer la spécificité des réactions sérologiques.

(8) Résumé de la communication orale présentée aux Journées des doctorants de l'Anses (Paris, 27-28 janvier 2011) :

Etude épidémiologique des pestiviroses en alpage

MARTIN Claire.

De nombreux cas d'infections d'ongulés sauvages par des pestivirus ont récemment été rapportés, laissant suspecter un mode de transmission inter-espèces entre les ruminants sauvages et domestiques. L'objectif de cette étude a été de décrire l'épidémiologie des pestiviroses simultanément chez les ongulés sauvages et domestiques dans une zone alpine où des contacts inter-espèce sont connus comme importants. Des échantillons de sang et de rate ont été prélevés sur les animaux chassés au sein de six sociétés de chasse des Alpes du Sud. Pour les cheptels domestiques (ovins) étudiés, des prélèvements étaient envoyés par des vétérinaires lors de suspicions cliniques. Une RT-PCR conventionnelle, dirigée sur la région 5'UTR et conduite sur l'ensemble des prélèvements, a permis de caractériser et d'isoler deux souches virales, classées parmi les Border-Disease Virus, groupe BDV-6 ; issues chacune d'un élevage ovin et d'un chamois. L'étude de séroprévalence s'est effectuée à l'aide d'un kit ELISA (Synbiotics, Lyon, France) en échantillonnant 10% des sérums prélevés lors de la prophylaxie pour la brucellose et sur l'ensemble des sérums d'animaux sauvages. En 2009, tous les troupeaux domestiques (n=24) présentaient une séroprévalence positive, située entre 61,5 et 95,3%. Sur la même période, 29,7 % (CI95%: [19,6 – 39,8 %]) des 79 chamois testés et 25,9% (CI95%: [9,4 – 42 ,4 %]) des 27 chevreuils testés étaient séropositifs. Ces résultats semblent montrer un continuum viral circulant de façon importante entre les animaux sauvages et domestiques. Afin de confirmer ces résultats, des séroneutralisations croisées seront réalisées dans le but de déterminer la spécificité des réactions sérologiques.

(9) Résumé de la communication orale présentée aux Journées de l'Ecole Doctorale de l'Université de Nice Sophia Antipolis (Nice, 8-9 Septembre 2011).

PRESENTATIONS ORALES

Les pestivirus chez les ruminants sauvages et domestiques en alpage - Etude épidémiologique dans la région PACA et pathogénie chez l'isard

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Les alpages constituent des zones de fort contact entre ruminants sauvages et domestiques. Au sein de ces alpages, de nombreux cas d'infections d'ongulés sauvages par des pestivirus ont récemment été rapportés, laissant suspecter un mode de transmission inter-espèces entre les ruminants sauvages et domestiques.

Mon travail de thèse s'est articulé en deux points : Tout d'abord, une étude épidémiologique des pestiviroses a été mise en place simultanément chez des ongulés sauvages et domestiques dans une zone alpine où des contacts inter-espèce sont connus comme importants. Des échantillons de sang et de rate ont été prélevés sur les animaux chassés au sein de six sociétés de chasse des Alpes du Sud. Pour les troupeaux domestiques (ovins) étudiés, des prélèvements étaient envoyés par des vétérinaires lors de suspicions cliniques. Une RT-PCR conventionnelle, dirigée sur la région 5'UTR et conduite sur l'ensemble des prélèvements, a permis de caractériser et d'isoler deux souches virales, classées parmi les Border-Disease Virus, groupe BDV-6 ; issues chacune d'un élevage ovin et d'un chamois. L'étude de la séroprévalence (proportion d'animaux présentant des anticorps dirigés contre le virus) s'est effectuée à l'aide d'un kit ELISA (Synbiotics, Lyon, France) en échantillonnant 10% des sérums prélevés lors de la campagne de prophylaxie obligatoire pour la brucellose et sur l'ensemble des sérums d'animaux sauvages disponibles. En 2009, tous les troupeaux domestiques (n=24) présentaient une séroprévalence positive, située entre 61,5 et 95,3%. Sur la même période, 29,7 % (CI95%: [19,6 – 39,8 %]) des 79 chamois testés et 25,9% (CI95%: [9,4 – 42 ,4 %]) des 27 chevreuils testés étaient séropositifs. Ces résultats semblent montrer qu'il existe un continuum viral circulant de façon importante entre les animaux sauvages et domestiques. Une méthode sérologique (séroneutralisation) a été développée afin d'identifier de façon plus précise l'origine des infections (travaux en cours). Parallèlement à cette étude, une infection expérimentale a été conduite sur 4 isards (chamois pyrénéen) gestants afin de comprendre la pathogénie associée à une souche de pestivirus préalablement isolée sur des isards sauvages lors d'une épizootie dans les Pyrénées et d'évaluer les conséquences de cette infection sur la gestation. Sur 3 animaux inoculés, 2 animaux sont morts avec notamment des signes importants de diarrhée. Le troisième a avorté et a montré des signes de faiblesse. La virémie (présence de virus dans le sang) a duré pour 2 animaux jusqu'à leur mort (durée jamais décrite dans d'autres espèces) et la séroconversion a été tardive et faible. Les animaux témoins n'ont présenté aucun signe clinique durant toute l'expérimentation, et sont restés négatifs pour la recherche de virus et d'anticorps.

Ces travaux ont permis de montrer une séroprévalence des pestivirus élevée dans la région PACA chez les ongulés sauvages ou domestiques. Les isards semblent montrer une sensibilité élevée à l'infection, avec des mortalités et des avortements. L'infection par des pestivirus semble donc jouer un rôle important dans la dynamique des populations, provoquant des diminutions de taille de populations et de taux de fertilités.

Mots clés : Pestivirus, Epidémiologie, Pathogénie, Interface faune sauvage/faune domestique

(10) Résumé de la communication orale présentée aux journées du Groupe d'Etude pour l'Ecopathologie de la Faune Sauvage de Montagne (Juin 2009)

Etude expérimentale de la Border Disease chez l'isard (*Rupicapra rupicapra)* gestant

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TEXTE DU RESUME

Des pestivirus classés parmi les Border Disease Virus-4 (BDV-4) ont causé de fortes mortalités dans certaines populations d'isards espagnoles (*Rupicapra pyrenaica*) depuis le début des années 2000. Les signes cliniques associés à cette infection ont été caractérisés mais la pathogénie est encore mal comprise notamment chez les femelles gestantes. Le but de cette étude a été de décrire la pathologie d'une infection par un BDV-4 chez des isards et ses conséquences sur la gestation.
Trois isards femelles ont été inoculées durant le deuxième tiers de gestation avec une souche de BDV-4, préalablement isolée d'un isard sauvage. Un groupe témoin était constitué d'une quatrième femelle isard gestante, associée à une agnelle. L'évolution des signes cliniques, des valeurs hématologiques, de la virémie et de la sérologie a été quantifiée durant toute la durée de l'expérimentation. Des examens post-mortems (autopsie, histopathologie et quantification de l'ARN viral dans les organes) ont également été réalisés.
Aucune gestation n'a été menée à terme. Une femelle est morte à 24 jours post inoculation (jpi) sans montrer de signe clinique précurseur. Une deuxième femelle a présenté une diarrhée profuse de 13 jpi jusqu'à sa mort à 51 jpi. La troisième a avorté à 46 jpi et a été euthanasiée à 51 jpi. Les animaux témoins ont été euthanasiés à la fin de l'expérimentation. La virémie a commencé à 4 jpi pour tous les animaux et est restée positive jusqu'à leur mort. Des anticorps neutralisant les BDV-4 ont été détectés à partir de 12 jpi. A l'autopsie, une lymphadénomégalie généralisée a été observée, associée dans un cas à des pétéchies disséminées dans l'ensemble du tractus digestif.
Tous les organes des femelles inoculées (9 par animal) étaient positifs en RT-PCR en temps réel, sauf un prélèvement de peau. Les 3 fœtus issus de ces femelles étaient également positifs. Les principales lésions histologiques retrouvées sur les femelles inoculées étaient une encéphalite lymphohistyocytique modérée, associée à une déplétion lymphoïde modérée à sévère. La virémie, la sérologie et les examens post mortems des animaux témoins sont restés négatifs.
En conclusion, des lymphopénies importantes et un avortement ont été causés par l'infection avec le BDV-4 chez des isards gestants. La mort des animaux a été causée par des infections secondairesLes ARN viraux retrouvés dans les organes des fœtus infectés suggèrent que des animaux infectés persistents immunotolérants (IPI) peuvent naître chez cette espèce. Enfin, les résultats obtenus lors de cette étude pourraient expliquer les diminutions de population d'isard ou de chamois observées dans certaines régions.

(11) **TITRE DE LA COMMUNICATION :**

Etude des transmissions interspécifiques des pestivirus entre les ruminants sauvages et domestiques dans la région PACA : Bilan de 4 ans d'étude.

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TEXTE DU RESUME

Les transmissions inter-spécifiques sont régulièrement incriminées dans l'épidémiologie des pestiviroses des ruminants ; particulièrement sur les alpages où des contacts sont régulièrement décrits entre ongulés sauvages et domestiques. L'objectif de cette étude était de décrire l'épidémiologie des pestiviroses simultanément chez les ongulés sauvages et domestiques dans les Alpes du Sud où des contacts inter-espèces sont avérés.
Des échantillons de sang et de rate ont été prélevés sur les ruminants soumis à un plan de chasse (chamois, chevreuil, mouflon essentiellement) de 2003 à 2010. Sur les ovins transhumant, des sérums ont été échantillonnées à partir des prélèvements de prophylaxie pour la brucellose de 2009 à 2011. De plus, des écouvillons vaginaux ou des échantillons de rate étaient envoyés par les vétérinaires locaux lors de suspicion clinique (avortements).. Afin de mieux caractériser les réponses sérologiques, des séroneutralisations croisées ont été réalisées à partir de 12 sérums ovins, 12 sérums de chamois et 6 de chevreuils sur 7 souches de pestivirus de ruminants (dont 4 locales).
La totalité des troupeaux ovins testés (n=37) présentaient une séroprévalence positive, atteignant 76,6 % (IC95%: [74,3 – 78,8 %]) pour l'ensemble des 1387 sérums analysés. Dans la vallée de l'Ubaye (département des Alpes de Haute-Provence), sur la même période, 29,7 % [19,1 – 40,5%] des chamois et 25,9% [9,4 – 42,4 %] des chevreuils testés étaient séropositifs.
Les souches isolées d'ovins ont été classées parmi les génogroupes suivants : Border Disease Virus type 3 et Tunisian pour les Alpes Maritimes et BDV-6 pour les Alpes de Haute Provence. Sur 140 prélèvements d'animaux sauvages testés, seule une rate de cabri (prélevée en bordure des Alpes de Haute-Provence) était positive en PCR. La souche isolée a été classée parmi les BDV-6 (souche RUPI-05).
Les séroneutralisations croisées ont montré que les chamois avaient des titres en anticorps supérieurs contre la souche Rupi-05, alors que les moutons réagissent de façon homogène envers les différentes souches locales. De plus, ces derniers ont des titres supérieurs aux chamois (sauf pour la souche Rupi-05) pouvant laisser suspecter une circulation plus importante chez les moutons. Les titres des chevreuils sont restés négatifs.
En conclusion, une circulation active des pestivirus est présente dans la région PACA, chez les animaux sauvages comme domestiques. Dans les Alpes de Haute Provence, les souches isolées des différentes espèces sont classées parmi le même sous-type viral. Enfin, les ovins semblent être soumis à un taux d'infection plus important que les espèces sauvages. Une étude de la prévalence virale au sein de chaque espèce associée à des caractérisations virales permettrait de confirmer le sens de transmission entre les différentes espèces animales.

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