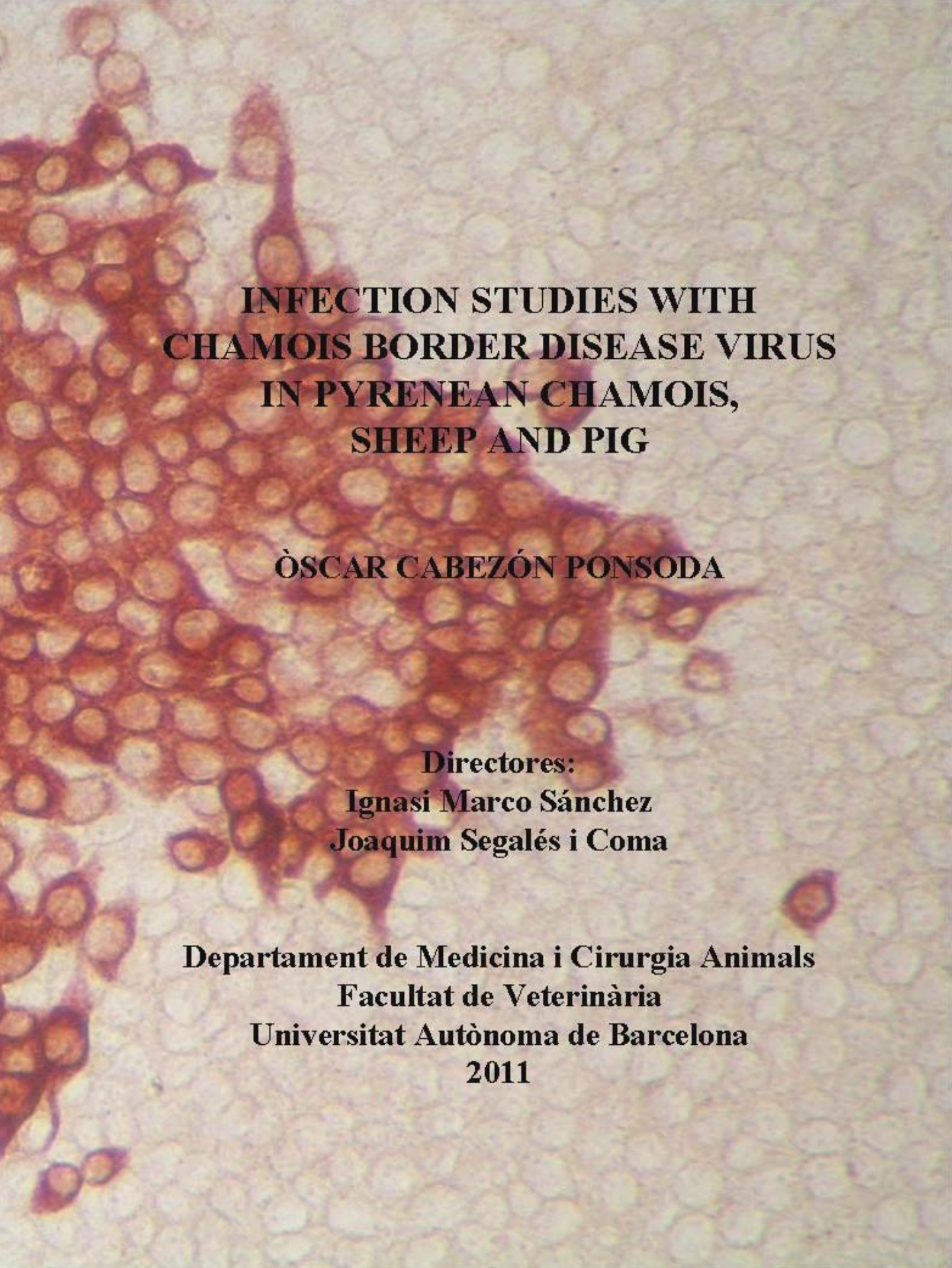

INFECTION STUDIES WITH CHAMOIS BORDER DISEASE VIRUS IN PYRENEAN CHAMOIS, SHEEP AND PIG

ÒSCAR CABEZÓN PONSODA

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Ignasi Marco Sánchez
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Departament de Medicina i Cirurgia Animals
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Los Doctores **Ignasi MARCO SÁNCHEZ** y **Joaquim SEGALÉS I COMA**, Profesores Titulares de Universidad de las Áreas de conocimiento de Medicina y Cirugía Animal y Sanidad y Anatomía Animal, respectivamente, de la Facultad de Veterinaria de la Universitat Autònoma de Barcelona,

HACEN CONSTAR,

Que la memoria titulada "**INFECTION STUDIES WITH CHAMOIS BORDER DISEASE VIRUS IN PYRENEAN CHAMOIS, SHEEP AND PIG**", presentada por Òscar Cabezón Ponsoda para la obtención del grado de Doctor en Veterinaria por la Universitat Autònoma de Barcelona, ha sido realizada bajo nuestra dirección y, considerándola satisfactoriamente finalizada, autorizamos su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos oportunos, firmamos el presente informe en Bellaterra, a 1 de Junio de 2011.

Firmado: Ignasi Marco Sánchez

Firmado: Joaquim Segalés i Coma

AGRADECIMIENTOS

No es habitual empezar los agradecimientos con alguien que no sea tu director de tesis, pero debo hacerlo. Y es que tengo que agradecer a Santiago Lavín primero, los 5 años que llevo en el SEFaS, y segundo, por la posibilidad de haber realizado esta tesis doctoral. Gracias, Santiago, por tener el convencimiento de que iba a ser así.

Sin duda, y también antes de mis dos directores de Tesis, tengo que mencionar a Rosa Rosell. A Rosa le tengo que agradecer todo. Ella ha sido y es el mayor aliado que he tenido en todos los trabajos realizados. Desde el primer día hasta hoy todo lo que ha hecho ha sido positivo para esta tesis. Ha tenido la habilidad de hacer de tercera directora aportando luz, soluciones y serenidad ante todos los problemas que han ido surgiendo durante estos años. ¡Muchas gracias jefa!

A mis dos directores de Tesis, Ignasi y Quim. Con ellos dos ha sido imposible que nada saliera mal. Ignasi es un tipo con las ideas meridianamente claras y para un estudiante de doctorado eso no tiene precio. El primer día que hablamos estoy seguro que sabía perfectamente qué íbamos a hacer y de qué manera, sin margen para el error, sin posibilidad de dar tumbos. Tengo la suerte, además, de que es una persona muy práctica, hecho que facilita el trabajo. Respecto a Quim, con él todo es fácil. Por muy liado, ocupado, preocupado, acelerado y omnipresente que le pueda haber visto, siempre que le he pedido consejo o ayuda se ha sacado de la chistera tiempo y dedicación absoluta, aportando un plus de calidad importante.

Durante uno de los primeros días de muestreo de los corderos en el P3, uno de éstos no tenía el día 'colaborador' y, Juan Carlos (técnico del NBS3, 2m de alto, 90kg, de Bilbao) y un servidor estuvimos más de lo que hubiera imaginado para cogerlo, pesarlo y sujetarlo. Recuerdo que mientras le 'sangraba' (sudando la gota gorda) pensé "*y con los rebecos como coño lo vamos a hacer??? Este Ignasi está loco*". 1 año después estábamos a 3 semanas del inicio del experimento y, efectivamente, disponer de rebecos como modelo experimental no iba a ser fácil. Era mayo y el hombre del tiempo anunciaba tormentas de nieve en todo el Pirineo (el peor invierno de los últimos 10)… y ni un bicho para la experimental. Esa fue la última semana con nieve. A la siguiente, todo el SEFaS se lanzaba, a contrarreloj, a capturar rebecos en Freser-Setcases para el experimento. En una sola batida salió todo a la perfección. Explico esto porque aunque en esta tesis solo se publica, solo se contempla y solo se describe la infección experimental a partir del día 0, un trabajo tanto o más descomunal se realizó entre dos y tres semanas pre-challenge. Es de justicia que agradezca aquí el trabajo que realizaron para mi tesis todo el SEFAS (Gregorio, Encarna, Emmanuel, Nora, Laura, Jorge, Ignasi, Esther, Santiago), los guardas, estudiantes de veterinaria y hasta algún voluntario (Nacho se pegó una sudada importante).

Por supuesto, he de tener unas palabras para Laura Fernández-Sirera ya que hemos compartido los Pestivirus estos años. He de confesarte, Laura, que al principio el laboratorio se te hacía un poco pesado (y a mí que se te hiciera pesado a ti) pero creo que al final los dos, como *'team'*, hemos estado a la altura del apasionante virus que

estudiamos. El trabajo que aquí se incluye ha sido el fruto de muchas horas trabajando codo a codo contigo (según tú, a codazos), buscando entre VNTs, macerados y PCRs las respuestas que no se encuentran en el campo. Sabes, Laura, que esta tesis también te pertenece.

Todos los estudios que se incluyen en esta tesis doctoral los he realizado en casi su totalidad en el Centre de Recerca en Sanitat Animal (CReSA). Y como es justo, a quien primero tengo que agradecer poder haber trabajado allí durante estos años es a Mariano Domingo. Él permitió poder desarrollar todo el trabajo experimental (que ha sido mucho, largo y exhaustivo) en ese excepcional centro de investigación.

Trabajar en el CReSA es, perdonen la expresión, una flipada. No solo por las infraestructuras sino por las personas tan brutalmente fascinantes que trabajan en él. Ahí es donde radica la fuerza investigadora de ese centro. La crisis económica azota a todo el mundo y el CReSA no es una excepción. Sin embargo, creo que con las ganas que le ponéis en todo, todo irá bien.

Es difícil personalizar mi admiración por la gente que allí he conocido. Sin embargo, me gustaría quitarme el sombrero ante la gente que trabaja en el NBS3. Todos - David, Valentí, Iván, Juan Carlos, Mari Ángeles, Conchita, Raquel Maeso, Raúl, etc. - creáis en mí un enorme sentimiento de respeto y admiración por cómo trabajáis y hacéis la vida más fácil a los que bajamos al P3 a trabajar. Solo espero no haberos dado mucha guerra o, por lo menos, espero no haber dado tanta como el rebeco RP10 (sí, aquel).

Obviamente, tengo que dedicarles unas líneas a los de 'viro'. Je je je. Núria Navarro, Cristina Riquelme, Iván y Esme. Me habéis ayudado TANTO que nunca os podré devolver todos los favores (podría estar rellenando vuestras cajas de puntas durante 10 años y todavía os debería favores). A veces no se tiene consciencia sobre el papel que jugáis los técnicos de laboratorio. Por mi parte deciros que, sin vosotros, no hubiera podido trabajar.

A los becarios con los que empecé (Maribel, Jordi, Eva) y a los que siguen todavía (Joan, Kate, Tufaria, Cristina, etc.): aunque nunca he sido Becario-Cresa… ha sido divertido compartir cabinas y charlas agradables sobre nuestros directores de tesis… jo jo jo…

Al resto de personal del CReSA: gracias por tratarme tan bien!!!

Esta Tesis está dedicada a varias personas.

> A mis padres, Roser y Ángel. Os tengo que dar las gracias por todo lo que os habéis esforzado por mí. Sé que mi madre sería la persona que más orgullosa estaría por esta tesis. A mi padre también le tengo que dar las gracias. Creo que fue él quien me metió en la cabeza mi pasión por la fauna.
>
> Aunque parezca difícil de creer, es posible que el origen de esta tesis esté muy lejos del Pirineo, de los rebecos y de los Pestivirus. En el fondo creo que todo

empezó en el mar de pinos de mi pueblo, Quintanar de la Sierra, en la Sierra del Urbión. Es allí donde mi hermano Carlos, mi prima Susana y yo dedicamos veranos enteros a *asilvestrarnos* en esos bosques llenos de ríos cristalinos, realizando 'estudios de ecopatología'. A vosotros dos os dedico esta tesis.

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Al resto de mi familia, les tengo que dar las gracias porque siempre que coincidimos se me escapa la risa: somos raros, los Cabezones.

INDEX OF CONTENTS

1. ABSTRACT

1. ABSTRACT

Since 2001 several outbreaks of disease associated with BDV infection have been reported in the Pyrenees (North-Eastern Spain), affecting Pyrenean chamois (*Rupicapra pyrenaica*) and entailing a major population reduction. However, the clinical disease observed in the affected chamois was not the typical one described in domestic animals. The present research aims to investigate the pathogenesis of the disease associated to chamois-BDV (ch-BDV) in naturally-infected chamois and in three experimental animal models: pig, sheep and Pyrenean chamois.

The shedding, distribution and quantification of ch-BDV in the organs of naturally infected chamois was investigated as a previous step to the experimental challenges in the three animal models. Sera and all tissue samples were positive to RT-PCR and virus isolation in all studied chamois. Also, nasal, oral and rectal swabs and urine were RT-PCR positive in almost all analyzed samples, confirming that the virus is shed through the main excretion routes. In addition, sera were tested for BDV antibodies using an ELISA and seroneutralization tests, with negative results. Sequence analysis of the 5' untranslated region (5'-UTR) confirmed that this virus was grouped into the BDV-4 genotype as reported in previous studies. The observation of a RT-PCR positive foetus in an adult female suggests that persistently infected animals could be possible.

The aim of the experimental infection in Pyrenean chamois was to reproduce the disease reported in chamois in the field and to study it under experimental conditions. Seven chamois (five seronegative and two seropositive against BDV) were inoculated with a BDV isolated from a naturally-infected chamois and three animals were kept as controls. The five seronegative infected chamois were viraemic from day 2 post inoculation (pi) until the day they died or the end of the experiment on day 34 pi, and developed neutralizing antibodies from day 18 pi until the end of the study. There was also a progressive decline in their white blood cell counts, especially marked in the neutrophil count. The most consistent histopathological lesions were in brain and lymphoid tissues, where non-suppurative meningoencephalitis and generalized moderate lymphocyte depletion were observed, respectively. Like naturally infected

chamois, experimentally infected animals also contained high doses of BDV in main putative excretion routes, which would explain the high transmission rate of the infection in free-ranging chamois populations in the Pyrenees. This experimental infection in chamois confirms that BDV is the primary agent of the disease that has been affecting chamois populations in recent years in the Pyrenees.

Clinical manifestations of Border Disease in healthy sheep acutely infected are mild or unapparent, but a few outbreaks with high mortality have been reported. Sheep were experimentally infected with ch-BDV and examined the susceptibility to the infection of this species. Clinical signs or histological lesions were not observed in inoculated lambs but BDV was detected in sera from the infected group from day 4 pi to day 10 pi. All infected lambs showed neutralizing antibodies at day 21 pi. Therefore, ch-BDV can infect domestic sheep through the oro-nasal route, developing a humoral response that completely eliminates the virus.

It is well documented that BDV can infect domestic and feral swine. In a previous study, seropositive wild boars against BDV were detected from the Pyrenees areas where chamois epizootics occurred. For this reason, experimentally the effects and dynamics of ch-BDV infection in domestic pig were studied. In this challenge all infected pigs were viraemic from day 3 to 14 pi, when all animals developed an antibody response. Clinical signs or histological lesions were not observed. Thus, the susceptibility of domestic swine to ch-BDV was demonstrated, representing a potential challenge to the monitoring of CSFV in wild swine populations.

2. RESUMEN

2. RESUMEN

Las poblaciones de rebeco pirenaico (*Rupicapra pyrenaica*) situadas en el Pirineo central y oriental se han reducido drásticamente debido a sucesivos brotes de enfermedad asociados a un Virus de la Enfermedad de la Frontera (BDV en sus siglas en inglés) desde el año 2001. Sin embargo, la enfermedad observada no presenta las características clásicas de la Enfermedad de la Frontera, descrita en las especies domésticas. La presente investigación tiene como objetivo investigar la patogenia de la enfermedad asociada al BDV del rebeco (ch-BDV) en rebecos infectados de manera natural y en tres especies infectadas experimentalmente: rebeco pirenaico, oveja y cerdo.

En primer lugar, investigamos la excreción, distribución y cuantificación del ch-BDV en los órganos de rebecos infectados de manera natural. El suero y los tejidos analizados fueron positivos mediante RT-PCR y aislamiento vírico en todos los animales estudiados. Los hisopos nasal, oral y rectal, así como la orina fueron también positivos mediante RT-PCR en casi todas las muestras analizadas, confirmando que el virus se elimina al ambiente a través de las vías de excreción analizadas. El estudio serológico no detectó anticuerpos en ningún rebeco utilizando técnicas de ELISA y seroneutralización. El estudio genético de la región no codificante 5' confirmó que el virus pertenecía al grupo BDV-4, tal y como se había descrito anteriormente en los brotes de enfermedad. La presencia de un feto positivo al ch-BDV mediante RT-PCR sugiere que sea posible la existencia de animales persistentemente infectados.

El objetivo de la infección experimental en el rebeco pirenaico fue la reproducción en condiciones experimentales de la enfermedad observada en los rebecos salvajes. Siete rebecos (cinco seronegativos y dos seropositivos a anticuerpos frente a BDV) fueron inoculados con una cepa BDV aislada de un rebeco de campo infectado. Tres animales más se mantuvieron como controles. Los cinco rebecos seronegativos infectados presentaron una viremia desde el día 2 post-inoculación (pi) hasta la muerte del animal o el fin del experimento en el día 34 pi, y desarrollaron anticuerpos neutralizantes a partir del día 18 pi. Los rebecos virémicos presentaron un descenso en el recuento

leucocitario, especialmente significativo en los neutrófilos. Las lesiones más significativas se observaron en el encéfalo y órganos linfoides, que presentaron una meningoencefalitis no supurativa y una moderada depleción linfoide generalizada, respectivamente. De manera similar a los rebecos infectados de manera natural, los rebecos de este experimento también presentaron virus en hisopo nasal, saliva y heces, hecho que explicaría la elevada capacidad de transmisión horizontal observada en las poblaciones de rebecos salvajes afectadas por las epizootias. Esta infección experimental confirma que el ch-BDV es el agente etiológico primario de la enfermedad que ha afectado a las poblaciones de rebeco en los últimos años en el Pirineo.

La Enfermedad de la Frontera en las ovejas cursa con un cuadro sintomático leve o subclínico, aunque se han descrito brotes de enfermedad con elevada mortalidad. En la infección experimental de oveja valoramos la susceptibilidad de esta especie al ch-BDV. No se observaron signos clínicos ni lesiones histopatológicas en los corderos inoculados aunque el BDV se detectó en suero entre los días 4 y 10 pi. Todos los corderos infectados mostraron anticuerpos neutralizantes a partir del día 21 pi. La conclusión principal de esta infección experimental fue que el ch-BDV infecta la oveja vía oro-nasal, desarrollando en ésta una respuesta humoral que elimina el virus.

El BDV infecta diferentes especies animales. Previamente a la infección experimental en cerdo, habíamos detectado jabalíes con anticuerpos frente a BDV en las zonas del Pirineo donde se habían producido los brotes de enfermedad en rebeco. El objetivo principal de la infección experimental en el cerdo fue estudiar bajo condiciones experimentales los efectos del ch-BDV y la dinámica de la infección en esta especie. Los cerdos infectados mostraron una viremia desde el día 3 hasta el 14 pi, a partir del cual los animales desarrollaron una repuesta humoral. No se observó sintomatología clínica ni lesiones histológicas. Esta infección experimental demostró la susceptibilidad del cerdo al ch-BDV, lo que podría representar un problema en el diagnóstico de la Peste Porcina Clásica en el jabalí.

3. INTRODUCTION

3. INTRODUCTION

3.1. Pestivirus

3.1.1. Taxonomy

The Family Flaviviridae comprises three genus of virus: genus Pestivirus, genus Hepacivirus and genus Flavivirus (ICTV, 2009). Inside genus Pestivirus, there are four recognised species: Classical Swine Fever Virus (CSFV), Bovine Viral Diarrhoea Virus (BVDV) types 1 and 2, and Border Disease Virus (BDV). Also, several pestivirus species have been proposed. In this sense, there is a tentatively species isolated from a giraffe (Fauquet et al., 2005), three proposed new species isolated from calf serum, cattle and buffalo (Schirrmeier et al., 2004; Stalder et al., 2005; Liu et al., 2009a), and two putative species isolated from pronghorn antelope (*Antilocapra americana*) from North America (Vilcek et al., 2005) and Bungowannah virus isolated from pigs in Australia (Kirkland et al., 2007). The last genetic comparison study has proposed the existence of 9 Pestivirus: BVDV-1, BDVD-2, BDVD-3, BDV, CSFV, Giraffe pestivirus, Tunisian sheep virus (TSV), Antelope and Bungowannah (Liu et al., 2009b).

Traditionally, Pestiviruses have been classified and named according to the affected species and the diseases they cause. However, Pestiviruses have the ability to cross species barriers and to infect a wide range of *Artiodactyla* species. Thus, several genetic comparison studies performed in the last years have attempted to classify the different pestivirus isolates into subgroups in the four accepted Pestivirus species (Becher et al., 1995, 1997, 1999, 2003; Paton et al., 1995; Vilcek et al., 1994, 1997; Liu et al., 2009b) and new pestivirus subgroups have been proposed recently (Hurtado et al., 2003, 2004; Thabti et al., 2005; Dubois et al., 2008; Oguzoglu et al., 2009; Vilcek et al., 2001).

3.1.2. Classical Swine Fever Virus

Classical Swine Fever Virus (CSFV) is the causative agent of Classical swine fever (CSF) or hog cholera. This virus infects domestic and wild swine and is distributed

worldwide, mainly in Asia, some Caribbean islands, some African countries, and South and Central America. In Europe, CSFV has been eradicated in most of Western European countries but it is still present in Eastern Europe. CSF is endemic in wild boar populations from Central and Eastern Europe (OIE, 2004).

CSFV is a highly contagious virus that causes a wide range of signs in infected pigs, depending on the viral strain. More virulent strains of CSFV cause acute disease consistent with multiorganic haemorrhagic disease, high fever, anorexia, conjunctivitis, constipation followed by diarrhoea, leukopenia and immunosupression leading to secondary infections (Moennig et al., 2003). These pigs use to die within one to three weeks. CSFV strains with less virulence cause chronic disease and the affected pigs experience intermittent fever, anorexia and diarrhoea, alopecia, and skin lesions. After one to three months of chronic disease, these animals usually die. Although CSFV has proven to infect cattle and goat experimentally (Loan and Storm, 1969; Shimizu and Kumagai, 1989), it is not considered to cross interspecies barrier under natural conditions (OIE, 2004).

3.1.3. Bovine Viral Diarrhoea Virus

Regarding the 'ruminant pestiviruses', BVDV comprises two species or genotypes referred as BVDV type-1, firstly isolated from cattle in 1954 (Baker et al., 1954), and BVDV type-2, identified in the 1990's in North America (Pellerin et al., 1994, Becher et al., 1995) that are different genetically (Nettleton and Entrican et al., 1995). In addition, BVDV has two biotypes referred as non-cytopathogenic (ncp) and cytopathogenic (cp) determined by their effects in cellular culture (Brownlie et al., 1984; Bolin et al., 1985). Although BVDVs can infect swine, their main hosts are domestic and wild ruminants, especially bovine species. Most BVDV infections worldwide are caused by ncp strains and they can cause acute or congenital infections in ruminant hosts.

The acute form of bovine viral diarrhoea (BVD) is characterized by a post-natal infection of an immunocompetent host and courses with a mild transient viraemia with

production of neutralizing antibodies. Clinically, acute ncp-BVDV infection courses with enteric disease consisting of diarrhoea, pyrexia and mild depression with a high mobidity and low mortality. However, the acute fatal haemorrhagic syndrome has been associated to ncp BVDV-2 (Carman et al., 1998).

The congenital infection occurs with the infection of the foetus before to the development of its immune competence. This infection causes persistently infected (PI) newborns, characterized by recognizing the virus as a self-antigen. This fact leads the virus to replicate inside the animal and being shed continuously to the environment. In the epidemiology of BVDV, these PI animals represent the major source of transmission of the virus within and among cattle herds causing significant losses in cattle farming worldwide (Brownlie et al., 1987).

In addition, there is another enteric syndrome associated to BVDV infection, namely mucosal disease (MD), which is a fatal infection that leads to the death of the infected animal. This syndrome is associated to cp BVDV biotypes. The presence of the cp biotype in the animals is the consequence of three possibilities: superinfection of a cp biotype in a PI animal (Bolin, 1995), recombination between ncp biotypes, or mutation of an already existant biotype (Loehr et al., 1998). MD presents low morbidity and high mortality and is characterized by diarrhoea, profound depression and death. At necropsy, erosions in the mucosa at various sites along the gastrointestinal tract are observed. Histological examination shows destruction of the gut-associated lymphoid tissue, which that it is replaced by inflammatory cells.

3.1.4. Border Disease Virus

BDV is one of the most widespread animal viruses in the world and has been described in a wide range of domestic and wild species of swine and ruminants (Nettleton and Entrican, 1995; Nettleton et al., 1998). BDV is enveloped, spherical, approximately 50 nm in diameter and its genome is a single-stranded positive-sense RNA molecule, of 12.5 kb long. The genome contains a single open reading frame (ORF) flanked by a 5' and a 3' untranslated regions (UTR) (Collett et al., 1988; Meyers et al., 1989; Ridpath

and Bolin, 1995) (Figure 3.1). The OFR encodes a polyprotein of 3,900 amino acids approximately that will be cleaved into twelve structural (S) and non-structural (NS) proteins. The structural proteins are the capsid protein C and the envelope glycoproteins Erns, E1 and E2 (Thiel et al., 1993). The NS proteins are p20 (Npro) that is an autoprotease, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Meyers and Thiel, 1996; Lackner et al., 2004). In addition, uncleaved E2-p7 and NS2-3 polypeptides are produced.

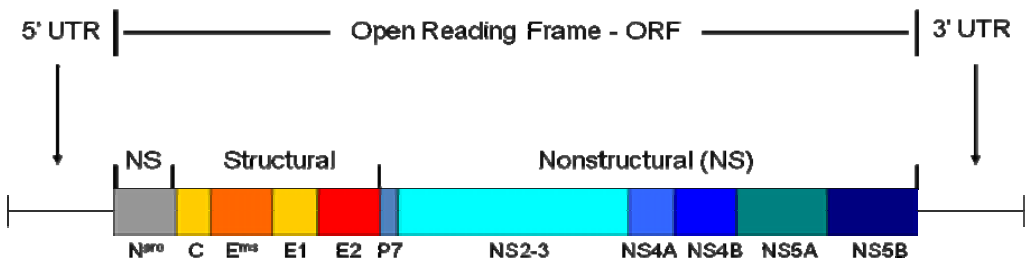

Figure 3.1. Representation of final protein products of the single open reading frame (ORF) and 5' and 3' untranslated regions (UTR) of a non-cytophatic Border Disease Virus.

In recent years, several genetic comparison studies of Pestivirus have attempted to classify the different pestivirus isolates into subgroups in the four accepted Pestivirus species. Results obtained from these studies have classified BDV in different groups inside this species (Becher et al., 1995, 2003; Vilcek et al., 1994, 1997; Hurtado et al., 2003; Arnal et al., 2004; Thabti et al., 2005; Dubois et al., 2008; Liu et al., 2009b; Oguzoglu et al., 2009). A recent classification of BDV was reported by Dubois (2008) and described six groups: BDV-1 to 6 (Figure 3.2)

In this sense, several studies carried out in Spain have provided information about seroprevalence of pestivirus, identification of new virus isolates and description of clinical features associated to BDV infections in ruminant flocks. These studies have concluded that BDV-4 is the genotype with main presence in sheep flocks in Spain (Hurtado et al., 2003, Valdazo-González et al., 2006, 2007, 2008).

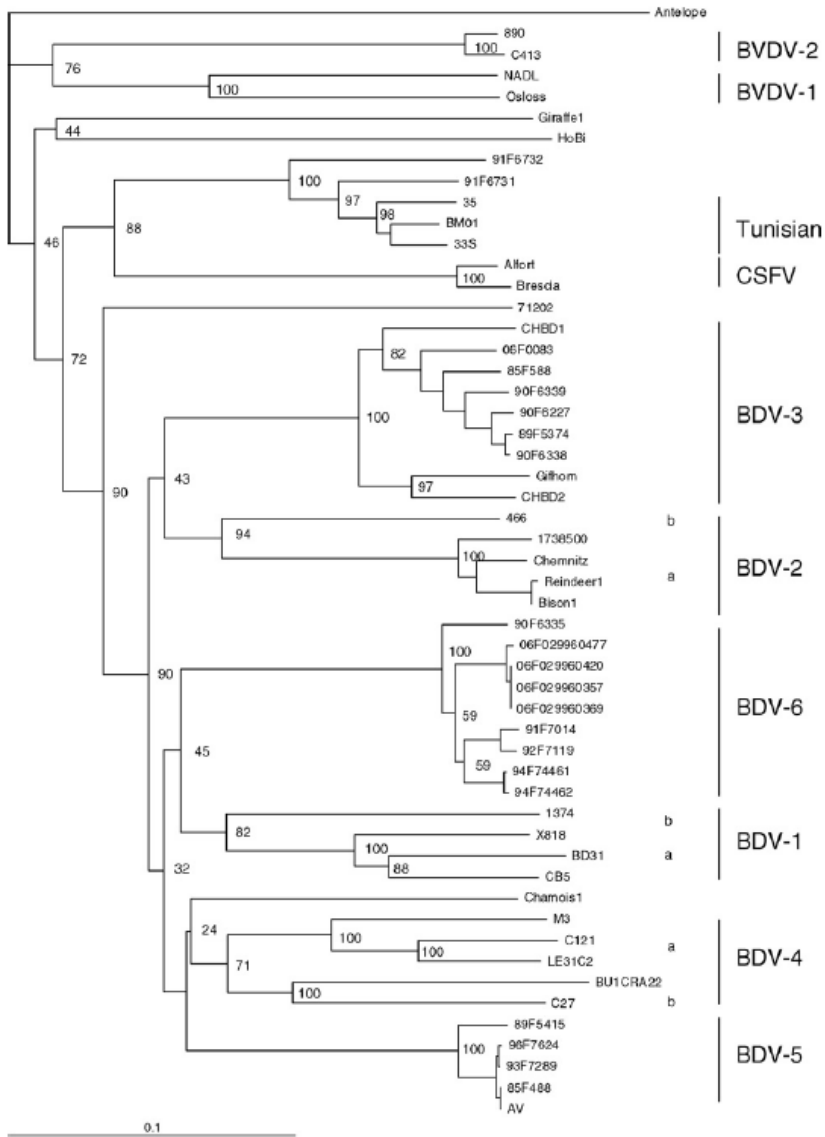

Figure 3.2. Neighbour-joining phylogenetic tree present in the research work of Dubois (2008). This phylogenetic tree is constructed using 489 nt from the Npro region of the pestivirus and proposes new BDV subgroups.

3.2. Border Disease

Border Disease (BD) was firstly reported affecting sheep in 1959 in the border region between Wales and England (Hughes et al., 1959). The current distribution of the virus is worldwide. Border disease is a viral disease observed mainly in sheep and rare and poorly reported in goat. Although classically BDV infections have been attributed to this two species, isolation of BDV or presence of antibodies have been reported in goat, cattle, swine and some wild-ruminants (Nettleton, 1990; Vilcek and Belák, 1996; Cranwell et al., 2007).

3.2.1. Pathogenesis and clinical features

BDV has two *via* of infection: horizontal and vertical transmission. Horizontal transmission of BDV among sheep causes acute infections characterized by a short-period of viraemia. The virus spreads among ruminants by the oro-nasal route and can be detected in serum between days 4 and 11 post-infection. After a short-period of viraemia, neutralizing antibodies appear in serum (Nettleton et al., 1998). Clinically, this acute infection is characterized by a mild pyrexia and transient lymphopaenia (Nettleton, 1990).

However, the success of BDV has been the vertical or congenital transmission from ewe to foetus. The infection of sheep during pregnancy originates different clinical situations. While the course of the infection of the pregnant ewe is clinically mild or unapparent and similar to the acute horizontal infection described above, the consequences for the foetus are of importance. The ability to cross the placenta and the infection of the foetus causes different consequences depending on the phase of the gestation when the infection occurs.

Although the death of the foetus/embryo can occur after BDV infection at any stage of gestation, it is at the first pregnancy stages when there is a higher probability of death. When it occurs, there is embryo resorption and usually it is undetectable. If the infection occurs before day 60 of gestation, before foetal immunocompetence, the BDV replicates

in the foetal tissues without control. The 50% of these infected foetuses die; if they survive and born alive, lambs are PI and characterized by the continuous replication and excretion of the virus. PI lambs display several clinical features but they usually appear as small and weak animals, being unable to stand, with characteristic neurological symptoms such as tremors. Also, PI lambs show fleece symptoms being more detectable in smooth-coated breeds, developing hairy fleeces with brown pigmentation usually in the back (Nettleton et al., 1998). If the infection of the foetus takes place after the day 80 of gestation, the immune system of the foetus is capable to eliminate the virus and the newborn will have pre-calostral antibodies against the virus (Nettleton, 1990).

In contrast to disease caused by CSFV or BVDV acute infections, BDV rarely causes epizootics associated to high-rate mortalities. However, some outbreaks of disease with high mortality have been associated to BDV acute infection in healthy sheep (Chappuis et al., 1984) and a mucosal disease-like syndrome in PI sheep (Monies et al., 2004).

3.2.2. Border disease in goats

Pestivirus infections in goat are rare and poorly reported. Although serological surveys in many countries have demonstrated widespread BD infection in goats (Nettleton, 1990), few isolates have been recovered from diseased goats (Loken et al. 1982; De Mia et al., 2005). Few experimental pestivirus infections in goats have been reported. In these, it has been proven that acutely infected goats do not transmit BDV to other animals, as occurs in cattle and sheep (Loken et al., 1990). Moreover, the survival rate of infected goat foetus is very low. Thus, it is believed that PI sheep and cattle are the main reservoir of pestiviruses (Loken, 2000).

3.3. Experimental BDV infections

The ability of Pestiviruses to produce cross-infections between *Artiodactyla* species and to persist in PI animals, have led to a wide geographical distribution and caused high

economic losses worldwide. In order to know the pathogenesis and pathological disorders caused by these viruses, several experimental infection studies have been performed of the different Pestiviruses in their different host species. A high percentage of experimental Pestivirus infections in ruminants use BVDV strains as innoculum due to the major economic and pathologic impact in ruminant populations worldwide. Thus, relatively a low number of experimental BDV infections is found in the literature other than those performed in sheep.

3.3.1. Experimental BDV infections in sheep

Experimental BDV infections in sheep have been performed since the 70's aimed at describing the pathogenesis and the lesions caused by the virus in adult and pregnant sheep. Also, these challenges were aimed at describing the relationship between the different clinical diseases and the different BDV strains. Recently, at the same time that new BDV groups have been identified (Becher et al., 2003; Dubois et al., 2008), these have been tested in new experimental trials (Edwards et al., 1995; Thabti et al., 2002; García-Perez et al., 2009). Almost all experimental infections of these new BDV groups in sheep have reproduced the disease observed under natural conditions. Only the experimental infection of lambs with the Aveyron strain showed unexpected results (Thabti et al., 2002). This strain caused an outbreak of haemorrhagic syndrome in 1983 in France resulting in death of 1500 ewes and 24000 lambs (Chappuis et al., 1984). In a subsequent experimental challenge, no severe disease as reported in 1984 outbreak was observed (Thabti et al., 2002). The hypotheses of the divergent results were attributed to the different breed of lamb used in the experiment and the several cellular passages of the virus (Thabti et al., 2002).

3.3.2. Experimental BDV infections in pigs

The interest to reproduce experimentally BDV infection in pigs was to study the potential lesions caused by this virus in swine, the immune response of this species to the virus and the potential diagnostic confusion with CSFV. To date, almost all BDV experimental infections in swine have demonstrated that this species is susceptible to

BDV and that this virus can produce an acute infection characterized by mild clinical signs and no histological lesions (Edwards et al., 1995), as observed under natural conditions. This infection is characterized by a short viraemia appearing antibodies after day 14 post-inoculation (pi). Moreover, Edwards (1995) demonstrated that although BDV can cause congenital infection, pathological lesions were rarely observed in piglets. However, Leforban (1992) observed haemorraghic lesions in piglets infected congenitally with the ncp BDV Aveyron strain and demonstrated that piglets infected '*in utero*' with BDV could excrete persistently the virus. When this virus infected non-pregnant pigs, they remained without clinical symptoms and induced an antibody response which protected against CSFV infection (Leforban et al., 1992).

3.3.3. Experimental BDV infections in goats

As reported in sheep, experimental BDV infections performed in goats showed a mild acute infection in adult animals with an immune response detectable within 2-5 weeks pi, which lasted for four years (Barlow et al., 1975). Also, parenteral inoculations of BDV in goat's offspring showed a similar immune response without significant clinical signs (Löken et al, 1990). In addition, these experiments showed that acute infected goats did not spread the virus to other animals as occurs in cattle and sheep. On the other hand, it was reported that adult goats were susceptible to BDV infection when cohabiting with PI ruminant species.

Congenital BDV infection has also been proved in goat, being the survival rate of the infected foetus very low (Löken et al., 1990). Interestingly, Löken (1990) found significant histological changes in CNS of goat's offspring infected parenterally (inoculated intracraneally) with BDV. Also, infiltration of the myocardium by lymphocytes was observed.

3.4. Ruminant Pestivirus infections in wildlife

3.4.1. Ruminant Pestivirus infections in wildlife other than chamois

Presence of ruminant Pestiviruses in wildlife is supported by numerous serological studies (Loken 1995; Vilcek and Nettleton, 2006; Marco et al., 2011; Fernández-Sirera et al., 2011) but the epidemiology of Pestivirus in wildlife is still unclear. In addition, isolation of Pestiviruses from these species is rarely reported. To date, free-ranging wild species with BVDV isolation are roe deer (*Capreolus capreolus*), eland (*Taurotragus oryx*), buffalo (*Syncerus caffer*), alpaca (*Lama pacos*), red deer (*Cervus elaphus*), pudu (*Pudu puda*) (Vilcek and Nettleton, 2006), serow (*Capricornis crispus*) (Harasawa et al., 2006), camel (Gen. Camelus) (Intisar et al., 2010), mule deer (*Odocoileus hemionus*) (Van Campen et al., 2001), and sika deer (*Cervus nippon*) (Gao et al., 2011). Also, BVDV strains were isolated from several captive wild ruminants (Doyle and Heuschele, 1983; Nettleton 1990; Becher et al., 1999; Uttenthal et al., 2005; Nelson et al., 2008). In addition, recent genetic studies have identified two new Pestivirus species isolated from two free-ranging ruminants: Pronghorn antelope (Vilcek et al., 2005) and Giraffe (*Giraffa camelopardalis*) (Becher et al., 1999).

BDV has only been isolated from three wild ruminant species. Two of them were from captive animals in a German zoo: reindeer (*Rangifer tarandus*) (Becher et al., 1999) and European bison (*Bison bonasus*) (Becher et al., 1999). The only free-ranging species from which BDV has been isolated is the Pyrenean chamois (*Rupicapra pyrenaica*) (Hurtado et al., 2004). BDV isolated from reindeer and bison were thought to be new Pestivirus species but recent studies have allocated these isolates into BDV-2 group (Becher et al., 2003). The BDV isolated from Pyrenean chamois has been classified into BDV-4 group (Arnal et al., 2004).

3.4.2. Epizooties associated to BDV in the Pyrenean chamois

Pyrenean chamois is a high-mountain ungulate. It belongs to the order *Artiodactyla*, sub-order *Ruminantia*, family *Bovidae*, sub-family *Caprinae*, and genus *Rupicapra*

(Pérez-Barbería y García-González, 2004). The genus *Rupicapra* has two species, the Pyrenean chamois and the Alpine chamois (*Rupicapra rupicapra*). Pyrenean chamois is widespread distributed in south-west Europe, where it is present as three subspecies: *R. p. pyrenaica*, *R. p. parva* and *R. p. ornata* inhabiting the Pyrenees (Spain, Andorra and France), the Cantabrian Mountains (Spain) and the Apennines (Italy), respectively (Shackleton, 1997; Pedrotti and Lovari, 1999). In the Spanish Pyrenees the distribution area is comprised from la Garrotxa in Catalonia to Valle del Roncal in Navarra (García-González, 2002). The altitudinal range of the species is 400-2,800 m (Palomo and Gisbert 2002). Chamois has social and economical importance since it is a game species.

The Pyrenean chamois is listed on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2010) as "least concern". Regarding *R. p. pyrenaica*, its status has greatly improved since 1990. The *R. p. pyrenaica* population estimated in 1989 was 15,500 animals (Shackleton 1997) and the same population in 2003 was to be at least 53,000 individuals (Herrero *et al.* 2004).

Its summer coat is a ruddy brown and in winter it is black/brown with darker patches around the eyes. Both males and females have backward-hooked horns up to 20 cm in length. Biometrical information about this species is: 100–110 cm of total length, 69-75 cm tall and 25-40 kg weight in males, and 20-32 in females (Delaunay, 1982; Pflieger, 1982; ANCGG, 1992; Weber, 2001).

The threats to this species vary depending of geographical distribution. However, in France and Spain disease is currently the most important threat. Pestivirus infections were firstly described in the Pyrenean subspecies in 2001, and sarcoptic mange outbreaks periodically cause local declines in the Cantabrian subspecies. Diseases like bacterial bronchopneumonia and contagious ecthyma are also present in chamois populations (Marco et al., 1992; Weber, 2001). Endemic diseases such as infectious keratoconjunctivitis also represent a potential threat to the survival of this ruminant.

During 2001 and 2002, an outbreak of an unreported disease associated with BDV infection (Arnal *et al*., 2004; Hurtado *et al*., 2004; Frölich *et al*., 2005) was detected in Alt Pallars-Aran, in the Pyrenees, entailing a 42 % reduction in the chamois population in the affected area (Marco *et al*., 2007). This was the first time a BDV has been associated with an outbreak of a high-mortality disease in a wild species. Pestivirus was detected in affected chamois with ELISA-antigen and RT-PCR tests, and immunohistochemistry studies detected positive staining in several tissue samples. A BDV (chamois BDV, ch-BDV) was isolated, characterized and causally associated to the outbreak (Hurtado *et al*., 2004; Arnal et al., 2004).

Three years later, in 2005, dozens of deaths in chamois populations of Alt Urgell-Cerdanya National Hunting Reserve were recorded and were associated to the same aetiological agent (Marco et al., 2009), reducing the chamois population of this area about 85.6%. After this second outbreak, the virus appeared in Cadí National Hunting Reserve in the same year 2005 entailing a reduction of 63% of the chamois population between 2005 and 2007. The last area where the virus has been detected is the Freser National Hunting. This case is interesting because no mass mortality has been observed in this area and ch-BDV was isolated from a single affected chamois in 2007 and the virus has been regularly isolated in healthy hunted animals. The hypothesis of the absence of mortality in this area can be related to the high seroprevalence observed (Marco et al., 2009). Recently, retrospective studies provided serological and genetic data that confirm the presence of ch-BDV in chamois populations since 1990 (Pioz et al., 2007; Marco et al., 2011)

Diseased chamois found in different outbreaks since 2001 showed clinical manifestations such as depression, weakness and movement difficulties, abnormal behaviour, with absence of flight reaction, and with skin alterations (alopecia and hyperpigmentation) (Figure 3.3). Gross-lesions found in post-mortem studies were cachexia, abscesses in different parts of the body and pneumonia. Microscopic lesions were found in the brain, mainly oedema, gliosis, spongiosis, and neuronal multifocal necrosis (Marco et al., 2007). A perivascular mononuclear inflammatory infiltrate was found in a few of them. Skin lesions included marked follicular atrophy, mild to

moderate epidermal hyperplasia with orthokeratotic hyperkeratosis, follicular hyperkeratosis, and hypermelanosis. In almost all diseased chamois, Pestivirus was detected by RT-PCR (Marco et al., 2007). Recently, Vilcek et al. (2010) described the full genome sequence of pestivirus ch-BDV strain.

Figure 3.3. Southern chamois with clinical signs of infection associated with a border disease virus: extensive alopecia and cachexia.

4. HYPOTHESIS AND OBJETIVES

4. HYPOTHESISI AND OBJECTIVES

The severe outbreaks of a new disease affecting Pyrenean chamois populations since 2001 were associated to a previously unreported BDV. To date, the epidemiological, clinical and histopathological findings observed in diseased chamois with BDV infection have not been reported in domestic ruminant BDV infection, with the exception of an isolated outbreak described in lambs in France (Chappuis et al., 1984). Several questions about the aetiology of the disease and the behaviour of this virus remained unanswered since the first outbreak was described. Epidemiological, pathological and virological studies of diseased chamois have been performed and several hypothesis regarding chamois BDV (ch-BDV) infection and the dynamics of the disease in chamois populations were proposed, some of which were considered to be answered with the studies presented in this Thesis.

Thus, this Thesis was aimed to determine the susceptibility of Pyrenean chamois, sheep and pig to the BDV isolated from naturally infected chamois in a previous outbreak of disease in the Pyrenees. The specific objectives were:

- To investigate the distribution and quantification of ch-BDV in the organs of naturally affected chamois and to determine whether the virus is shed via the main excretion routes.
- To investigate whether under experimental conditions ch-BDV causes in Pyrenean chamois the disease and lesions described in naturally infected animals and to describe the viral dynamics and the humoral immune response.
- To investigate the susceptibility of domestic sheep to postnatal infection with ch-BDV isolated from naturally infected chamois and to monitor the infection dynamics of inoculated lambs during one month.
- To investigate the susceptibility of domestic pig to infection with ch-BDV, as well as to monitor the dynamics of the virus infection and antibody response.

5. STUDIES

STUDY I

Border disease virus shedding and detection in naturally infected Pyrenean chamois (*Rupicapra pyrenaica*)

J Vet Diagn Invest 22, 744-747

INTRODUCTION

Border disease (BD) is a congenital infectious disease caused by Border disease virus (BDV, Gen. *Pestivirus*, Fam. Flaviviridae), an enveloped, positive-sense, single-stranded RNA virus that mainly affects small ruminants. In 2001 and 2002 an outbreak of disease associated with a BDV genotype-4 (from here afterwards, ch-BDV) infection was reported in a Pyrenean chamois (*Rupicapra pyrenaica*) population in the central Pyrenees (Catalonia, North-Eastern Spain) (Hurtado *et al*., 2004; Arnal et al., 2001, Marco et al., 2007). Since this severe epizootic episode, the disease spread to the Eastern Pyrenees causing further severe outbreaks with even higher mortality rates leading chamois populations to collapse (Marco et al., 2009). Ten years after the first outbreak, affected chamois populations are recovering slowly than expected being this disease the major health concern in Pyrenean chamois currently.

The clinical presentation of BD in livestock is characterized by the presence of barren ewes, abortions, stillbirths and birth of weak lambs and persistently infected (PI) animals. BD in healthy post-natally infected sheep is mild or unapparent, developing a short viraemia that ends when neutralizing antibodies appear in serum (Nettleton *et al*., 1998). However, Chappuis et al. (1984), reported an outbreak of disease with high mortality associated to acute infection with BDV in healthy sheep in Aveyron region (France) and a mucosal disease-like syndrome has been described occasionally in PI sheep (Monies *et al*., 2004; Hilbe *et al*., 2009).

Although BDV infection has been studied widely in domestic animals, there are no studies on virus excretion and organic distribution in diseased chamois and other wild animals. The aim of this study is to investigate the distribution and quantification of the virus in the organs of affected chamois and to determine whether the virus is shed via the main excretion routes.

MATERIALS AND METHODS

Animals and samples

In 2006, eight Southern chamois (seven males and one pregnant female) from one to eight years old with clinical signs consistent with BDV infection were studied. All chamois were cachectic and in an agonic stage of illness. Most of the eight animals showed alopecia with skin hyperpigmentation. Moreover, several degrees of neurological alterations (depression, weakness and difficulty in moving) were observed in all of the animals. The chamois were captured in the Cadí National Hunting Reserve (Eastern Pyrenees, Catalonia, Spain), during a severe outbreak of the disease (Marco et al., 2009). All animals were sent to the Veterinary Faculty's wildlife facilities of the Universitat Autònoma de Barcelona and euthanized in accordance with animal care guidelines. Complete necropsy studies were performed in all animals.

Before necropsy, nasal, oral and rectal swabs were collected and a 20 ml blood sample taken from the jugular vein of each animal, using 10ml syringes and 0.8 x 25 mm needles; 10ml of blood was placed in a tube with lithium heparin as anticoagulant. The remainder was placed in a serum collection tube. At necropsy, urine and tissue samples (spleen, kidney, brain, lymph node, skin, thyroid gland, intestine, liver, lung, bone marrow and testicle) were obtained. Tissue samples were taken from the spleen of the pregnant female's foetus, that was estimated to be in the last third of gestation. Separate sterile instruments were used for various animals and samples in order to avoid cross-contamination. Nasal, oral and rectal swabs were resuspended with 2 ml of sterile PBS supplemented with antibiotic. Blood samples from serum collection tubes were centrifuged at 1200g for 15 minutes, and sera obtained. Tissue samples were homogenized and resuspended in sterile MEM-E + antibiotic (1g of tissue + 9ml MEM-E). All samples were stored at -20ºC until tested.

Laboratory analyses

Amplification of viral RNA was conducted by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Previously, viral RNA had been extracted directly from tissue homogenates, swabs and sera by a commercial kit (Macherey Nagel Nucleospin Viral RNA Isolation). RT-PCR was performed to detect pestiviral RNA (5'UTR region) using previously described primers 324 and 326 (Vilcek et al., 1994, 1997) and a commercial kit (One-Step PCR kit, Quiagen).

In order to quantify the virus from the samples of the studied animals, oral, nasal and rectal swab samples, sera, urine and tissue homogenates were titrated directly on sheep foetal thymoid (SFT-R) pestivirus free monolayer cell line in a 96 wells tissue culture plate. Viral replication was monitored by the Immuno Peroxidase Monolayer Assay (IPMA) with a polyclonal home-made pestivirus antibody (OIE, 2004). Virus titration were perfomed as described previously (Reed and Muench, 1938).

Sera samples from all eight animals were tested for the presence of Pestivirus antibodies against p80 with a commercial sandwich enzyme-linked immunosorbent assay test (ELISA, Pourquier, Montpellier, France) following the procedure described by the manufacturer. A Virus Neutralization Test (VNT) was performed in the sera to detect anti-BDV antibodies isolated from the chamois. Titers of 1:10 or higher were considered as positive.

Analyses of 5'UTR region -243 pb fragment- of the virus sequence were performed in all the chamois studied. Amplified DNA from one sample from each animal was purified (Minelute Gel Extraction Kit, Quiagen) and sequenced. Purified amplicons were analyzed with the Big Dye Terminator v.3.1 Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3130xl Genetic Analyzer (Applied Biosystems).

The phylogenetic tree was calculated by the neighbour-joining method (Saitou and Nei, 1987) using an automatic root location. To test the reliability of the branches in the tree

a bootstrap analysis of 1000 replicates was performed by creating a series of bootstrap samples.

RESULTS

Pestivirus genome was detected by RT-PCR in sera (8/8; 100%), oral (6/7; 85.7%), nasal (7/7; 100%) and rectal (5/7; 71.4%) swabs, urine (7/7; 100%) and the 100% of tissue samples of all studied chamois. Also, the spleen of the foetus tested positive by RT-PCR (Table 5.1.1).

Isolation and titration of virus in different samples are expressed in Table 5.1.1. Briefly, BDV was isolated in sera (8/8; 100%), oral (2/7; 28.6%), nasal (4/7; 57.1%), and rectal (1/7; 14.3%) swabs, skin (6/7; 85.7%), small intestine (2/6; 33.3%) and in the 100% of the other main organs analyzed. Infectious BDV could not be isolated from urine samples and spleen foetus. The higher presence of infective virus was observed in serum samples with 6 out of 8 sera with titres higher than 6,5 $TCID_{50}$/ml.

Both ELISA and virus neutralization (VNT) tests for Pestivirus antibodies produced negative results.

Genetic analysis of the 5'UTR of the viral genome detected in the studied chamois showed that this pestivirus is included in the BDV-4 genotype, closely related to the other virus previously isolated from sick Pyrenean chamois (Hurtado et al. 2004, Arnal et al. 2004, Pioz et al. 2007, Marco et al. 2007). The sequences are deposited in GenBank under the following names and accession numbers: CADI-1 (AM905918), CADI-7 (AM905924), CADI-8 (AM905925), CADI-9 (AM905926), CADI-10 (AM905927), CADI-11 (AM905928), CADI-12 (AM905929), CADI-13 (FN397676) (Figure 5.1.1).

DISCUSSION

Pestivirus infection has been reported in many species of wild ruminants, being associated for the most part to a Bovine Viral Diarrhoea Virus (BVDV-1) (Vilcek and Nettleton, 2006). BDV infection and fatal disease has been described only in a captive bison, a reindeer from a German zoo, and in free-ranging Southern chamois during a disease outbreak (Becher et al., 1999; Arnal et al., 2004; Hurtado et al., 2004; Marco et al., 2007, 2009).

In sheep with BDV infection, pestivirus have been demonstrated to exist in almost every organ, indicating generalized infection. Continuous excretion has been mainly via nasal secretions and saliva, making the oronasal route the main vector of naturally-spreading pestivirus among adult individuals (Loken, 1995, Radostis 2000). In the diseased chamois of our study, the presence and isolation of the virus in sera, in all tissues and in all excretion routes concur with sheep studies, confirming that these chamois were in a viraemic state of the infection.

Presence of BDV in all the oral, nasal and rectal swabs, urine and foetal samples was succesfully detected by the RT-PCR. However, virus was not isolated in several of these samples. When virus was isolated the titres observed were lower than the titres observed in the main organs (Table 5.1.1). These differences might be explained by the fact that the virus content in these samples was low or below the detection level by virus isolation. Nevertheless, detection by RT-PCR of BDV in all of oral, nasal and rectal swabs, and urine samples supports the hypothesis of diseased chamois with a significative level of excretion of virus. This would explain the high horizontal transmission rate of the virus, the most probable cause of the latest epidemics observed, in certain cases accompanied by a high-speed epidemic wave and extremely high mortality (Marco et al., 2007, 2009).

Also, the detection of a BDV in the foetus of a viraemic female chamois indicates that vertical transmission of Border Disease Virus may occur in this species, as in pestivirus-infected domestic ruminants. Confirmation of PI status of a ruminant requires

identification of virus in two separate samples with a minimum of 21 days (Nettleton, 1998). Nevertheless, we cannot confirm that the chamois of our study were PI because none of the animals could be retested on a second occasion due to their agonic stage of illness. The high viraemia, shedding of BDV and the lack of antibodies in the chamois would indicate that they were PI animals. However, the epidemiological pattern of the mentioned outbreaks, with high-speed waves and very high mortality –as much as 85%- (Marco et al., 2007, 2009), seems to indicate fatal acute infection with a high horizontal transmission rate.

In sheep, acute infections of lambs and non-pregnant mature animals produce no obvious pathological changes. Only slight fever and mild leucopenia are associated with a short-lived viraemia, after which virus-neutralising antibodies appear in serum (Nettleton and Willoughby, 2007). Although high mortality in adult sheep is rare, highly pathogenic BDVs have been reported to cause high mortality in antibody-free populations (Chappuis et al., 1984). Thus, we presume that the same thing could have happened in the outbreaks associated with a BDV in the chamois populations of the Central Pyrenees.

Table 5.1.1. RT-PCR study and infectious BDV titres in sera, swabs and main organs of eight Southern chamois with Pestivirus infection. BDV titres are expressed as $\log_{10}$ $TCID_{50}$/ml of sera, swab and gr of tissue. ( ) non tested, (+) positive, (-) negative.

Cham Code	Sex	Age (years)	Test	Leuc.	Urine	Nasal Swab	Oral Swab	Rectal Swab	Spleen	Kidney	Brain	Lymph. Node	Skin	Thyroid Gland	Small intestine	Liver	Lung	Bone Marrow	Testicle	Foetus
1 10/06	Male	6	RT-PCR	+	+	+	+	+	+	+	+	+	+			+	+	+	+	
			Isolation	5.7	-	2.1	-	-	4.3	5.5	4.7	4.2	5.8			4.5	5.0	4.3	5.5	
2 30/06	Male	1	RT-PCR	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+		
			Isolation	6.4	-	2.1	-	-	3.2	3.1	4.0	3.8	-	4.8	-	4.1	3.8	5.5		
3 26/06	Male	5	RT-PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
			Isolation	>6.5	-	3.3	<1.5	-	3.6	3.5	4.5	5.2	3.5	4.2	-		4.4			
4 31/06	Male	6	RT-PCR	+	+	+	+	-	+	+	+	+	+	+	+	+	+			
			Isolation	>6.5	-	2.3	-	-	3.3	3.1	4.8	3.5	2.7	4.0	3.3	3.8	5.3			
5 48/06	Male	2	RT-PCR	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
			Isolation	5.5	-	-	-	-	3.9	4.2	5.0	3.3	4.5	4.3	3.3		5.3			
6 49/06	Male	4	RT-PCR	+	+	+	+	+	+	+	+	+	+		+	+	+			
			Isolation	3.0	-	-	<1.5	<1.5	4.7	4.2	5.2	4.7	4.3		-	3.9	5.0			
7 55/06	Male	8	RT-PCR	+	+	+	-	+	+	+	+	+	+	+	+					
			Isolation	>6.5	-	-	-	-	3.7	3.8	4.8	4.5	3.8	3.7	-					
8 22/06	Fem.	8	RT-PCR	+					+	+										+
			Isolation	4.6					5.1	5.1										-

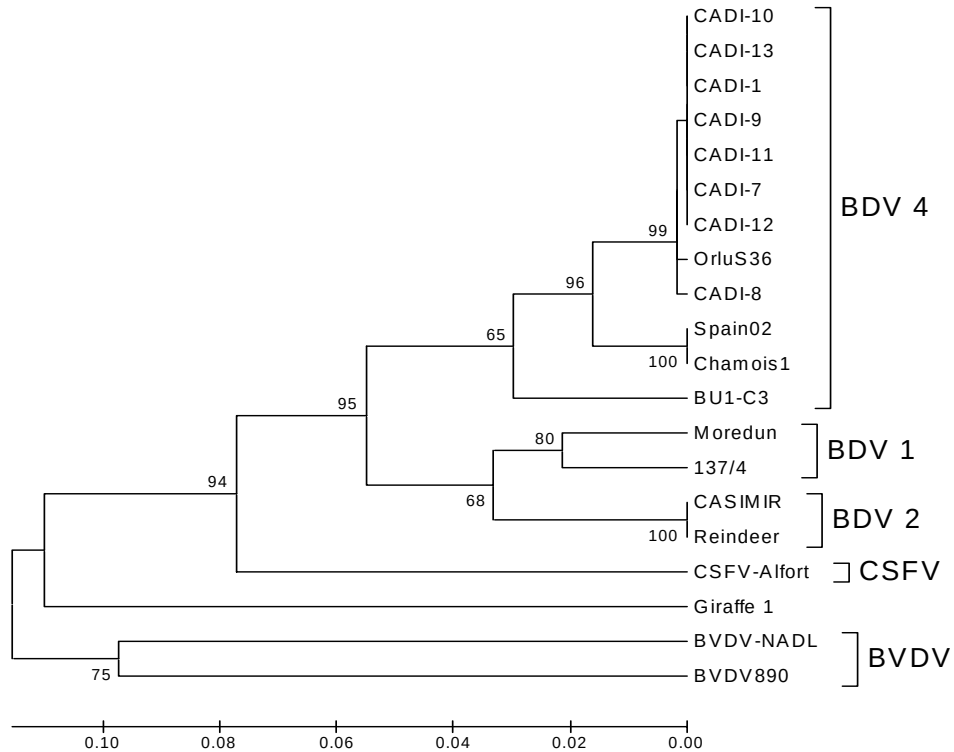

Figure 5.1.1. Unrooted neighbour-joining phylogenetic tree based on the 5'UTR sequence among pestiviruses. Chamois strains were enclosed in a differenciated group into BDV-4. The numbers on the branches indicate the bootstrasp values (in percent; 1000 replicates). Sequences of strains were taken from GenBank with the following accession numbers: Chamois-Spain02 (AY641529), Chamois-1 (AY738080), OrluS36 (DQ898293), BU1-C3 (DQ361068), CASIMIR (AB122085), Reindeer (AF144618), 137/4 (U65052), Moredun (U65023), Alfort (X87939), Giraffe1 (AF144617), NADL (M31182), 890 (U18059), CADI-1 (AM905918), CADI-7 (AM905924), CADI-8 (AM905925), CADI-9 (AM905926), CADI-10 (AM905927), CADI-11 (AM905928), CADI-12 (AM905929), CADI-13 (FN397676).

STUDY II

Experimental infection with chamois Border Disease Virus causes long-lasting viraemia and disease in Pyrenean chamois (*Rupicapra pyrenaica*)

J Gen Virol (under revision)

INTRODUCTION

Border Disease, caused by BDV, is characterized by reproductive manifestations in adult sheep and goats. The infection between days 50 and 60 of gestation causes births of immunotolerant PI lambs that excrete the virus during all life, being the most important source of BDV into ruminant breeds (Vilcek *et al*., 1994). Although severe outbreaks of BD in small ruminant populations are unusual, occasional severe epizooties have been described in sheep (Chappuis et al., 1984; Campbell et al., 1995; Thabti et al., 2005).

Traditionally, genus Pestivirus includes four species: *Bovine viral diarrhea virus* type 1 (BVDV-1) and type 2 (BVDV-2) affecting cattle, BDV that infects small ruminants, and *Classical swine fever virus* (CSFV) that affects swine (Becher *et al*., 1997). Interspecies transmission of BVDV and BDV among several domestic species has been reported in the last years, leading to the detection of antibodies against pestivirus in many other domestic and wild Artiodactyla species (Vilcek and Nettleton, 2006). Despite pestiviruses are widespread and present in many populations of free-living ruminants (Loken, 1995), BDV isolation has been only reported occasionally (Becher *et al*., 1997), and it has never been associated to disease previously to the outbreaks described in Pyrenean chamois. The outbreak of the disease associated with BDV infection (Hurtado et al., 2004; Arnal et al., 2004; Frölich et al., 2005) detected in 2001 in Pyrenees entailed a 42% reduction in the chamois population in the affected area (Marco et al., 2007). Since that date, further severe outbreaks of BDV infection in Southern chamois have been reported in the Catalan Pyrenees (Marco et al., 2009). To the authors' knowledge, the disease observed in chamois is the first epizootic episode associated to virus described in wild ruminants in Europe.

Clinical and post-mortem studies of diseased chamois revealed a previously unreported clinical presentation of infection with BDV, consisting of cachexia, alopecia with skin hyperpigmentation and several degrees of neurological alterations such as depression, weakness and difficulty in moving (Marco *et al*., 2007). Although several studies of affected chamois associated the observed disease with this virus, current knowledge

about the disease in chamois is still limited and little is known about its virulence and virus dissemination (STUDY-I). Unanswered questions such as pathogenesis and dynamics of the infection in chamois are essential for the understanding of the dynamics of this virus in wildlife ruminant populations and between them and livestock in Pyrenees. In this sense, interspecies ch-BDV infection would be a diagnostic challenge for the control of other pestiviruses in livestock (STUDY-III, STUDY-IV) and other wildlife species. Moreover, information about the behaviour of this virus in infected chamois, which is associated to fatal outbreaks of disease entailing severe ecological and economic consequences, is therefore of importance for the conservation and management of this species. The three objectives of the present work were to demonstrate if ch-BDV causes the illness and lesions described in naturally-infected chamois; to describe the pathogenesis of the disease, the organ lesions and the infection dynamics; and to asses if previously acquired humoral immunity against pestiviruses protects Pyrenean chamois from ch-BDV infection and disease occurrence.

MATERIALS AND METHODS

Animals

Ten female Pyrenean chamois, between 1 and 8 years of age were captured by drive-net (López-Olvera et al., 2009) in the Freser-Setcases National Hunting Reserve (Northern-Eastern Spain, 42º22'N, 2º09'E). All the animals were confirmed negative by Reverse Transcription – Polymerase Chain Reaction (RT-PCR) to the presence of pestivirus in sera ten days before and at the day of challenge. Chamois number (No.) 1, 2, 3, 4, 5, 8, 9 and 10 were antibody negative (Ab-neg) and chamois No. 6 and 7 were positive (Ab-pos) against BDV according to a Virus Neutralization Test (VNT) (OIE, 2004).

Innoculum

A non-citopathogenic (ncp) ch-BDV isolated from a diseased chamois found in the Pyrenees in 2005 was used as innoculum. Ch-BDV was cultured in single and double

passages in the pestivirus-free Sheep Fetal Thymoid (SFT-R) cell line (provided by the Friedrich-Loeffler-Institut, Island of Riems, Germany). The titre of virus was determined by end-point titration in SFT-R cell line obtaining a $1x10^7$ tissue cultured infectious dose 50 ($TCID_{50}$) ml^{-1} of virus. To asses phylogenetic homology between this virus and the BDV isolated in the same and previous outbreaks, amplified DNA from spleen homogenate and virus resulting from cellular culture was purified (Minelute Gel Extraction Kit, Quiagen) and sequenced. Purified amplicons were analyzed with the Big Dye Terminator v.3.1 Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence of the 5'UTR of the virus used in this experimental infection was deposited in the GenBank under the name CADI-6 and accession number AM905923. The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using an automatic root location. To test the reliability of the branches in the tree, a bootstrap analysis of 1000 replicates was performed by creating a series of bootstrap samples. The virus isolated from the chamois was located in the same BDV genotype (BDV-4) described in previously outbreaks in chamois populations (Marco et al., 2009).

Experimental design

After two weeks of adaptation to captive conditions the animals were housed in level-3 biosafety facilities of the experimental station of Centre de Recerca en Sanitat Animal (CReSA, Universitat Autònoma de Barcelona, Spain). In order to prevent the adverse effects of stress associated to captivity, enanthate perphenazine (1.5 mg/kg) was intramuscularly administered weekly. Also, all the chamois were treated with a single dose of 2.5 mg/kg of tulathromycin (Draxxin®, Pfizer Animal Health, Montreal, Canada) and a single oral dose of 2.5 mg/kg of toltrazuril 5% (Baycox®, Bayer Animal Health GmbH, Leverkusen, Germany) to prevent opportunistic bacterial and parasitic infections. Knowing the serological status of the chamois concerning BDV antibodies, animals were assigned into two groups. Animals No. 1 to 7 were challenged with $1x10^7$ $TCID_{50}$ of the ch-BDV CADI-6 AM905923. The total virus dose was thawed immediately before the inoculation and diluted in a total volume of 2 ml of Eagle's Minimal Essential Medium (EMEM). The inoculum was administered by a combination

of nasal catheter (0.5 ml in each nostril) and 1 ml was administered orally. Animals No. 8 to 10 (control group) were inoculated with 2 ml of sterile EMEM by the same routes. The animals were observed daily for clinical signs. Rectal temperature was monitored three times per week. They were weighted on day 0 post-inoculation (pi) and at the moment of the necropsy, which were programmed to be performed on day 34 pi. Prior to initiation of the study, criteria for euthanasia were established. Chamois displaying any or a combination of the following signs, e.g., complete anorexia, recumbence with inability to rise, or signs of severe dehydration, were euthanized. Animal care activities and study procedures were conducted in accordance with the guidelines of the Good Experimental Practices, under the approval of the Ethical and Animal Welfare Committee of the Universitat Autònoma of Barcelona.

Sample collection

To monitor viraemia and antibody response, blood samples were obtained from the jugular vein on days 0, 2, 5, 8, 11, 18, 25 and 34 pi. Blood samples were placed in commercial serum separator tubes and were centrifuged at 1200 g during 15 minutes to obtain serum. Sera were stored at -80ºC until processing. Nasal, oral and rectal swabs were collected on the same days of blood sampling and urine swabs at necropsy in 2 ml of sterile phosphate buffered saline (PBS) 6% supplemented with penicillin 10,000 U/mL, streptomycin 10,000 µg/mL, and nystatin 10,000 U/mL antibiotics. Samples were mixed vigorously and stored at -80ºC until analyzed. At necropsy, 1.0 g of each sampled tissue was homogenized in 9 ml of EMEM 6% supplemented with penicillin 10,000 U/mL, streptomycin 10,000 µ/mL, and nystatin antibiotics 10,000 U/mL, and stored at -80ºC.

In order to determine the white blood cell count (WBC), blood samples were placed in a commercial tube containing tripotassium ethylenediaminetetraacetic acid (EDTA) as anticoagulant and were counted by manual method. The differential leukocyte count was performed by identifying 200 leukocytes on blood smears stained with a commercial Diff-Quick-like stain (Química Clínica Aplicada, Amposta, Spain).

RT-PCR

Total viral RNA was extracted directly from 150 µl of sera, swabs, urine and tissue samples by a commercial kit (Macherey Nagel Nucleospin Viral RNA Isolation, Düren, Germany.) according to the manufacturer's procedure. Two microlitres of the purified RNA were used for RT-PCR with the Qiagen One-Step RT-PCR Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out at 50°C for 30 min. Thereafter, the RNA/DNA hybrid was denatured at 95°C for 15 min and the DNA amplified in 36 cycles (60 s 94°C, 60 s 56°C, 60 s 72°C), following a final extension time of 7 min at 72ºC. Thirty picomoles of primers 324 (5' ATGCCCWTAGTAGGACTAGCA 3'; W = A or T) and 326 (5' TCAACTCCATGTGCCATGTAC 3') (Vilcek et al, 1994) were used for the amplification reaction.

Virus isolation

In order to confirm that RNA detected by RT-PCR was due to the presence of viable replicating infectious ch-BDV CADI-6 and to quantify it, sera of viraemic animals were subjected to virus isolation and end-point titration on monolayer SFT-R cell line. The titration was performed by 10-fold dilutions in 96-microtiter plate cultures and replication was monitored by the immunoperoxidase monolayer assay (IPMA) (OIE, 2004) with a polyclonal home-made pestivirus antibody. Virus titration was performed according to Reed and Muench (1938). Furthermore, swab samples of challenged chamois (No. 1 to 7) from all days of collection were cultured in SFT-R cell line to confirm the presence of viable replicating virus.

Virus Neutralization Test

Sera from all animals and dates of collection were tested for the presence of antibodies against homologous BDV CADI-6 with VNT, performed under procedure described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2004). Briefly, serum samples were diluted 1:10 with sterile EMEM, heat-inactivated (56ºC, 30 min) and 10-fold diluted in 96-well plates (50 µl/well). After addition a volume of 50 µl

containing 100 $TCID_{50}$ of the homologous BDV, the plates were incubated at 37ºC during 1 hour. Finally, $2{,}8x10^4$ Madin–Darby bovine kidney cells (MDBK) (100 µl) were added per well. Replication was monitored by the IPMA method as described above. Titres were expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures, calculated according to the method of Reed and Muench (1938).

Pathologic examination and immunohistochemistry (IHC)

Necropsies and tissue sampling were performed according to a standard protocol. On day 34 pi, all chamois were euthanized with a lethal injection of barbiturate. At necropsy, tissue samples collected for histopathologic examination included brain, tonsils, four lymph nodes (superficial cervical, superficial inguinal, mediastinal and mesenteric), spleen, digestive tract (esophagus, reticulum, rumen, omasum, abomasum, duodenum, jejunum, ileum and colon), pancreas, liver, respiratory tract (nasal turbinates, trachea and lung), thyroid gland, adrenal gland, kidney, ovaries and uterus, heart, skin (ear and eyelid) and bone marrow from proximal femur and sternum. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin according to standard procedures. The bone marrow sample from the sternum was decalcified before embedding in paraffin. Four sections of the brain were evaluated: (1) telencephalon (basal ganglia), (2) telencephalon/diencephalon (hipoccampus, thalamus, hypothalamus), (3) mesencephalon (superior colliculus), and (4) metencephalon (cerebellum and pons). Lesions were classified into four categories: (0) no lesions; (1) mild gliosis, occasional glial nodules and mild histiocytic perivascular infiltrates (2) diffuse gliosis, frequent glial nodules, non-suppurative lymphohistiocytic meningoencephalitis; (3) diffuse gliosis, frequent glial nodules, non-suppurative lymphohistiocytic meningoencephalitis, neuronal degeneration and necrosis, and spongiosis. A histopathological evaluation of the bone marrow included a qualitative and subjective evaluation of the cellularity and the myeloid:erythroid series ratio (decreased, normal, increased) and a quantitative evaluation of the number of megakaryocytes per high-power field (400x). Ten different high-power field areas were evaluated in each chamois.

To demonstrate the presence of pestiviral antigen, immunohistochemical staining was done using the monoclonal antibody 15C5 (IDEXX Laboratories, Inc., Westbrook, ME, USA) against the conserved envelope glycoprotein gp48 of pestiviruses. This antibody has been used previously in cases of ovine abortion (Thür et al., 1997) and naturally infected chamois with ch-BDV (Arnal et al., 2004). Briefly, the immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections with the avidin-biotin-peroxidase complex technique as previously described (Haines et al., 1992), modified with the use of a tyramide signal amplification (TSA) system (Perkin Elmer) according to the manufacturer's directions to optimize the detection of the BDV antigen in the tissues. Sections were counterstained with haematoxylin. An intestinal sample from a case of BVD was used as positive control. The same sections with no primary antibody added were used as negative controls as well as samples from one of the control animals.

Statistical analysis

To explore whether body temperature and WBC counts were influenced by pestivirus infection, a set of mixed models in which both dependent variables were explained by the interaction between the treatment group (as fixed effect: "viraemic" vs. "non-viraemic") and the time in days (as fixed nominal factor) were fitted. Since chamois were sampled over the study period, we considered each individual was as random factor for controlling pseudo-replication (Zuur et al, 2009). The relationship between WBC and both date of sampling and treatment group was non-lineal (Skewness = 1.38, kurtosis = 4.55, Shapiro – Wilk normality test = 0.86, p-value < 0.01, residual pattern from a lineal mixed model) and therefore an additive modeling based on the cubic regression splines method (mgcv 1. 6 - 1 version package, see Wood, 2008) was used. Finally, the consequences of pestivirus infection on the body weight comparing the weight variation (e.g., (weight at day 0 – weight at necropsy)*100 / weight at day 0) between viraemic and non-viraemic chamois were explored. Since individual weight variation were normally distributed (Shapiro – Wilk normality test = 0.96, 0 = 0.86)

comparisons were made using an unpaired t-student test. All statistical analyses were performed using R version 2.12.0. (R Development Core Team, 2010).

RESULTS

Clinical signs

Clinical signs which could unequivocally be attributed to the virus were not observed during the adaptation or post-challenge periods. However, chamois No. 2 and 4 died acutely on day 18 pi and 20 pi, respectively, presenting haemorragic diarrhoea with pasty dark faeces on perineal region and tail. In addition, chamois No. 3 showed mild coughing and was found dead on day 24 pi.

Mean body temperatures in viraemic chamois were higher than in non-viraemic chamois during the experiment, ($F = 3.93$, edf = 2.6, p-value = 0.015, 10% of explained deviance), especially from day 8 pi when the mean body temperature of the viraemic chamois increased in 0.77 degrees with respect to their counterparts (Mean = 39.79ºC, SE = 0.18, Min = 38.65, Max = 41.84 for viraemic vs., Mean = 39.02ºC, SE = 0.17, Min = 37.86, Max = 41.12 for non-viraemic chamois). All chamois lost between 13 to 25% of body weight during the adaptation period to captivity. From day 0 of the experiment to the day of necropsy, viraemic chamois continue losing an additional 4% on average, while non-viraemic chamois recovered 15% of their initial weight ($t = 6.96$, p-value < 0.001).

Virological examination

BDV was detected by RT-PCR in sera of chamois Nos. 1 to 5 from day 2 pi until they died or were euthanized. BDV was isolated and titrated from sera samples from all these chamois from day 2 pi until the last day of sampling, showing an increase in the virus titres until day 11 pi with a maximum titre of $10^{6.8}$ $TCID_{50}$/ml (Table 5.2.1). From day 18 pi until the death/euthanasia of the animals, titres of virus decreased in one animal

(No. 1), disappeared in another (No. 3) and remained with similar titres in the other three (No. 2, 4, 5) viraemic chamois. The two Ab-pos challenged chamois (Nos. 6 and 7) and all control animals (Nos. 8 to 10) were negative to the presence of BDV in sera samples at all days of sampling by RT-PCR and virus isolation.

BDV RNA was detected by RT-PCR in oral, nasal and rectal swabs in chamois No. 1 to 5 from day 2 pi until the end of the study. Animals No. 6 and 7 were negative with the exception of nasal swabs on days 25 pi for chamois No. 6 and 34 pi for animals No. 6 and 7 (Table 5.2.2). Swab samples were negative to RT-PCR in all control chamois.

All tissues collected at necropsy and urine from viraemic animals (Nos. 1 to 5) were positive to the presence of BDV by RT-PCR. On the contrary, chamois Nos. 6, 7 (challenged animals), 8, 9 and 10 (control animals) were negative.

Antibody response

Two of the viraemic chamois (Nos. 2 and 3) had low neutralizing antibody titres against homologous BDV CADI-6 on day 11 pi. On day 18 pi, all viraemic chamois (Nos. 1 to 5) had seroconverted and remained antibody-positive until the end of the study (Table 5.2.1). Challenged Ab-pos chamois No. 6 and 7 had neutralizing antibodies against homologous BDV during all the study but an increase of neutralizing antibodies against this homologous BDV was not observed. The three Ab-neg control animals tested negative to VNT during all the study.

Haematological analysis

WBC decreased in viraemic chamois ($F = 5.68$, edf = 2.5, p-value = 0.003 for the interaction between "time" and "treatment group"), being 40% of the observed variability in the WBC explained by the effect of BDV ($F = 9.59$, edf = 2.8, p-value = 0.00018, R^2 = 40 %). Severe alterations in the neutrophil differential count were observed in the viraemic chamois. Chamois No. 4 and 1 had a complete absence of neutrophils on blood smears on days 18 and 34 pi, respectively. Chamois No.5 had a

transient neutropenia detected on days 18 (477/µL) and 25 (722/µL) pi and recovered by day 34 pi (4495/µL). No left shift was observed at any time.

Evaluation on platelet numbers was not feasible because of moderate to intense aggregation. However, a significant absence of platelets was observed in chamois No. 4 on day 18 pi, and very low numbers of platelets were seen in chamois No.1 at the end of the experiment (day 34) and in No.2 on day 11 pi.

Gross pathological evaluation

At necropsy, chamois No. 2 and 4 had lesions of haemorrhagic diathesis affecting mainly the gastrointestinal (GI) tract with fluid to thick pasty haemorrhagic contents from the duodenum to the rectum (Figure 5.2.1). Haemorrhages were mainly seen in subcutaneous tissue, pericapsular tissue of lymph nodes, serosal surface of GI tract, mucosal surface of reticulum and pyloric region of abomasum, epicardium and endocardium, especially pronounced in left ventricle, and mucosa of the urinary bladder. In Chamois No.4, the wall of the large intestine was diffusely haemorrhagic and there were two small focal areas of mucosal erosion in the rumen pillar, and also 5 to 10 pinpoint yellow foci in the liver. A small amount of clear yellow fluid was noted in the peritoneal cavity and pericardial sac. In order to rule out an haemorrhagic enterotoxaemia, an ELISA test for the detection of α−, β− and ε− toxins and a structural protein of *Clostridium perfringens* (Bio-K 095, Bio-X Diagnostics, Jemelle, Belgium) was performed in sera samples from these two animals. Both chamois were negative.

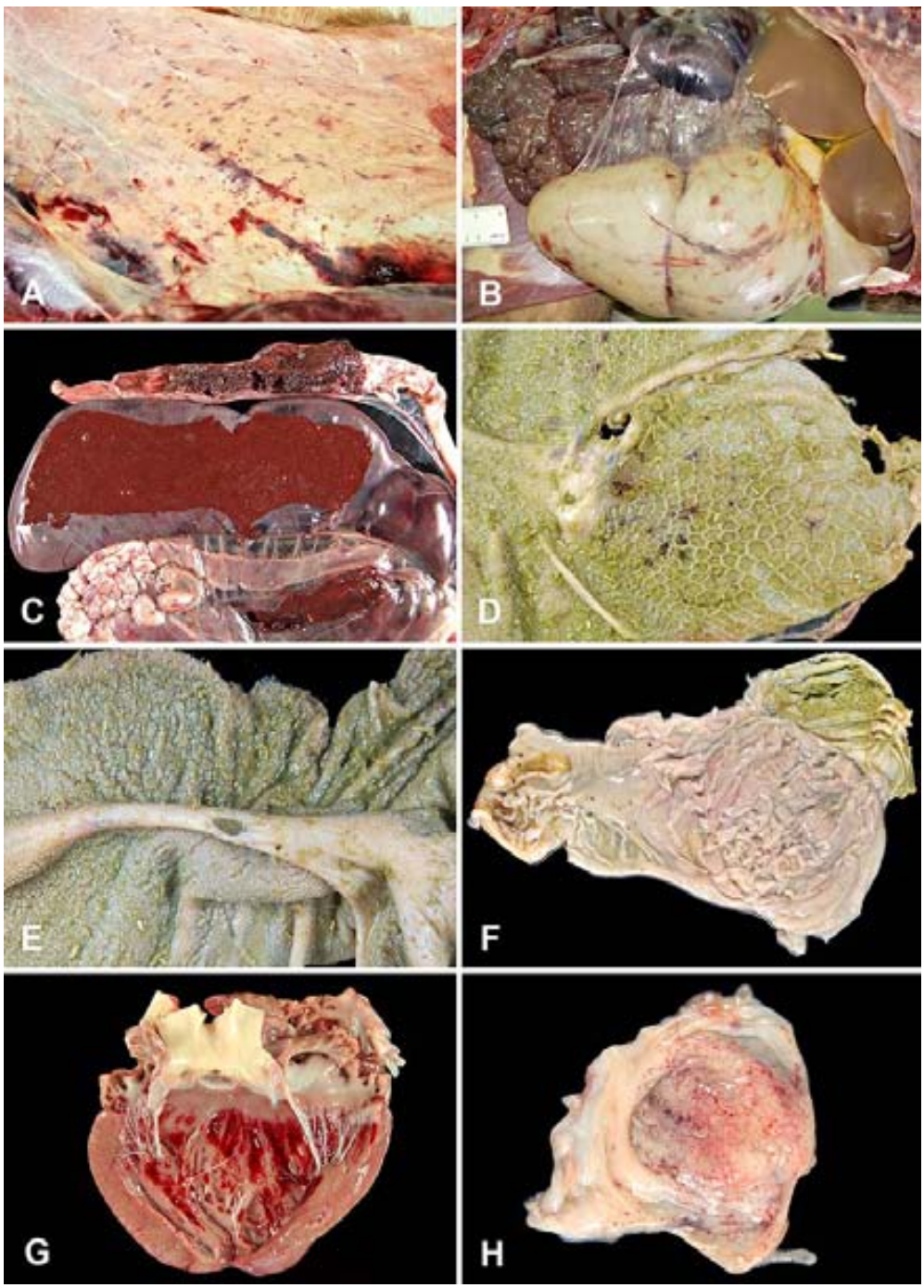

Figure 5.2.1. Border Disease Virus Experimental Infection in Chamois (Case No. 4). Died of haemorrhagic diathesis at 20 days post inoculation with A. Multifocal subcutaneous haemorrhages, B. Serosal and transmural haemorrhages in the gastrointestinal tract, C. Haemorrhagic intestinal contents, D. Mucosal haemorrhages in reticulum, E. Ulcers in the rumen pillars, F. Mucosal haemorrhages in abomasum, G. Endocardium, and H. Urinary bladder.

Chamois No. 3 died at day 24 pi with a severe bacterial fibrinous bronchopneumonia. In chamois No.1 and 5, only a mild lymphadenomegaly was observed at necropsy on day 34 pi (Figure 5.2.2).

In the rest of chamois no significant gross lesions were observed. All chamois (viraemic and non-viraemic) had mild nodular verminous pneumonia in the caudal lung lobes and different degrees of old fibrous adhesions in cranio-ventral lung lobes.

Histopathological and immunohistochemical evaluation

Microscopic lesions and antigenic detection of the virus by immunohistochemical staining were only seen in viraemic chamois. The main and most consistent lesions in viraemic chamois were seen in the brain, lymphoid tissues and bone marrow. Positive immunohistochemical staining was observed in all tissues. It was brown to dark brown and coarsely to finely granular within the cytoplasma of cells. The presence of viral antigen was not necessarily related with the presence of lesions.

All five viraemic chamois had a non-suppurative meningoencephalitis with gliosis (Figure 5.2.3). Lesions were almost generalized but the severity was higher in the telencephalon, diencephalon and mesencephalon than in the metencephalon, being the latest area unaffected or only mildly affected. Lesions in chamois No. 2 were mild (grade 1) with only two small microglial aggregates in the caudate nucleus and very few histiocytic periarteriolar cuffs in telencephalon. Brain lesions in the other four viraemic chamois were more prominent and were graded as 2 in chamois No. 1, 4 and 5, and as 3 in chamois No. 3. Antigenic detection of the virus was most prominent in neuroglial cells, perivascular histiocytes, epithelial cells of the choroid plexus and ependymal cells. In neurons was seen only occasionally and only in chamois No. 1, 3, and 5.

Changes in lymphoid tissue, both lymph nodes and spleen, were seen in all viraemic chamois. These alterations were characterized by moderate lymphoid depletion with loss of lymphoid follicles and decreased lymphoid density in both follicular and

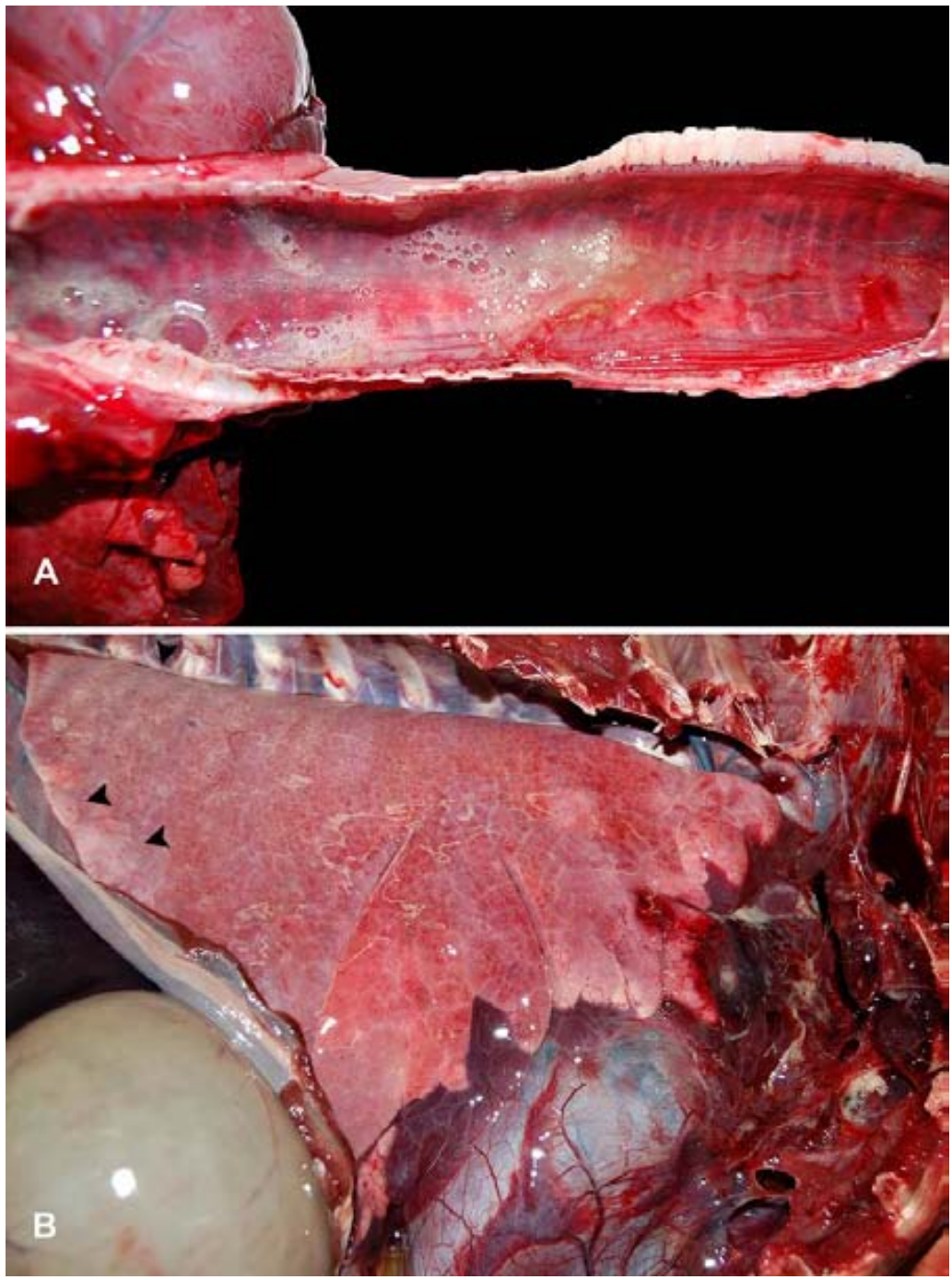

Figure 5.2.2. Border Disease Virus Experimental Infection in Chamois (Case No.3). Died with a concomitant bacterial broncopneumonia at 24 days post inoculation. A. Abundant mucus in trachea. B. Fibrinosuppurative cranioventral bronchopneumonia. The ventral portions of the cranial and middle lobes are dark red with fibrin over the pleural surface. The dorsal portion of the caudal lung is relatively normal but it has nodular grey areas of verminous pneumonia (arrowheads) and fibrin tags over the pleura.

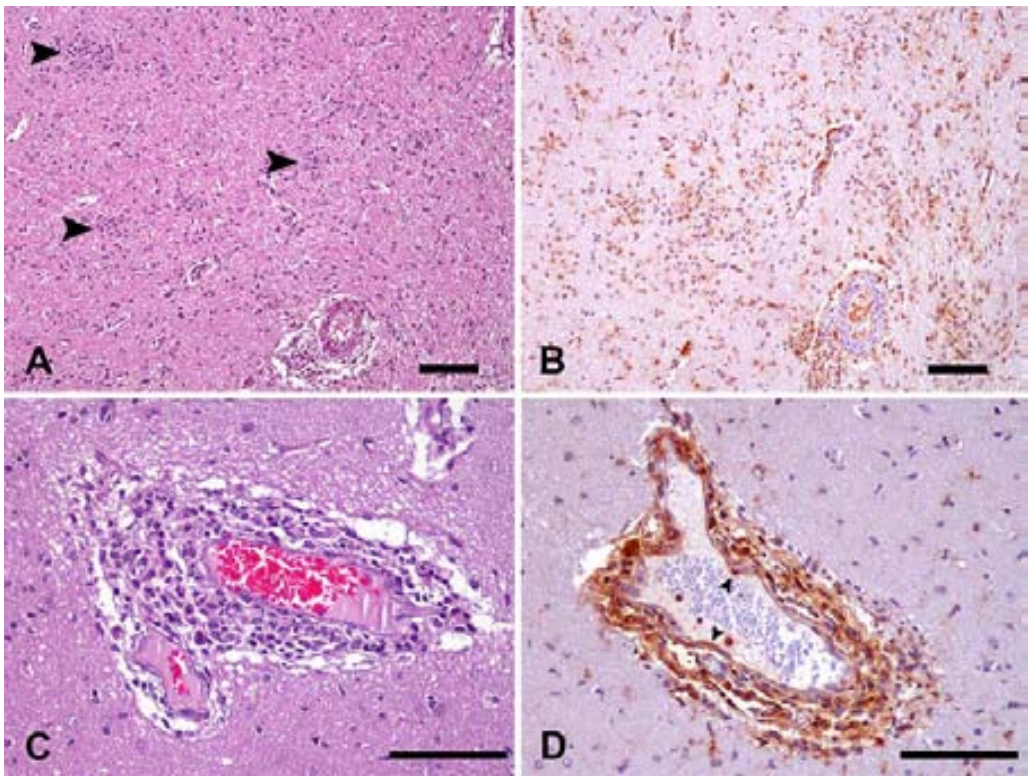

Figure 5.2.3. Histopathologic lesions and associated detection of *Pestivirus* antigen in brain of chamois experimentally infected with Border Disease Virus (Chamois No. 1, 34 days post inoculation). A. Gliosis and glial nodules (arrowheads). Lesions such as this were sparsely scattered throughout grey and white matter (HE). B. Same area as A. *Pestivirus* antigen (brown reaction) is detected in the cytoplasm of glial cells and perivascular inflammatory mononuclear cells (IHQ). C. Thick perivascular inflammatory cuffing with mainly histiocytic and lymphocytic cells (HE). D. *Pestivirus* antigen (brown reaction) is detected in the cytoplasm of mononuclear cells, scattered endothelial cells (arrowhead) and intravascular leukocytes (IHQ). Bar = 100µm.

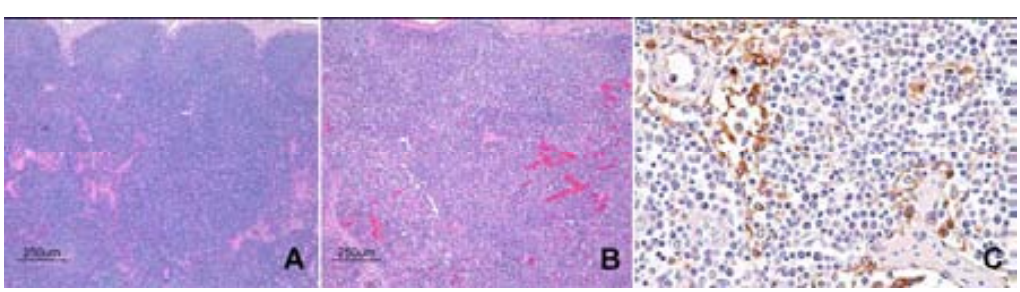

Figure 5.2.4. Histopathologic lesions and associated detection of *Pestivirus* antigen in lymph node of chamois experimentally infected with Border Disease Virus. (A) Control chamois, (B) viraemic chamois, (C) IHC of viraemic chamois lymph node.

interfollicular areas and paracortical areas or periarteriolar sheaths (Figure 5.2.4). Differences were seen between those chamois that died earlier in the study and those that were euthanized at the end. In the formers (chamois No. 2, 3 and 4), the follicular architecture was still evident but there were numerous apoptotic bodies and tingible body macrophages, especially in medullary sinusoids. In chamois No. 1 and 5, many follicles were lost but apoptotic bodies and tingible body macrophages were not present. Within an animal, changes in the different lymphoid tissues were generalized (with no obvious differences of severity among them). In chamois No. 4, pericapsular acute haemorrhages and intrasinusoidal erythrocytes, as a result of draining a region of hemorrhage, were prominent. Positive immunostaining was present mainly in fibroblastic reticular cells, sinus histiocytes and follicular dendritic cells. Staining in a few lymphocytes was faint.

Cellularity of the bone marrow was normal to increased in viraemic chamois when compared to that in non-viraemic chamois. However, the myeloid:erythroid ratio was altered in viraemic chamois with a decrease in the myeloid components. Especially, in chamois No. 1, 3 and 4, there were essentially only myeloblasts (early precursors), while metamyelocytes, band neutrophils or mature granulocytes were absent or scarce. The image was consistent with a depletion of the bone marrow granulocyte reserves and a maturation arrest. These findings coincided with a severe neutropenia in peripheral blood in these chamois. Chamois No. 5, who had had a previous transitory neutropenia, showed a recovery or hyperplasia in the myeloid component in the bone marrow at the time of necropsy. A normal maturation process (cells in different stages of maturation) was seen in the erythroid line in both viraemic and non-viraemic chamois (Figure 5.2.5).

In viraemic chamois megakaryocytes (MKs) showed both changes in number and morphology. The mean number of MKs per high power field (HPF) (x400) in non-viraemic chamois was 3.9 (range from 3.1 to 5.1). The number of MKs per HPF in viraemic chamois was within this range in chamois No. 2 (3.9) and No. 5 (3.3) and markedly decreased in No. 1, 3, and 4, with 2.3, 1.0 and 0.3, respectively. Tight clusters of MKs, suggesting a megakaryocytic hyperplasia, were only seen in chamois No. 2.

Morphological changes in MKs were present in all viraemic chamois and these included an increase of cells with hypereosinophilic cytoplasm, an increase of cells with

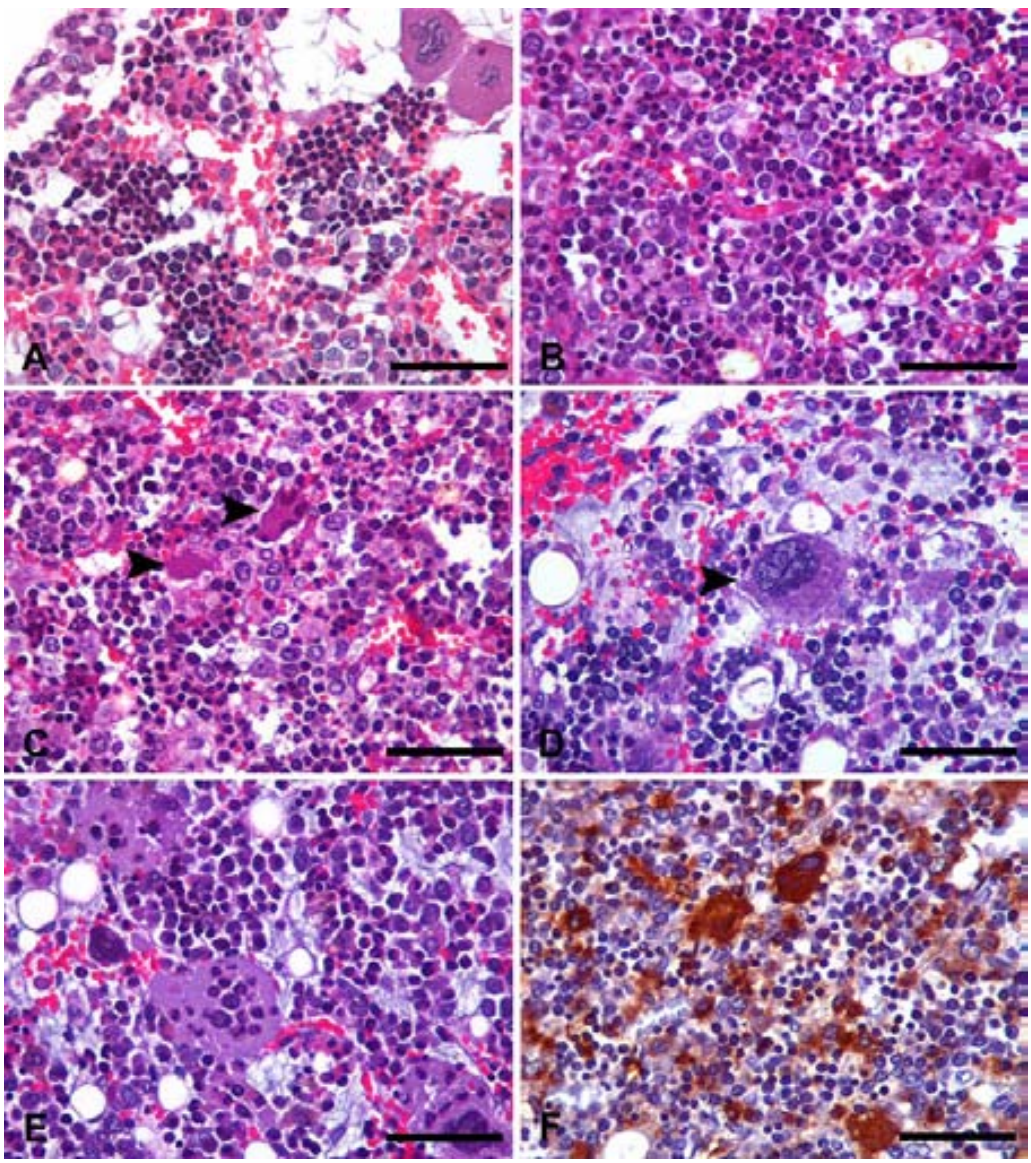

Figure. 5.2.5. Histopathologic lesions and associated detection of *Pestivirus* antigen in bone marrow of chamois experimentally infected with Border Disease Virus. A. Bone marrow from a control chamois. B. Bone marrow from a viraemic chamois (No.1), 34 days post inoculation (dpi) with increased overall cellularity, depletion of granulocyte reserves and a maturation arrest. C (chamois No.1, 34 dpi) and D (chamois No.2, 18 dpi). Morphological changes of Megakaryocytes (MKs) in viraemic chamois: hypereosinophilic cytoplasm and hyperchromatic nuclei (arrowheads in C) and atypical nucleated MK, cloud-nuclei cell, (arrowhead in D). E. Emperipolesis (viraemic chamois No. 5, 34 dpi). F. Detection of *Pestivirus* antigen in the cytoplasm of hematopoietic cells including MKs (viraemic chamois No. 1, 34 dpi). Bar= 50 µm.

apoptotic nuclei (hyperchromatic or condensed and lobulated), and atypical nucleated MKs like cloud-nuclei cells which are large megakaryocytes with hypolobulated nuclei. Megakaryocytic emperipolesis (intact neutrophils in the cytoplasm of megakaryocytic cells) was seen only in viraemic chamois, and it was especially prominent in chamois No. 5 and to a lesser extend in chamois No. 2 (coinciding with the fact that these two chamois where the only ones of the viraemic chamois with neutrophils in the bone marrow). Viral antigen in bone marrow was detected in all viraemic chamois mainly in stromal cells but also in hematopoietic cells including MKs.

Most severe lesions were seen in those animals that died with haemorrhagic diathesis (Chamois No. 2 and 4). In these animals multifocal extravasation of red blood cells, especially in the serosa, were seen throughout the intestinal tract. Focal areas of transmural haemorrhages were seen in the large intestine of animal No. 4. In chamois No. 2, multifocal mild superficial erosion, occasional crypt loss, a moderate increase of leukocytes in the lamina propria and oedema were also seen in the jejunum. Mild mononuclear, lymphocytic and histiocytic, interstitial inflammatory infiltrates were seen in the lamina propria of reticulum-rumen of 3 out of 5 of the viraemic chamois.

Positive immunohistochemical staining was multifocal to diffuse in the stratified squamous cell epithelium of the upper gastrointestinal tract (oesophagus to omasum) but most intense in the basal cell layer. In the abomasum, it was present multifocally in the glands. In the intestines it was present faintly and multifocally in the apical cytoplasm of epithelial cells, and in Brunner's glands of the duodenum. Throughout the entire GI tract positive staining was constant in inflammatory cells (mainly histiocytic cells) of the lamina propria, endothelial cells of small and large vessels, both arteries and veins, stromal fibroblastic cells, and multifocally in smooth muscle cells, and occasionally in glial cells of myenteric plexus.

Hepatic lesions were only seen in chamois No. 4 and were mild, with only occasional foci of acute hepatocellular necrosis with bacteria seen in one of those foci. In all viraemic chamois staining was present in Kupffer cells and sinusoidal endothelial cells. Inflammatory cells in portal areas and bile epithelium were also occasionally positive.

Chamois No.3 had a severe active bacterial fibrino-suppurative bronchopneumonia. The rest of chamois (viraemic and non-viraemic) had small focal areas of chronic lesions of bronchopneumonia in cranio-ventral lung lobes characterized by bronchiectasia (dilated bronchi filled with mucus) and peribronchial atelectasia, and a caudal nodular histiocytic or granulomatous verminous pneumonia, but no differences in severity were seen among them. Antigenic positive staining was seen throughout the respiratory tract (nasal turbinates, trachea and lungs). In the pseudostratified ciliated epithelium BDV antigen was seen preferentially in the basal cell layer. Positive cells were also present in ductular epithelial cells of the glands, mioepithelial cells and inflammatory cells in the lamina propria, alveolar macrophages, both in alveoli and wall of the alveoli, and possibly pneumocytes, and mesothelial cells of the pleura.

In the kidney, mild chronic multifocal interstitial and perivascular lymphoplasmacytic and histiocytic infiltrates were present in 4 out of 5 of the viraemic chamois (No. 2, 3, 4 and 5) and only in one of the non-viraemic chamois (No.8). Immunohistochemical staining was present in cells of the juxtaglomerular apparatus, multifocal in mesangial cells, occasional in tubules of both cortical and medullar areas, inflammatory mononuclear cells, multifocal to diffuse in the transitional epithelium of the pelvis and stromal cells. Positive inflammatory cells present in interstitial areas were not associated to positive tubular cells.

In the urinary bladder, a marked increase of mucous cells and a mild to moderate mononuclear inflammatory infiltrate was seen in chamois No. 1, 4, 5 (2 and 3 were not sampled) and 8. Immunohistochemical staining was present in the transitional epithelium, mainly in the basal cell layer, stromal fibroblastic cells, inflammatory cells and endothelium of vessels, and multifocally in myocytes of the muscle wall.

All viraemic chamois were females. Changes present in genital tract were considered mild and only seen in chamois No. 2 where a small focal area of necrosis was seen in the granulose cell layer in a developing follicle, and in chamois No. 4 with perifollicular haemorrhages. Viral antigen in the ovary was detected in the germinal epithelium or epithelial surface that covers the cortex, stromal cells of the dense connective tissue, the

theca layer of developing follicles and in some, in the granulosa cell layer too, especially in those atretic follicles. In the uterus, antigen was detected diffusely in the epithelium and stromal cells of the endometrium, and multifocally in the myometrium. In both, ovary and uterus, staining was also present in the endothelium of vessels.

Both viraemic and non-viraemic chamois had irregular areas of slight hypotrichosis in the flanks and lateral aspect of thighs but there were not obvious differences in severity between the two groups. Microscopically, all animals had mononuclear perivascular inflammatory infiltrates in the superficial dermis, which were mild or minimum in non-viraemic chamois and moderate in viraemic ones. In chamois No. 2 this inflammatory infiltrate was more prominent; there were also small dermal haemorrhages and occasional foci of epithelial necrosis in follicular bulbs. Viral antigen was detected multifocally to diffusely in the surface and follicular epithelium, sebaceous glands (mainly in basal cells), only in occasional apocrine gland cells, fibrocytes in the dermal connective tissue, vessels, and inflammatory cells present.

In the heart, small focal areas of myocyte replacement with mature fibrous tissue were seen in chamois No.1, 3, 5 and 8. Acute subendocardial and myocardial hemorrhages were seen in chamois No. 2 and 4. Positive staining was seen in stromal or fibroblastic cells, and histiocytic inflammatory cells present in those areas of fibrosis seen. Viral antigen was also present in the endothelial cells of some arteries and arterioles and multifocally in the endocardium. In all tissues, viral antigen staining was seen in some endothelial cells, vascular smooth muscle cells, and intravascular or circulating monocytes.

In sections from the thyroid gland, adrenal gland and pancreas there were no lesions in any of the viraemic chamois. Positive antigenic staining was seen multifocally in thyroid follicular epithelial cells, multifocally to almost diffuse in the fascicular layer of the adrenal gland, and in the islet cells, and occasionally in individual or small groups of exocrine cells in the pancreas.

DISCUSSION

This study reproduces experimentally the infection of ch-BDV in chamois, and confirms that this virus is the aetiological agent of the disease described in Pyrenean chamois populations since 2001 (Marco et al., 2007, 2009).

Virological and clinico-pathological data of the inoculated chamois of the present study were unexpected when compared to reported pestivirus infections in domestic and wild ruminants. Post-natal BDV infections in immunocompetent animals are normally subclinical and viraemia is transient and difficult to detect, usually seen between days 4 and 11 pi (Nettleton *et al*., 1998). In accordance with this, experimental infections in pigs and lambs with the same ch-BDV followed this pattern (STUDY-III, STUDY-IV). However, in the experimental infection of chamois the virus appeared in serum on day 2 pi and persisted until the animals died or were euthanized at day 34 pi. The high titres of viable replicating infectious virus detected in sera and the continuous shed of virus by nasal, oral, faecal and urinary routes agree with previously reported naturally-infected Pyrenean chamois (STUDY-I). This virus excretion in experimentally infected chamois may facilitate a high horizontal virus transmission rate which explains the rapid spread of the infection during the different outbreaks reported to date along the Central and Eastern Pyrenees. This is supported by the fact that chamois No. 6 and 7 (seropositive challenged chamois that did not become viraemic) were positive by RT-PCR in nasal swabs at days 18 and 34 pi, most probably by transmission from viraemic chamois. Noteworthy, the absence of viraemia in these two Ab-pos challenged chamois would confirm that previously acquired humoral immunity is protective against ch-BDV systemic dissemination.

Viral RNA was widely distributed in all organs of the viraemic chamois as reported in naturally-infected ones (STUDY-I). In experimentally acutely infected sheep with the same ch-BDV, a transient and mild viraemia was described and presence of viral RNA was only detected at day 7 pi in mesenteric, mediastinal and prescapular lymph nodes, tonsil, and brain (STUDY-III). Therefore, the high capacity of dissemination and replication of this virus in almost all organs of acutely infected chamois is

unprecedented. Long-lasting viraemia, continuous shedding of the virus via oral, nasal and rectal routes, and generalized viral tissue distribution observed in the chamois of the current study have only been described in PI animals (Vilcek *et al*., 1994, Nettleton et al., 1998). While PI animals are infected *in utero* and do not develop antibodies, chamois of the present study were post-natally infected and developed neutralizing antibodies.

Antibody response in the experimentally infected chamois was similar to that of acute BDV infection (Nettleton et al. 1998) and to that of experimental infection with ch-BDV in sheep and pig (STUDY-III, STUDY-IV). However, viral elimination was not achieved in most animals, with the exception of chamois No. 2 that neutralized the virus in blood by day 34 pi, although RNA and antigen detection by RT-PCR and IHC, respectively, was confirmed at the time of euthanasia. It is interesting to note that in this animal, antigen distribution was not diffuse and more multifocal than in any other viraemic chamois and for example it was not present in the stratified squamous epithelium of the upper GI tract.

Clinical signs and severe disease following acute BDV infection in immunocompetent animals are very rare. An outbreak of severe disease characterized by a haemorrhagic syndrome and leukopenic enterocolitis with high mortality has been described in sheep and growing lambs (Chappuis *et al*., 1984). It was associated with a new BDV strain (Aveyron) that produced profound leukopenia and death in 50% of 3 to 5 month-old lambs. However, further experimental infection in lambs with this highly pathogenic strain was not able to reproduce the disease (Thabti et al, 2002). In chamois, severe clinical signs have been observed in naturally infected animals, such as neurological signs, chronic wasting and alopecia (Marco et al. 2007). In our experimental infection, none of the viraemic chamois showed neurological signs. However, it was difficult to assess them because the animals were under the effects of the perphenazine in captive conditions. Nevertheless, the lesions observed in brain were similar to those described in naturally infected chamois (Marco et al., 2007), suggesting that these symptoms would have occurred if they were not tranquilized and the experiment would have lasted for long. Non-suppurative meningoencephalitis and focal and diffuse gliosis was similar

in both, naturally and experimentally infected chamois, but spongiosis, which is consistently seen in naturally infected animals was seen only mildly in one of the chamois of the present study. This result may indicate that this lesion could be the result of a longer, persistent infection, an hypothesis that needs to be further explored. The neurotropism of pestiviruses in congenital infections is well-documented (Maxie and Youssef, 2007; Bielefeldt-Ohmann et al., 2008) but it has never been described in post-natal infections with BDV and only suspected in one case of BVDV infection in a Heifer (Blas-Machado et al., 2004). Brain lesions in chamois resemble those produced by classical swine fever virus (Gómez-Villamandos et al., 2006).

Cachexia and alopecia was not observed in viraemic chamois, but weight loss was significantly different between viraemic and non-viraemic animals. In the skin, mild to moderate mononuclear perivascular to interstitial inflammatory infiltrate in the dermis was observed, as described in naturally infected animals, but follicular atrophy and increase telogenization were not prominent. Occasional follicular epithelial necrosis was seen only in one of the chamois. These findings suggest that those atrophic lesions would appear in more persistent infections, delaying the growth of the new coat, but essentially the pathogenesis remains unclear and would deserve further investigation.

Secondary infections are common findings in naturally infected chamois with BDV (Marco et al., 2007, Fernández-Sirera et al., 2011), as well as in other pestiviral infections in domestic animals, and they have been related to the immunosupression caused by the virus (Brackenbury et al., 2003). In the viraemic chamois of this study, leukopenia with neutropenia and depletion in lymphoid tissue suggest that immunossupression and predisposition to secondary infections are very likely. Neutropenia was related to a depletion of the granulocyte reserves and to a maturation arrest in the bone marrow. The depletion of the bone marrow granulocyte reserves can be caused by sequestration of neutrophils in spleen or suppurative inflammation. This situation could have happened in chamois No.3, which had a severe cranio-ventral bronchopneumonia; however, no evidence of significant suppurative inflammation was detected macroscopically or histologically in any of the other viraemic chamois. The mechanism induced by ch-BDV to arrest the maturation in this cell line was not evident.

Neutropenia in other pestiviral infections has been specifically attributed to suppression of granulopoiesis or decreased proliferative capacity of bone marrow progenitor cells in highly virulent BVDV-2 (Keller et al., 2006) and to apoptosis of the bone marrow neutrophil-lineage cells in CSF (Summerfield et al., 2001). Neutropenia is transient in most viral infections, and this was the case in chamois No. 5, in which the neutrophil count recovered by day 34 pi. In fact, neutropenia is not a common in naturally infected chamois,while lymphopenia is most consistently found (Fernández-Sirera et al., 2011). Lymphoid follicles were depleted and almost no discernible in viraemic chamois, but this was not reflected in the lymphocyte peripheral counts.

Severe hemorrhagic diathesis was seen in the viraemic chamois that died of acute diarrhoea. In another report, BDV was associated with a hemorrhagic syndrome in acutely infected adult animals (Chappuis et al, 1984), but the mechanism was not elucidated. This condition has been reported in BVDV-2 and CSFV infections and may be associated to thrombocytopenia, which could be multifactorial and a consequence of platelet activation and aggregation and defective production in bone marrow (Walz et al., 1999 and 2001; Bautista et al., 2002; Gómez-Villamandos et al., 2003). In the present study, thrombocytopenia was also suspected as the most likely cause of the haemorrhages. Although platelet count could not be precisely evaluated, the absence of platelet detection in these two cases of haemorrhagic diarrhoea was considered very indicative of a thrombocytopenic status. In addition, significant alterations in the bone marrow were observed. Chamois No. 4 had the lowest count of megakaryocytes per HPF, which is similar to BVDV and CSFV haemorragic syndromes (Walz et al., 1999; Gómez-Villamandos et al., 2003).

The wide distribution of the antigen in the organs of viraemic chamois detected by IHC is resembled to that described for severe acute BVDV-2 infections (Ellis et al., 1998; Liebler-Tenorio et al, 2003). In chamois No 2, after reaching a high titre of neutralizing antibodies, a partial clearance of the virus was noted, and only multifocal immunostaining in tissues was observed. Similarly to experimentally infected chamois, despite the wide distribution of the viral antigen, the presence of lesions in severely BVDV-2 infected cattle is restricted to a few tissues (Liebler-Tenorio, 2005).

In conclusion, the present study confirms that ch-BDV is the aetiological agent of the illness that has been affecting Pyrenean chamois in the Pyrenees since 2001, entailing a significant reduction of its populations. The long-lasting viraemia and high load of virus excretion may explain the high-transmission rate and spread of the disease during the reported outbreaks.

Table 5.2.1. Virus Neutralization Test (VNT) and virus titration in SFT-R cell line of sera from viraemic chamois. In VNT, sera were tested against homologous virus and titres are expressed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures. Titres of virus are expressed as log_{10} $TCID_{50}$/ml of sera. (-), negative; n.a., non-available.

Chamois	2 dpi		5 dpi		8 dpi		11 dpi		18 dpi		25 dpi		34 dpi	
	VNT	virus	VNT	virus	VNT	virus	VNT	virus	VNT	Virus	VNT	virus	VNT	virus
RP 1	-	3.0	-	4.6	-	5.8	-	6.8	160	3.8	640	3.3	640	3.3
RP 2	-	1.9	-	4.6	-	6.0	20	6.0	2560	-	n.a.		n.a.	
RP 3	-	1.9	-	3.9	-	5.3	20	5.0	80	5.0	n.a.		n.a.	
RP 4	-	1.9	-	4.6	-	6.3	-	6.7	80	6.0	n.a.		n.a.	
RP 5	-	2.3	-	3.9	-	6.5	-	6.5	80	6.0	80	6.0	80	6.3

Table 5.2.2. RT-PCR of oral (O), nasal (N) and rectal (R) swabs from challenged chamois. -, negative; +, positive; n.a., non-available.

Chamois	2 dpi			5 dpi			8 dpi			11 dpi			18 dpi			25 dpi			34 dpi		
	O	N	R	O	N	R	O	N	R	O	N	R	O	N	R	O	N	R	O	N	R
RP 1	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RP 2	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+		n.a.			n.a.	
RP 3	-	+	-	-	-	-	+	+	+	+	+	+	+	+	+		n.a.			n.a.	
RP 4	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+		n.a.			n.a.	
RP 5	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RP 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
RP 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-

STUDY III

Experimental infection of lambs with Border disease virus isolated from a Pyrenean chamois.

Vet Rec 167, 619-621

INTRODUCTION

Sheep flocks with Border disease are characterised by barren ewes, abortions, stillbirths, the birth of weak lambs and the presence of persistently infected animals. Sheep acutely infected with BDV usually show no clinical signs and have a short period of viraemia, detectable in serum between days 4 and 11 postinfection, when neutralising antibodies appear in the serum (Nettleton and others 1998). However, severe outbreaks of disease with high mortality associated with acute BDV infection have occasionally been reported (Chappuis and others 1984).

The Pyrenean chamois (*Rupicapra pyrenaica*) is a wild small ruminant found in the Pyrenees (south-west France, Andorra and northern Spain). Since 2001, severe outbreaks of disease associated with BDV genotype 4 (BDV-4) infection have been reported in the Catalan Pyrenees, resulting in a severe reduction of the chamois population (Hurtado and others 2004, Marco and others 2009). This study aimed to evaluate the susceptibility of domestic sheep to a BDV-4 isolate from a naturally infected chamois, and to monitor the infection dynamics in inoculated lambs over a period of one month.

MATERIAL AND METHODS

Animals and samples

Eleven four-month-old Lacaume lambs (five males and six females) were challenged by oronasal inoculation with 1 x 107 TCID50 of a non-cytopathogenic BDV isolated from a diseased chamois (GenBank accession number AM905923). Five more lambs (three males and two females) were kept as negative controls. The animals were examined for clinical signs and weighed three times per week during the experiment. Differences between the mean rectal temperatures of the inoculated and control groups were analysed by analysis of variance using PROC MIXED of SAS. Values of $P<0.05$ were considered significant.

Laboratory analyses

Whole blood and nasal, oral and rectal swabs were collected from all animals on days 0, 2, 4, 7, 10, 14, 17, 21 and 28 postinoculation (PI) and stored at –80°C until tested. The animal care and study procedures were conducted in accordance with the guidelines of good experimental practice, under the approval of the Ethical and Animal Welfare Committee of the Universitat Autonoma de Barcelona. Lambs were euthanased and examined postmortem on day 7 (one control lamb and two inoculated lambs), day 14 (one control, two inoculated), day 21 (one control, three inoculated) and day 28 pi (two controls, four inoculated). At postmortem examination, urine swabs and tissue samples (tonsil, mesenteric, mediastinal and prescapular lymph node, small and large intestine, lung, thyroid, adrenal gland, kidney, urinary bladder, testis/ovary and uterus, brain, heart, ear skin and bone marrow) were collected. Each sample was fixed by immersion in 10 per cent neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with haematoxylin and eosin.

The presence of virus in the sera of all the animals on the sampling days was screened by a commercial sandwich ELISA (BVD/MD 80 – Antikorper; Synbiotics) according to the manufacturer's instructions. This kit detects p125/p80 antigen of bovine viral diarrhoea virus (BVDV) and BDV. The sample index (SI) in individual serum samples was calculated as: SI = 0.5 x (OD sample–ODP), where OD is the average of the optical densities and ODP is the average of the positive control optical densities. The reaction of the ELISA was considered valid when the optical density obtained with the positive control is 0.3 and optical density obtained with the negative control is <0.5 x ODP.

Sera, oral, nasal, rectal and urine swabs and tissue homogenates were tested by RT-PCR using the pan-pestivirus primers 324 and 326 (Vilcek and others 1994) and a commercial kit (One-Step PCR kit; Qiagen). Before the RT-PCR, viral RNA was extracted from samples using a commercial kit (Nucleospin Viral RNA Isolation; Macherey Nagel) according to the manufacturer's instructions. Furthermore, three passages of all the sera, oral, nasal, rectal and urine swabs were performed in the sheep fetal thymoid cell line SFT-R (provided by the Friedrich-Loeffler Institut, Germany) in

order to isolate the virus. Viral replication was monitored by an immunoperoxidase monolayer assay using a home-made polyclonal pestivirus antibody (OIE 2004). Sera from all the animals at different collection times were tested for the presence of antibodies to pestivirus with a commercial blocking ELISA (Pourquier) and virus neutralisation test (VNT) (OIE 2004). The VNT was performed against four pestivirus isolates: homologous BDV-4 (CADI-6), and the reference pestiviruses BDV-Moredun, BDV-137/4 and BVDV-1 NADL.

RESULTS

No clinical signs or significant gross or histopathological lesions were observed during the study period in any of the inoculated or control lambs. The ELISA to detect viral antigen did not detect pestivirus in any of the serum samples throughout the experiment. However, BDV was detected by RT-PCR in serum samples on day 4 pi in lamb 8 and on day 10 pi in lamb 5. Isolation of pestivirus from serum was achieved on the third passage in SFT-R cells on day 4 pi in samples from lambs 5 and 8, and on day 7 pi in lambs 3, 6 and 7. None of the oral, nasal or rectal swabs and tissues from the inoculated or control lambs were ELISA-positive. However, pestivirus was detected by RT-PCR and isolated on the third passage in SFT-R cells in a nasal swab from lamb 5 on day 4 pi and a rectal swab from lamb 2 on day 7 PI. The two lambs euthanased on day 7 pi (lambs 10 and 11) had positive RT-PCR results in several tissue samples: lamb 10 was PCR-positive in tonsil and mesenteric lymph node, and lamb 11 was positive in tonsil, mediastinal and prescapular lymph nodes and brain.

All tissue samples collected at postmortem examination on days 14, 21 and 28 pi were negative by RT-PCR. The ELISA to detect antibodies to pestivirus was positive on day 17 (in three of seven lambs), 21 (four of seven) and 28 pi (two of four) (Table 5.3.1). However, the VNT detected antibodies earlier: all the infected lambs except lamb 2 had neutralising antibodies on day 14 PI. On day 21 and 28 PI, all the remaining inoculated lambs showed significant neutralising antibody titres. Maximum VNT titres were observed on day 28 pi (Table 5.3.1).

DISCUSSION

There are several reports on experimental BDV infections in sheep but, this report is unusual in that it reports an experimental infection in lambs with a BDV isolate from a wild species. The lambs that were challenged were subclinically infected and did not develop the severe clinical signs that have been observed in Pyrenean chamois affected by the same BDV-4 isolate (Marco and others 2007). Thus, these results provide further evidence that acute infections of non-pregnant sheep with virtually all BDV isolates tested so far produce no obvious pathological changes (Nettleton and Willoughby 2008).

In the present study, no clinical signs were observed, and virological and serological examination of the inoculated lambs indicated that an acute BDV infection occurred. Five of the inoculated lambs had a short period of viraemia (one to six days), and in six of 11 animals the viraemia was undetectable by RT-PCR or virus isolation. Thabti and others (2002) obtained similar results in lambs experimentally infected with BDV, with virus detected from day 4 to 9 PI. Garcia-Perez and others (2009) detected virus from day 2 to 15 pi and reported a longer period of viraemia (one to 10 days). In that experiment, two of 13 infected ewes remained RT-PCR-negative for the presence of BDV in blood throughout the study; similar to the results of the present study, Garcia-Perez and others (2009) did not detect the virus with antigen ELISA.

BDV was detected only in one nasal and one rectal swab from the lambs in the present study, which further suggests that the viraemia caused by the chamois BDV-4 isolate was short. The presence of the virus in different lymphoid organs, observed in the two lambs euthanased on day 7 PI, and in the brain in one of them, has not been reported in acutely infected sheep, but has been described in cattle acutely infected with BVDV (Marshall and others 1996, Liebler-Tenorio and others 2002, 2004).

The seroconversion observed in this experimental trial confirms that all the lambs were infected, and is consistent with reports of previous acute BDV infections in sheep (Nettleton and others 1998, Thabti and others 2002). The VNT displayed higher

sensitivity than the ELISA, detecting antibodies on day 14 PI, coinciding with the end of the period of viraemia; in contrast, the ELISA detected antibodies only from day 17 PI, and in two inoculated lambs no antibodies were detected at all by ELISA at the end of the study (day 28 PI). The VNT showed higher titres against the challenge strain compared with the other pestivirus strains, but cross-reactivity with both BDV and BVDV strains was noted. In conclusion, a BDV-4 isolate from a chamois was able to infect domestic lambs subclinically via the oronasal route; the lambs developed a short viraemia, and a humoral response two weeks after inoculation.

Table 5.3.1. Virus neutralization (VNT) study of sera of infected lambs. Sera were tested against homologous and heterologous BDV isolated from chamois CADI-6, and reference strains of BDV (137/4 and Moredun Strains) and BVDV (BVDV-1 NADL). Titres are expresed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures; (-): samples negative to VNT and ELISA; (POS) samples positive to antibody ELISA.

Lamb	Day 14 pi					Day 17 pi					Day 21 pi					Day 28 pi				
	CADI-6	137/4	Mored.	NADL	ELISA	CADI-6	137/4	Mored.	NADL	ELISA	CADI-6	137/4	Mored.	NADL	ELISA	CADI-6	137/4	Mored.	NADL	ELISA
1	10	-	-	-	-	40	-	-	-	-	80	5	-	-	-	640	5	-	5	-
2	-	-	-	-	-	20	5	-	-	-	80	5	-	-	-	320	5	-	5	-
3	10	-	-	-	-	80	10	10	-	POS	160	10	10	-	POS	320	10	10	5	POS
4	5	-	-	-	-	80	-	-	-	-	320	5	-	-	POS	320	10	-	0	POS
5	20	-	-	-	-	80	-	-	-	-	80	-	-	-	-					
6	10	-	-	-	-	80	-	-	-	POS	160	5	-	5	POS					
7	40	-	-	-	-	80	-	5	-	POS	80	-	5	-	POS					
8	20	-	-	-	-															
9	40	-	-	-	-															

STUDY IV

Experimental infection of pigs with Border disease virus isolated from Pyrenean chamois (*Rupicapra pyrenaica*)

J Vet Diagn Invest 22, 360-365

INTRODUCTION

Interspecies transmission of pestiviruses among several domestic species has been reported previously. BVDV virus infects not only cattle but also sheep, pigs and goats (Graham et al., 2001; Pratelli et al., 2001), CSFV experimentally infect cattle and goats (Loan and Storm, 1968; Shimizu and Kumagai, 1989) and BDV have been isolated from several domestic species like sheep, goat, cattle and swine (Paton, 1995; Becher et al., 1997; De Mia et al., 2005; Rosell et al., 2008a).

Cross-species transmission of pestivirus between wildlife and domestic livestock, and the ecology of transmission is a subject of recent concern. While some investigators theorize that an independent cycle of pestivirus infections occur in wildlife (Frölich, 1995; Frölich et al., 2005), others describe transmission of pestiviruses from wild animals to susceptible domestic animals on pastures (Marco et al., 2007; Passler et al., 2009). Moreover, pestivirus isolates have been reported in several wild ruminants (Hurtado et al., 2004; Frölich et al., 2005; Vilcek and Nettleton, 2006).

Clinically, BDV infection of sheep is characterized by reproductive disease (Nettleton et al., 1998). Acute infection of immunocompetent animals is usually subclinical and viraemia is transient and difficult to detect. Severe BD epizootics are unusual but, under some conditions, a severe haemorrhagic syndrome has been reported in sheep (Chappuis et al., 1984; Thabti et al., 2005).

Pyrenean chamois (*Rupicapra pyrenaica*) is a small mountain ruminant belonging to the family Bovidae and subfamily Caprinae. During 2001 and 2002, an outbreak of a disease associated with BDV infection (Hurtado et al., 2004; Frölich et al., 2005) occurred in the central Pyrenees, which resulted in a 42 % reduction in the chamois population in the affected area (Marco et al., 2007). Classical signs of illness associated to this virus in chamois outbreaks were cachectic condition of the animals, presence of pallid mucous membranes due to a low red blood cells counts in comparison with reference values, alopecia with skin hyperpigmentation and several degrees of neurological alterations such as depression, weakness and difficulty in moving. Since

then, additional outbreaks of severe BDV disease in Pyrenean chamois have been reported in the Catalan Pyrenees (Marco et al., 2009).

During the same period, wild boars from the same area were found seropositive as determined by ELISA test against CSFV (Rosell et al., 2008b). The antibodies were determined to be due to BDV infections by a comparative virus neutralization test (VNT). If pigs were infected with chamois BDV, the presence of cross-reactive antibodies to CSFV detected by the CSFV would pose a diagnostic challenge for the control of CSF. Therefore, the objective of the present study was to assess the susceptibility of the domestic pig to infection with a BDV isolated from naturally infected chamois, as well as to monitor the dynamics of the BDV infection and antibody response in experimentally inoculated pigs during a one month period.

MATERIALS AND METHODS

Animals

Thirty-six female one-month-old Large White x Landrace pigs were housed in the level-3 biosafety facilities of the experimental station of Centre de Recerca en Sanitat Animal (CReSA, Universitat Autònoma de Barcelona, Spain). Sera from all pigs were tested for the presence of pestiviruses and antibodies using the commercial ELISA BVD/MD 80 – Antikörper (Pourquier, Montpellier, France), VNT, and Reverse Transcription – Polymerase Chain Reaction (RT-PCR) (Vilcek et al., 1994, 1997) tests. All samples analyzed were negative.

Virus and innoculum preparation

The BDV strain used to infect the animals was isolated from the spleen of a diseased chamois found in the Pyrenees in 2005, which showed the classical signs of illness associated to BDV infection (Marco et al., 2007). The spleen sample was homogenized in sterile Eagle's sterile Minimal Essential Medium (EMEM) (1g of tissue + 9 ml

EMEM) 6% supplemented with penicillin, streptomycin and nistatine antibiotics and tested for pestiviral RNA by RT-PCR. A BDV strain was isolated in the pestivirus-free sheep foetal thymoid (SFT-R) cell line. The virus was titrated directly in SFT-R pestivirus free monolayer cell line in a 96 wells tissue culture plate. Viral replication was monitored by the Immune Peroxidase Monolayer Assay – IPMA (OIE, 2004) with a polyclonal home-made pestivirus antibody. Virus titration was performed as described previously (Reed and Muench, 1938) obtaining a titre of $1x10^7$ $TCID_{50}$.

To asses phylogenetic homology between this virus and the BDV isolated in the same and previous outbreaks, amplified DNA from spleen homogenate and virus resulting from cellular culture was purified using Minelute Gel Extraction Kit, Qiagen Inc., Valencia, CA., and sequenced. Purified amplicons were analyzed with the Big Dye Terminator v.3.1 Kit (Applied Biosystems, Foster City, CA.) and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA.). Sequence of the 5'UTR of the virus used in this experimental infection was deposited in the GenBank under the name CADI-6 and accession number AM905923. The phylogenetic tree was calculated by the neighbour-joining method (Saitou and Nei, 1987) using an automatic root location. To test the reliability of the branches in the tree a bootstrap analysis of 1000 replicates was performed by creating a series of bootstrap samples. The viral sequence from the chamois isolate was located in the same BDV-4 genotype described in previously outbreaks in chamois populations (Figure 5.4.1).

Experimental design

After an acclimatization period of seven days, pigs were randomly assigned to two groups reared in separated isolation units. Twenty pigs were inoculated by oro-nasal natural route with $1x10^7$ $TCID_{50}$ of Border Disease Virus re-suspended in 2 ml of EMEM. The inoculum was administered by a combination of nasal catheter (0.5 ml in each nostril) and 1ml was administered orally. Sixteen pigs were inoculated with 2 ml of sterile EMEM by the same route to serve as negative controls.

Pigs were observed daily for clinical signs. Rectal temperature was monitored three times per week and weight was measured weekly during the experiment. Whole blood was collected at days 0, 3, 7, 10, 14, 21, and 31 post-inoculation (pi). Blood samples were collected from the cranial cava vein, placed in serum collection tubes and centrifugated at 1,200 rpm during 15 minutes. Nasal, oral and rectal swabs were also collected the same days as blood samples, and resuspended in 2 ml of sterile PBS supplemented with antibiotic. Sera samples and swabs were stored at – 80 ºC until tested. Necropsies were performed on days 0 (2 control pigs - CP), 3 (2 CP, 3 inoculated pigs - IP), 7 (2 CP, 3 IP), 14 (3 CP, 3 IP), 21 (2 CP, 3 IP) and 31 (5 CP, 8 IP) post-inoculation. At necropsy, urine swabs and tissue samples (spleen, kidney, brain, lymph node, skin, thyroid gland, intestine, liver, lung, bone marrow and testicle) were collected.

Animal care activities and study procedures were conducted in accordance with the guidelines of the Good Experimental Practices, under the approval of the Ethical and Animal Welfare Committee of the Universitat Autònoma of Barcelona.

Virological examination

Viral RNA was extracted directly from 150 µl of sera and swab samples diluted in PBS by a commercial kit (Macherey Nagel Nucleospin Viral RNA Isolation, Düren, Germany) according to the manufacturer's procedure. RT-PCR was performed to detect pestiviral RNA (5'UTR region) using previously described pan-pestivirus primers 324 and 326 (Vilcek et al., 1994; 1997) and a commercial kit (One-Step PCR kit, Qiagen Inc., Valencia, CA).

In order to verify the homology between the virus detected in infected pigs and the innoculum, RNA present in a positive serum sample was amplified, purified and sequenced as described above.

Serological tests

Three commercial ELISA tests were used to detect anti-pestivirus antibodies in sera from all animals and sample collection days. The three ELISA tests were performed following the procedure described by the manufacturer. The ELISA BVD/MD 80 – Antikörper test (Pourquier, Montpellier, France) detected antibodies against p80 protein of BVDV and BDV. The inhibition percentage (IP) in individual sera samples was calculated as: IP = ODS / ODN x 100, where OD is the optical density of the negative (N) control, and of the sample (S). Samples with %IP ≥ 50, 40-50%, and ≤ 40%, were considered negative, weakly positive and positive for BDV antibodies, respectively. The reaction of the ELISA BVD/MD 80 – Antikörper was considered valid when the negative control had a minimum mean OD 450 value of 0,800 and the percentage of competition of the positive control was less than 20%.

The ELISA HerdChek® Classical Swine Fever Virus (CSFV) Antibody Test Kit (IDEXX Laboratories, Inc., Westbrook, ME) and the ELISA Ceditest® CSFV 2.0 (Cedi-Diagnostics B.V., Lelystad, The Netherlands), detected antibodies against E2 protein of CSFV. The IP in individual sera samples in ELISA HerdChek® Classical Swine Fever Virus (CSFV) Antibody Test Kit was calculated as: Bloking% = ((NC*X* – sample A_{450}) / NC*X*) x 100, where NC*X* is the negative (N) control mean. Samples with blocking percentage ≥ 40%, 30 – 40%, and ≤ 30% were positive, weakly positive and negative, respectively. The reaction of the test was considered valid when the negative control had a minimum mean OD 450 value of 0.50 and the percentage of competition of the positive control was more than 50%.

The IP of the sera samples in ELISA Ceditest® CSFV 2.0 was calculated as IP = 100 – ((ABS sample / ABS N) x 100), where ABS is absorbance. Samples with blocking percentage < 40% and ≥ 40% were considered negative and positive respectively. The reaction of the test was considered valid when the N control absorbance mean was >1.0 and the positive control IP was > 80%. A VNT test was used for the detection and quantification of anti-BDV antibodies in serum of inoculated and control pigs. The VNT was performed against four pestiviruses: the homologous BDV (CADI-6), and the

reference pestiviruses CSFV-Alfort, BDV-137/4 and BVDV-NADL. Virus neutralization test was performed following the procedure described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2004). Titres of 1:10 or higher were considered as positive. Viral replication was monitored by the IPMA (OIE, 2004) with a polyclonal home-made pestivirus antibody.

Histopathological studies

All tissue samples collected during necropsy (spleen, kidney, brain, lymph node, skin, thyroid gland, intestine, liver, lung, bone marrow and testicle) were fixed by immersion in formalin and subsequently embedded in paraffin. Tissue sections were cut at 4 μm, stained with haematoxylin and eosin and examined for the potential presence of lesions.

Statistical analysis

Differences in mean temperatures between inoculated and control groups were analysed by ANOVA using PROC MIXED of SAS® System (SAS Inc., Cary, NC). P values less than 0.05 were considered significant.

RESULTS

No clinical signs were observed during the whole experimental period. There were not statistically differences of mean temperatures between inoculated and control pigs during all the infection period ($P>0.05$).

At post-mortem examination, gross or histopathological lesions were not found in any inoculated or control pig. Prevalence of viraemic pigs at different days post-inoculation was 20 % (4 out of 20 pigs) on day 3 pi, 29.41 % on day 7 pi (5 out of 17 pigs), 100 % on day 10 pi (14 out of 14 pigs) and 85.71 % on day 14 pi (12 out of 14 pigs). The mean duration of viraemia was 7.9 days (± 3.36 SD) (Figure 5.4.2).

Border disease virus was detected in oral and/or nasal swabs from day 3 pi in 9 of 20 pigs (45%) to day 7 pi in 10 of 17 pigs (58.82%). BDV was not detected in rectal or urine swabs from any inoculated pig. Sera and all swab types from control pigs at all sampling points were negative for BDV by RT-PCR. The sequencing of a BDV isolated from one inoculated pig on day 10 pi had 100% identity with the BDV strain used for inoculation.

Antibodies to BDV were detected in 1 of 14 pigs (7.14%) on day 14 pi, 3 of 11 pigs (27.27%) on day 21 pi and 7 of 8 (87.5%) on day 31 pi using the ELISA BVD/MD 80. The other two ELISA tests to detect antibodies to CSFV detected CSFV antibody positive serum samples from only 1 pig on days 21 and 31 pi (Table 5.2.1).

Neutralizing antibodies to BDV were detected in serum samples from almost all pigs on day 14 pi and in all pigs on day 21 pi. Maximum titers were observed on day 31 pi (min. 80, max. 640) (Table 5.2.1). Higher titers of neutralizing antibodies were observed against the challenging BDV strain compared to other pestivirus strains (BDV-137/4 and BVDV-NADL) on days 21 and 31 pi. Neutralizing antibodies to CSFV-Alfort were not detected in inoculated animals at any time point during the experiment.

DISCUSSION

To our knowledge, this is the first experimental infection of swine with BDV isolated from a wild ungulate. There are many experimental infections with pestiviruses, mainly CSFV and BVDV, in swine. Nevertheless, there are few studies on experimental infections with BDV viruses in pigs and even less information on the clinical signs and pathogenesis (Leforban et al., 1992; Depner and Schirrmeier, 2002).

The pathogenesis of pestivirus infection in pigs is highly variable according to the strains involved. Classical swine fever virus infection is associated with clinical signs and disease (OIE, 2004). However, BVDV infections in pigs lead to mild or unapparent disease (Fernelius et al., 1973a, 1973b; Stewart et al., 1980; Walz et al., 2004). Pigs

inoculated by oro-nasal route with the CADI-6 BDV isolate did not develop clinical disease, and statistically differences between mean rectal temperatures of control and inoculated groups was not observed. The results support previous data that BDV is unlikely to induce clinical disease in pigs (Leforban et al., 1992).

Classical swine fever virus, BVDV and BDV are shed by infected animals in oro-nasal and lacrimal secretions, urine, feces and semen (OIE, 2004). Despite the presence of virus in oral and nasal swabs between days 3 and 7 pi in inoculated pigs of the present study, it is likely that those results do not only represent an active secretion of virus through this two routes, but viral replication at the inoculation region as well.

The four species of pestiviruses infects swine; however, the viremia depends on the species and strain of the virus (Loken, 1995, OIE, 2004). In CSFV infections, viremia usually starts between day 2 and 15 pi (OIE, 2004), while in BVDV infections viremia is detected within the first week of the infection (Fernelius et al., 1973a, 1973b; Stewart et al., 1980; Walz et al., 2004; OIE, 2004). Furthermore, the results of experimental BDV infections in pigs indicated low or undetectable virus in blood (Depner and Schirrmeier, 2002). In the present study, BDV-inoculated pigs were positive for the presence of virus in sera from day 3–14 pi; a longer period of viraemia than that previously reported. The reasons of this relatively long-term viraemia are unknown, but maybe due to the high inoculating dose and/or a characteristic of replication of the specific BDV used in the challenge.

The serological data indicate that all inoculated pigs were infected with BDV and an antibody response was developed against the homologous virus. The humoral immune response of pestivirus infected swine has been previously reported to be highly variable with respect to onset relative to infection. Some authors reported detectable BVDV antibody levels 5 weeks after infection in sows, while others showed evidence of seroconversion within 3 weeks after the inoculation of pigs with a BDV (Leforban et al., 1992; Walz et al., 2004). In the present study, swine seroconverted by day 14 pi and antibodies were maintained until the end of the study (day 31 pi), with titres as high as 640 in some pigs as did the ELISA and VNT tests. The detection of antibodies by VNT

coincided with the end of RT-PCR positive serum samples. It is important to note that higher titres to the BDV challenge were found compared with the other pestivirus used in VNT. There was some cross-reactivity with other BDV and BVDV strains detected by VNT; however, no CSFV-specific antibodies were detected in the serum of any of the infected pigs.

The three commercial ELISA tests were much less sensitive than the VNT in detecting antibodies. The detection of antibody positive pigs with the ELISA-BVD/MD/BD test increased from day 14 pi (7.7%) until the end of the study at day 31 pi (87.5%). One pig out of 14 that was detected seropositive by ELISA-BVD/MD/BD test at day 14 pi was seronegative by VNT. The most likely explanation for this situation is that this serum was a false-positive sample detected by the ELISA. Interestingly, a single pig was antibody positive on days 21 and 31 pi by both CSFV ELISAs. This animal was only positive to the ELISA BVD/MD/BDV test at day 31-pi. Detection of BDV antibodies in swine serum by CSFV-ELISA could present a diagnostic challenge for CSFV sero-diagnosis. This study further confirms VNT as the reference laboratory test to discriminate antibodies to the various species of pestiviruses. Therefore, the current circulating pestivirus strains should be included in the routine VNT.

In conclusion, the present study confirms that the BDV isolated from naturally infected chamois can also infect the domestic pig. The infected animals developed a relatively long viraemia with seroconversion two weeks after inoculation. The results support the horizontal transmission of BDV between chamois and wild boar suspected to have occurred during the BDV epizootic in chamois (Rosell et al., 2008b). Further, the findings suggest that this BDV strain could be transmitted from wild boar to domestic pigs. This study agrees with previous studies that indicate transmission of BDV from ruminants to swine occurs when both species are in close contact (Leforbanet al., 1992; Roic et al., 2007). Taking into account that the wild boar is a reservoir of different viral agents for the pig (Ruiz-Fons et al., 2008), the potential transmission from the wild to the domestic pig would represent a diagnostic challenge due to the cross-reactivity for other pestiviruses in the ELISA-based tests interfering with routine official diagnostics tests for CSF.

Table 5.4.1. Virus neutralization (VNT) titres on sera of inoculated pigs. Sera were tested against homologous and heterologous BDV strains isolated from chamois and reference strains of BDV, BVDV and CSFV. (*) Titres are expresed as the reciprocal of the highest dilution that neutralized 100 tissue culture infective doses (100 $TCID_{50}$) in all cultures; (†), samples negative to VNT. (‡)1, positive to ELISA against BVD-BD (Pourquier); 2, positive to ELISA against CSFV (IDEXX); 3, positive to ELISA against CSFV (Cedi-Diagnostics B.V.).

Pig ID	Day 14 pi					Day 21 pi					Day 31 pi				
	BDV CADI 6	BDV 137/4	BVDV NADL	CSFV Alfort	ELISA‡	BDV CADI 6	BDV 137/4	BVDV NADL	CSFV Alfort	ELISA	BDV CADI 6	BDV 137/4	BVDV NADL	CSFV Alfort	ELISA
1	10*	-	-	-	-	40	-	5	-	-	320	5	20	-	1
9	-†	-	-	-	-	80	10	-	-	-	160	20	10	-	1
10	-	-	-	-	1	80	5	-	-	1	640	5	10	-	1
24	10	-	-	-	-	40	5	-	-	-	320	5	5	-	1
29	10	-	-	-	-	80	5	-	-	2,3	80	20	10	-	1,2,3
32	10	-	-	-	-	40	-	-	-	1	320	5	5	-	1
33	10	-	-	-	-	320	-	-	-	-	640	5	10	-	-
36	10	-	-	-	-	40	5	-	-	-	320	5	5	-	1
8	5	-	-	-	-	160	-	-	-	1					
22	10	-	-	-	-	160	-	-	-	-					
28	20	-	-	-	-	160	-	-	-	-					
5	10	-	-	-	-										
21	5	-	-	-	-										
26	10	-	-	-	-										

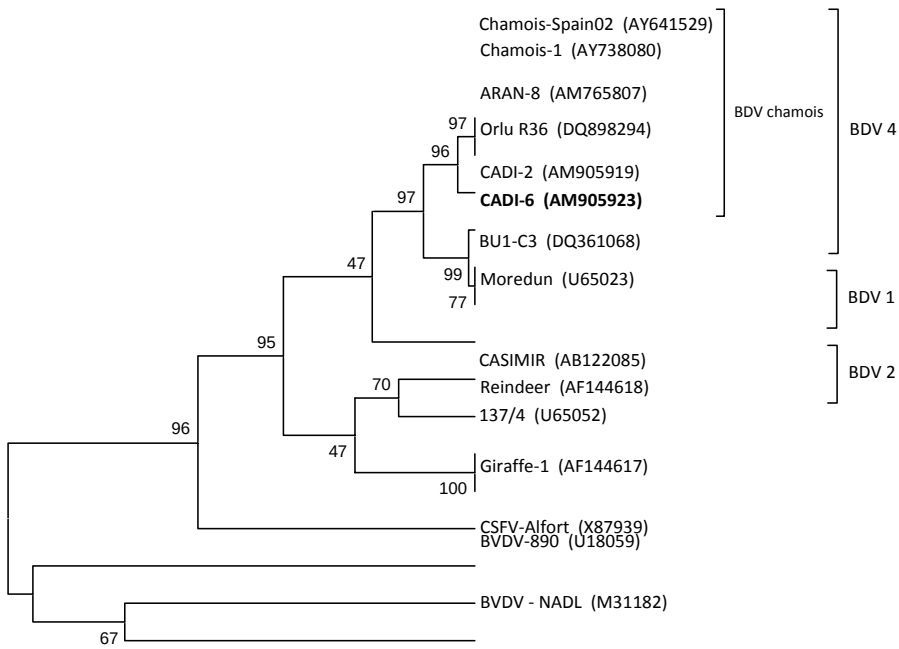

Figure 5.4.1. Unrooted neighbour-joining phylogenetic tree based on the 5'UTR sequence among pestiviruses. Chamois strain CADI-6 AM905923 used to experimentally infect swine was enclosed in a differenciated group into BDV4. The numbers on the branches indicate the bootstrasp values (in percent; 1000 replicates). GenBank accession numbers are indicated in brakets.

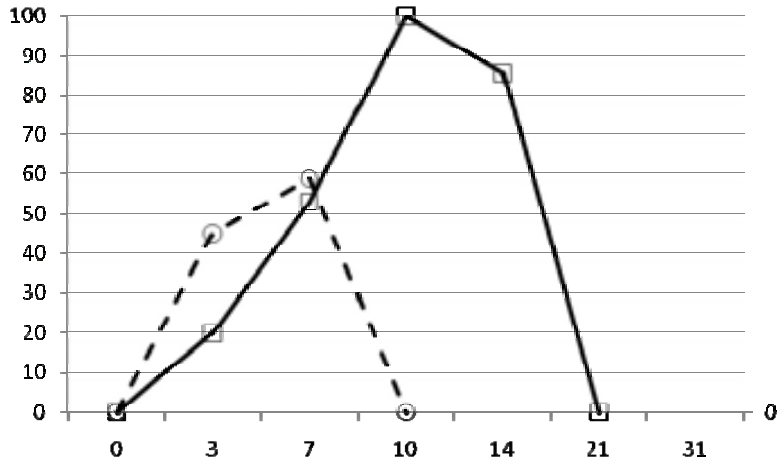

Figure 5.4.2. Infection dynamics of inoculated piglets. Percentage RT-PCR pigs positive in oral or nasal swabs (○) and in serum (□).

6. GENERAL DISCUSSION

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Disease outbreaks associated to ch-BDV that have affected Pyrenean chamois populations since 2001 entailed a significant reduction of individuals in a vast area of the Pyrenees, being the main threat for its populations (Marco et al., 2009). Epidemiological, clinical and pathological studies have already been performed (Arnal et al., 2004; Hurtado et al., 2004; Frölich et al., 2005; Marco et al., 2007, 2009, 2011). However, several questions concerning the virus and the infection/disease in chamois and other animal species remain without answers. Therefore, the objectives of the four studies presented in this Thesis were to elucidate some of these questions.

The STUDY-I aimed to investigate the distribution and quantification of ch-BDV in the organs of naturally affected chamois and to determine whether the virus was shed via the main excretion routes. After confirming that this virus was widely present in all organs with high titres, the STUDY-II aimed to reproduce experimentally the disease caused by the ch-BDV in the species affected in field, Pyrenean chamois.

Since Pyrenean chamois and sheep share communal pastures in Pyrenees, the objective of the STUDY-III was to elucidate the susceptibility of domestic sheep to postnatal infection with ch-BDV and to monitor the infection dynamics of inoculated lambs during one month.

During the outbreaks of disease in chamois populations, wild boars from the same areas were found seropositive as determined by ELISA test against CSFV (Rosell et al., 2008b). The antibodies were determined to be due to BDV infection with a comparative virus neutralization test (VNT). Thus, the STUDY-IV aimed to describe the susceptibility of the domestic pig to infection with ch-BDV, as well as to monitor the dynamics of the infection and antibody response.

Clinical signs and severe disease following acute BDV infection in immunocompetent domestic animals are very rare (Nettleton et al., 1998). Clinically, both challenged pigs and sheep from our studies did not develop any symptom or sign of disease. Neither,

gross or histopathological lesions were observed, agreeing with previously reported studies. In chamois, severe clinical signs have been observed in naturally infected animals, such as neurological signs, chronic wasting and alopecia (Marco et al. 2007). Also, Chappuis (1984) reported an outbreak of severe disease in sheep characterized by a haemorrhagic syndrome and leukopenic enterocolitis with high mortality. It was associated with a new BDV strain (Aveyron) that produced profound leukopenia and death in 50% of 3 to 5 month-old lambs. However, a subsequent experimental infection in lambs with this highly pathogenic strain was not able to reproduce the disease (Thabti et al, 2002).

In our experimental infection in chamois, none of the viraemic animals showed neurological signs. Nevertheless, the lesions observed in brain were similar to those described in naturally infected chamois (Marco et al., 2007), suggesting that these symptoms would have occurred if they were not tranquilized and the experiment would have lasted for long. Cachexia and alopecia was neither observed in viraemic chamois, but weight loss was significantly different between viraemic and non-viraemic animals.

Regarding histopathological findings in experimentally infected chamois, the most prominent lesions were a non-suppurative meningoencephalitis and gliosis, similar to what has been previously reported in naturally infected chamois. The neurotropism of pestiviruses in congenital infections is well-documented (Maxie and Youssef, 2007; Bielefeldt-Ohmann et al., 2008) but it has never been described in post-natal infections with BDV and only suspected in one case of BVDV infection in a Heifer (Blas-Machado et al., 2004). Brain lesions in chamois resemble those produced by classical swine fever virus (Gómez-Villamandos et al., 2006). The wide distribution of the antigen in the organs of viraemic chamois detected by IHC is similar to that described for severe acute BVDV-2 infections (Ellis et al., 1998; Liebler-Tenorio et al, 2003). In one chamois, after reaching a high titre of neutralizing antibodies, a partial clearance of the virus was observed, and only multifocal immunostaining in tissues was observed. Similarly to experimentally infected chamois, despite the wide distribution of the viral antigen, the presence of lesions in severely BVDV-2 infected cattle is restricted to a few tissues (Liebler-Tenorio, 2005).

Additionally, in the viraemic chamois of our study, leucopoenia with neutropenia and depletion in lymphoid tissue were detected suggesting that immunossupression and predisposition to secondary infections are very likely. Secondary infections are common findings in naturally infected chamois with BDV (Marco et al., 2007; Fernández-Sirera et al., 2011) as well as in other pestiviral infections in domestic animals, and they have been related to the immunosupression caused by the virus (Brackenbury et al., 2003). In our study, one chamois developed a severe bronchopneumonia and severe haemorrhagic diathesis was seen in two viraemic chamois that died of acute diarrhoea. In previous studies, Chappuis (1984) reported that BDV was associated with a hemorrhagic syndrome in acutely infected adult sheep but the mechanism was not elucidated. In addition, this condition has also been reported in BVDV-2 and CSFV infections and may be associated to thrombocytopenia, which could be multifactorial and a consequence of platelet activation and aggregation and defective production in bone marrow (Walz et al., 1999 and 2001; Bautista et al., 2002; Gómez-Villamandos et al., 2003). In the experimentally infected chamois of our study, thrombocytopenia was also suspected as the most likely cause of the haemorrhagic diarrhoea.

Data obtained from the challenge performed in chamois confirmed that ch-BDV is the main aetiological agent of the outbreaks observed in Pyrenean chamois populations since 2001. This is in agreement with the epidemiological and pathological studies performed in naturally-infected populations. One of the unanswered questions by field studies was the duration of the viraemia in free-ranging diseased chamois. This was difficult to elucidate because the timing of infection was unknown and most of the diseased chamois that could be captured were in an agonic state and did not survive more than a few hours or days, despite they were hospitalised and treated. Only on one occasion one chamois was hospitalised for more than one month and remained viraemic during all the time until death (Hurtado et al., 2004). The long-lasting viraemia observed in the experimentally infected chamois was unexpected when compared to reported pestivirus infections in domestic animals. Post-natally acute infections of BDV in domestic ruminants develop a short viraemia between days 4 and 11 pi (Nettleton *et al*., 1998). While pigs and lambs from our experimental infections showed this short viraemia that agree with previously reported studies, the inoculated chamois developed

a long-lasting viraemia, being the ch-BDV detected in sera from day 2 pi until the last day of experiment (day 34 pi in some animals). This chronic infection may be related with the clinical symptoms observed in naturally-infected chamois, such as weakness, skin alopecia and hyperpigmentation, and several degrees of cachexia (Marco et al., 2007).

It is noteworthy that inoculation of seropositive chamois did not result in viraemia. Thus, these data would confirm that previously acquired humoral immunity is protective against ch-BDV infection.

Regarding the quantification and distribution of the virus in diseased chamois, both naturally and experimentally infected chamois had a wide distribution of ch-BDV in most organs by RT-PCR and isolation techniques. Also, the detection of the virus in the foetus of a viraemic female chamois indicated that vertical transmission of BDV may occur in this species, as in domestic ruminants.

Interestingly, the continuous shed of virus by nasal, oral, faecal and urinary routes in experimentally infected chamois agree with the data observed in naturally-infected chamois from our first study (STUDY I), demonstrating that the viraemic chamois have a high capability of dissemination of the virus in field. Thus, this condition may facilitate a high horizontal virus transmission rate, which would explain the rapid spread of the infection during the different outbreaks reported to date along the Central and Eastern Pyrenees (Marco et al., 2007).

Long-lasting viraemia, continuous shed, and generalized viral tissue distribution observed in the chamois of the current study have only been described in persitent infected (PI) animals infected *in utero* (Vilcek *et al*., 1994, Nettleton et al., 1998). The immunological mechanisms by which ch-BDV is capable to produce a long-lasting viraemia and chronic continuous shedding of ch-BDV in the experimentally infected chamois has not been elucidated in our experiments and investigation in immunology of the Pyrenean chamois and genetic characterization of ch-BDV from different outbreaks and geographic areas is necessary to improve the understanding of this concern.

Regarding the challenges performed in pig and sheep, the short viral shedding through nasal and oral secretions and faeces agree with the short viraemia detected in serum. However, the viraemia was longer in infected pigs, when compared with sheep, which suggests that this species has a better capability of virus dissemination than sheep. The excretion of the virus observed in pigs and, to a lesser extent in sheep, demonstrates that sheep and swine species are potential disseminators of the virus to other areas

The significant neutralising humoral response observed from day 14 pi in experimentally infected lambs was consistent with previous BDV acute infections of sheep (Edwards et al., 1995, Nettleton et al., 1998, Thabti et al., 2002). Data collected from this challenged confirm that sheep is sensitive to ch-BDV. However, antibody response in these species is capable to eliminate the virus.

All infected pigs of the experimental study developed antibodies against the homologous virus from day 14 pi, which were able to neutralize and clear the virus. It is important to note that we observed significant higher titres against the ch-BDV than to the other pestiviruses used in the VNT. In this test, some cross-reactivity with other BDV and BVDV strains but not with CSFV was observed. Interestingly, a single pig was antibody positive on days 21 and 31 pi by both CSFV ELISAs. Detection of BDV antibodies in swine serum by CSFV-ELISA could represent a diagnostic challenge for CSFV sero-diagnosis.

Humoral response of challenged chamois was similar to that described in acute BDV infection (Nettleton et al. 1998) and to that of experimental infection with ch-BDV in sheep and pig (STUDIES III and IV). Unexpectedly, viral neutralization was not achieved in most of the viraemic chamois, with the exception of one animal that neutralized the virus in blood by day 18 pi. It is interesting to note that in this animal, antigen distribution was not diffuse and was more multifocal than in any other viraemic chamois and was not found in some tissues, such as the stratified squamous epithelium of the upper GI tract.

The results of this Thesis confirmed that ch-BDV is the aetiological agent of the disease described in Pyrenean chamois since 2001. The description of the pathogenesis of the disease and the pathological lesions in chamois contribute to the field of virology with an unreported behaviour of a BDV in ruminant species. Although the pig and sheep have not developed clinical disease, both species are susceptible to ch-BDV infection and could play a role in the maintenance and transmission of this infection.

7. CONCLUSIONS

7. CONCLUSIONS

1.

Ch-BDV is present in high titres and widely distributed in organs from naturally infected chamois. The presence of virus in excretion routes (oro-nasal, urine and faeces) would explain the high transmission rate of the disease in free-ranging Pyrenean chamois populations.

2.

The detection of ch-BDV in the foetus of a viraemic chamois infected naturally confirms the vertical transmission of the virus in this species and suggests that PI animals could exist in free-ranging populations.

3.

Ch-BDV infects domestic pig and sheep, causing a short viraemia that ends with the production of neutralizing antibodies. Also, ch-BDV does not cause clinical disease or histological lesions in these species.

4.

Pig and sheep shed the virus through oronasal route, urine and faeces, confirming that both species are potentially able to disseminate the ch-BDV in the field.

5.

Ch-BDV produces a long-lasting viraemia in experimentally infected chamois. This long-lasting viraemia has only been described in persistently infected ruminants and never reported in post-natally infected animals.

6.

Experimentally infected chamois develop a humoral response to ch-BDV, detected at day 14 pi that lasts at least 16 days more. However, this humoral response does not neutralize the virus.

7.

Ch-BDV is confirmed to be the aetiological agent of the disease that has been affecting Pyrenean chamois in the Pyrenees since 2001.

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8. REFERENCES

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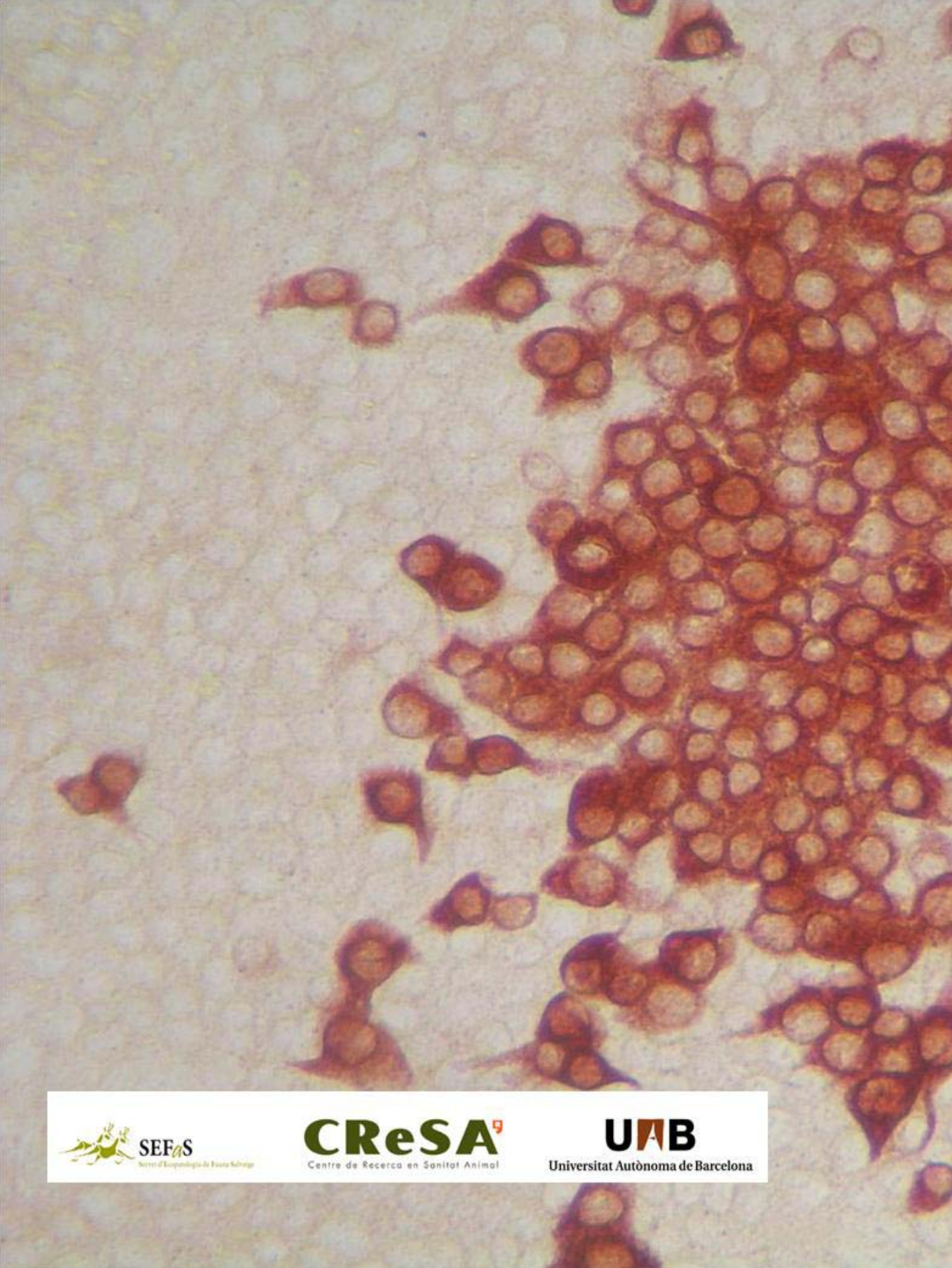

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