Epidemiology, vaccination and infection in wild ruminants with bluetongue virus

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CERTIFIQUEN:

Que la tesi titulada: "***Epidemilogy, vaccination and infection in wild ruminants with bluetongue virus***" presentada per **Cristina Lorca Oró** s'ha realitzat sota la seva direcció en el Centre de Recerca en Sanitat Animal (CReSA), per l'obtenció del grau de Doctora en Medicina i Sanitat Animals per la Universitat Autònoma de Barcelona.

I per tal que consti als efectes oportuns, signen el present certificat a Bellaterra (Barcelona), a 3 de setembre de 2012.

Ignacio García Bocanegra
Director

Jorge Ramón López Olvera
Director

Mariano Domingo Álvarez
Director

Cristina Lorca Oró
Doctoranda

Queda prohibido llorar sin aprender,

levantarte un día sin saber qué hacer,

tener miedo a tus recuerdos...

Queda prohibido no sonreír a los problemas,

no luchar por lo que quieres,

abandonarlo todo por miedo,

no convertir en realidad tus sueños...

(Queda prohibido – Alfredo Cuervo Barrero)

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LIST OF ABBREVIATIONS

AHS	African horse sickness
ANOVA	analysis of variance
BSL3	biosafety level 3
BT	bluetongue
BTV	bluetongue virus
CI	confidence interval
CPE	cytopathic effect
Ct	threshold cycle
dpi	days post-inoculation/infection
DIVA	differentiation/discrimination of infected from vaccinated animals
dpv	days post-vaccination
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
EHD	epizootic haemorrhagic disease
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunosorbent spot assay
HGB	haemoglobin
HTC	haematocrit
IFN-γ	gamma interferon
IM	intramuscular
IUCN	International Union for Conservation of Nature
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MEM	minimal essential medium
mpv	months post-vaccination

NS	non-structural (protein)
OD	optical density
OIE	World Organisation for Animal Health (*Office International des Epizooties*)
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PLT	platelets
R	coefficient of regression
RASVE	Veterinary Sanitary Alert Network (*Red de Alerta Sanitaria Veterinaria*)
RBC	red blood cells
RNA	ribonucleic acid
RT-qPCR	real time reverse transcriptase polymerase chain reaction
SC	secreting cells
SPSS	Statistical package for social sciences
SNT	serum neutralisation test
$TCID_{50}$	median tissue culture infectious dose
TMB	3,3',5,5'-tetramethylbenzidine
VERO	African green monkey kidney cells
WBC	white blood cells

Cridem qui som i que tothom ho escolti.
I en acabat, que cadascú es vesteixi
com bonament li plagui, i via fora!,
que tot està per fer i tot és possible.

(Ara mateix – Miquel Martí i Pol)

RESUM

La llengua blava (LB) és una malaltia transmesa per vectors *Culicoides* de declaració obligatòria causada pel virus de la llengua blava (VLB) i considerada emergent i re-emergent a Europa. Aquesta malaltia afecta principalment a remugants domèstics i salvatges i també camèlids, causant importants pèrdues econòmiques en el sector ramader. La implicació de diferents hostes i vectors en el cicle de transmissió del VLB dificulta el control de la malaltia. El cicle de transmissió del virus està condicionat per factors externs, com són el canvi climàtic i l'alteració dels ecosistemes, els quals han afavorit l'expansió dels vectors en les últimes dècades. Entre les mesures de control, destaquen la vacunació, la restricció de moviment d'animals susceptibles en zones de risc i els programes de vigilància epidemiològica, tant d'animals domèstics com de vectors. Tot i que les vacunes comercials actuals han demostrat ser efectives en remugants domèstics, l'elevat nombre de serotipus del VLB presents (fins a 26 descrits actualment), fa que sigui complicat desenvolupar una vacuna universal que ofereixi protecció creuada. Totes aquestes variables han fet impossible fins al moment l'eradicació d'aquesta malaltia.

La majoria d'espècies de remugants salvatges presents a Europa, si no tots, són susceptibles a la infecció pel VLB, que és majoritàriament asimptomàtica. Aquest fet fa que tinguin importància com a possibles reservoris i transmissors del virus, tant entre animals salvatges com de salvatges a domèstics. La informació relativa a la seva implicació en el cicle de transmissió del virus entre animals salvatges i domèstics és encara escassa. Això, juntament amb el fet que els programes de vacunacions massives s'apliquin exclusivament a remugants domèstics, posa de manifest la necessitat de portar a terme estudis addicionals a fi de determinar el paper dels remugants salvatges en l'epidemiologia de la LB.

La present tesi s'ha centrat en l'estudi de la LB en els remugants salvatges presents a la Península Ibèrica. El seu contingut segueix l'estructura típica d'un treball científic. Comença amb una Introducció, en la qual es realitza una breu revisió actual sobre la LB i el VLB, seguit d'un apartat amb els Objectius que seran tractats en cada capítol. A continuació es presenten dues Seccions estructurades en cinc

Capítols, que corresponen a articles científics en diferent estat de publicació (tres publicats i dos enviats). A l'apartat de Discussió general es pretén donar una breu visió del conjunt de capítols i, finalment, s'ennumeren totes les Conclusions obtingudes d'aquesta tesi doctoral.

En la primera secció de la tesi (Epidemiologia) s'han realitzat estudis serològics i virològics retrospectius per tal d'aportar més informació de l'evolució de la LB en les espècies de remugants salvatges presents a la Península Ibèrica. Aquests estudis indiquen que aquestes espècies estan implicades en el manteniment del VLB i que poden actuar com a reservoris del virus a la Península Ibèrica. En la segona secció (Vacunació i infecció experimental) s'ha demostrat la susceptibilitat a la infecció amb els serotipus 1 i 8 del VLB del cérvol (*Cervus elaphus*) i la cabra salvatge (*Capra pyrenaica*). A més, s'ha avaluat la protecció induïda per una dosi (en cabra salvatge) o dues dosis (en cérvol) vacunals enfront la inoculació experimental amb soques homòlogues del virus. Finalment, s'ha realitzat un estudi longitudinal del desenvolupament d'anticossos neutralitzants fins a 18 mesos després de la immunització a la cabra salvatge.

RESUMEN

La Lengua azul (LA) es una enfermedad de declaración obligatoria causada por el virus de la lengua azul (VLA) y considerada emergente y reemergente en Europa. Esta enfermedad afecta especialmente a rumiantes domésticos y salvajes y también camélidos, causando importantes pérdidas económicas en el sector ganadero. La implicación de diferentes hospedadores y vectores en el ciclo de transmisión del VLA dificulta el control de la enfermedad. Dicho ciclo está condicionado por factores externos como son el cambio climático y la alteración de ecosistemas, los cuales han favorecido la expansión de los vectores en las últimas décadas. Entre las estrategias de control destacan la vacunación, la restricción del movimiento de animales susceptibles en zonas de riesgo y los programas de vigilancia epidemiológica tanto de animales domésticos como de vectores. Aunque las vacunas comerciales actuales han demostrado ser efectivas en rumiantes domésticos, el elevado número de serotipos del VLA presentes (hasta 26 descritos actualmente), hace que sea complicado desarrollar una vacuna universal que ofrezca protección cruzada. Todas estas variables han hecho imposible hasta el momento la erradicación de la enfermedad.

La mayoría de especies de rumiantes salvajes presentes en Europa, si no todos, son susceptibles a la infección por el VLA, que es mayoritariamente asintomática. Esto los hace importantes como posibles reservorios y transmisores del virus, tanto entre animales salvajes como de salvajes a domésticos. La información relativa al estudio de su implicación en el ciclo de transmisión entre salvajes y domésticos es todavía escasa. Ésto, unido al hecho de que los programas de vacunaciones masivas se apliquen exclusivamente a rumiantes domésticos, pone de manifiesto la necesidad de llevar a cabo estudios adicionales con el fin de determinar el papel de los rumiantes salvajes en la epidemiología de la LA.

La presente tesis se ha centrado en el estudio de la LA en rumiantes salvajes presentes en la Península Ibérica. Su contenido se organiza siguiendo el orden habitual de un trabajo científico. Comienza con un apartado de Introducción, en el que se realiza una breve revisión actual sobre la LA y el VLA, seguido de un

apartado de los Objetivos que se abordarán en cada capítulo. A continuación se presentan dos Secciones estructuradas en cinco Capítulos, que corresponden a artículos científicos en diferente estado de publicación (tres aceptados y dos enviados). En el apartado de Discusión general se pretende dar una breve visión del conjunto de capítulos y, para finalizar, se enumeran todas las Conclusiones obtenidas en la tesis doctoral.

En la primera sección de la tesis (Epidemiología) se han realizado dos estudios serológicos y virológicos retrospectivos con el fin de aportar más información sobre la evolución de la LA en las especies de rumiantes salvajes presentes en la Península Ibérica. Estos estudios indican que estas especies están implicadas en el mantenimiento del VLA y que pueden actuar como reservorios del virus en la Península Ibérica. En la segunda sección (Vacunación e infección experimental) se ha demostrado la susceptibilidad a la infección con los serotipos 1 y 8 del VLA en el ciervo (*Cervus elaphus*) y la cabra montés (*Capra pyrenaica*). En estos experimentos, se ha evaluado la protección inducida por una dosis (en cabra montés) o dos dosis (en ciervo) vacunales frente a la inoculación experimental con cepas homólogas del virus. Finalmente, se ha realizado un estudio longitudinal del desarrollo de anticuerpos neutralizantes hasta 18 meses después de la inmunización en la cabra montés.

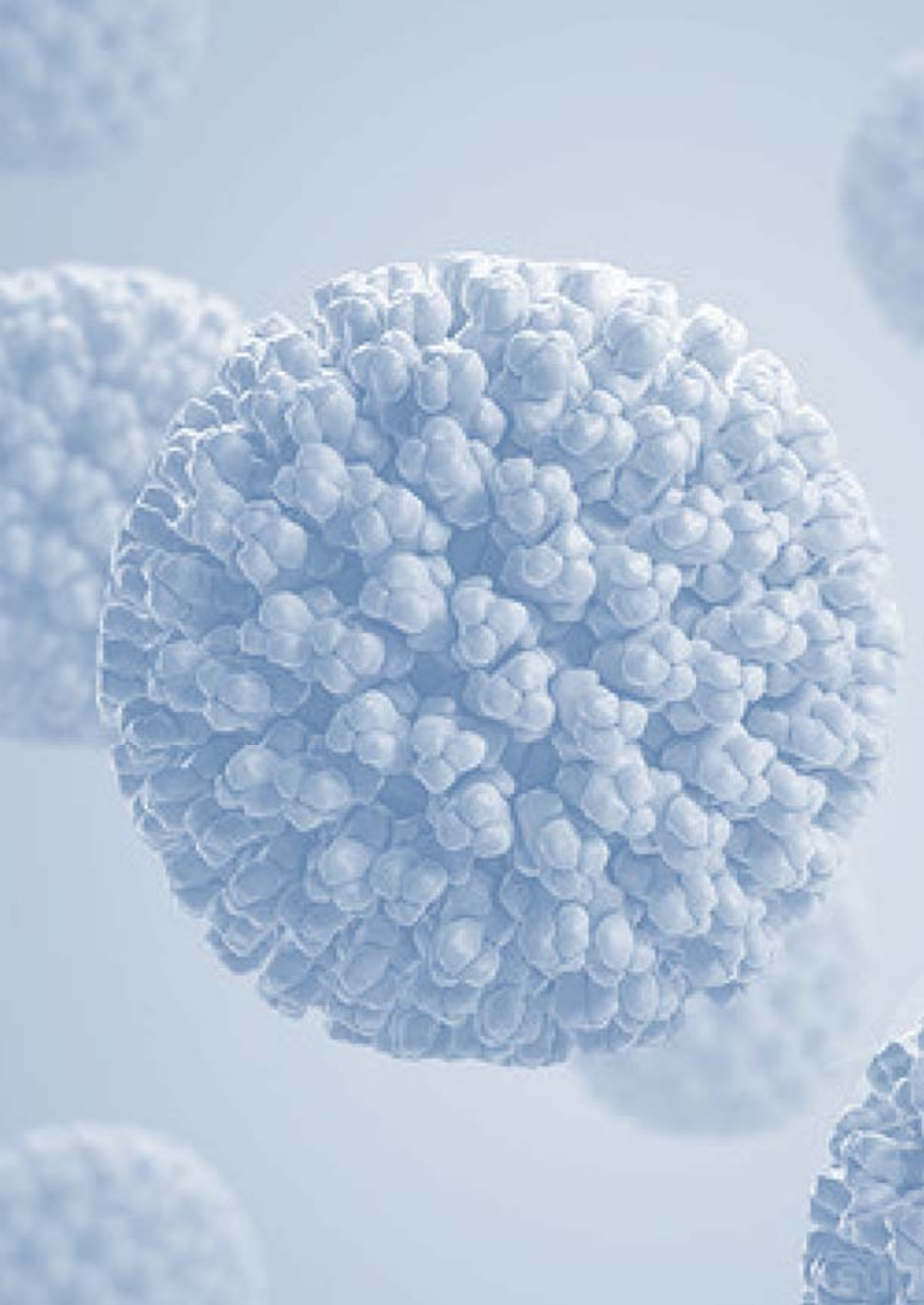
SUMMARY

Bluetongue (BT) is a reportable disease caused by bluetongue virus (BTV) considered emerging and re-emerging in Europe. BT affects especially domestic and wild ruminants and also camelids, causing important economic losses in the animal industry. The implication of different hosts and vectors in the transmission cycle of BTV makes difficult to control the disease. The transmission cycle is affected by external factors, as climate change and ecosystems' alteration, which have favored vector expansion in the last decades. Among the control measures, vaccination, restricted movement of susceptible hosts during risk periods and epidemiologic surveillance programs including livestock and vectors are the most implemented. Although available commercial vaccines have proven to be effective in domestic ruminants, the high number of BTV serotypes (up to now, 26 described) makes difficult the development of a universal vaccine able to confer cross-protection. All these factors have made impossible the eradication of this disease.

Most, if not all, wild ruminant species present in Europe are susceptible to BTV infection, although it is mainly asymptomatic. This fact makes wild species important as potential reservoirs and transmitters among wildlife or from wild to domestic ruminants. Data related to the implication of wild ruminants in the BTV transmission cycle between domestics and wildlife is still limited. This point, and also the fact that mass vaccination campaigns are applied exclusively to domestic ruminants highlights the need to carry out additional studies with the aim of determining the role of wild ruminants in the epidemiology of BT.

The present thesis is focused in the study of BT in wild ruminants present in the Iberian Peninsula. The structure is the typical of a scientific paper. It starts with an Introduction, which contains a brief review of BT and BTV, followed by the Objectives that will be developed in each chapter. Afterwards, there are two Sections structured in five Chapters. All the studies are published or submitted to publish in international peer-reviewed journals. In the General discussion section is given a summary of the main findings and, finally, all the Conclusions obtained are listed at the end of the thesis.

In the first section of the thesis (Epidemiology) two retrospective serological and virological studies have been carried out in order to provide new information regarding the evolution of BT in wild ruminant species present in the Iberian Peninsula. These studies indicate that wild ruminants are implicated in maintaining BTV, and they may play a relevant role as BTV reservoirs in the Iberian Peninsula. In the second section (Vaccination and experimental infection), the susceptibility to BTV-1 and BTV-8 infection has been demonstrated in red deer (*Cervus elaphus*) and Spanish ibex (*Capra pyrenaica*). Moreover, the efficacy of two commercial vaccines has been evaluated by means of specific neutralising antibodies and absence of viraemia in both species, vaccinated and experimentally inoculated with homologous strains. Finally, it has been carried out a longitudinal study of the development of neutralising antibodies until 18 months post-immunization in Spanish ibex.



1. General Introduction

1.1. Bluetongue

Importance

Bluetongue (BT) is an arthropod-transmitted viral disease of ruminants, caused by bluetongue virus (BTV). Due to its economic impact, BT is an *Office International des Epizooties* (OIE)-listed disease. Economic losses associated with BTV infection are caused directly through reductions in productivity and death and, more importantly, indirectly through trade losses due to animal movement and cattle semen export restrictions, and the costs of implementing control measures, including diagnostic tests [Schwartz-Cornil *et al*., 2008].

History

BT was described for the first time in Cape Colony, South Africa, as “fever” or “malarial catarrhal fever”, after the introduction of Merino sheep at the end of the 18th century. The term of “bluetongue” was introduced from the English translation of the Afrikaans name “Blaauwtong” [Henning, 1956; Spreull, 1905]. Local farmers used this word to describe the cyanotic tongue of heavily affected sheep. In 1905, the aetiological agent was identified. In 1944, it was discovered that members of the *Orbivirus* genus, including both BTV and African horse sickness virus (AHSV), were transmitted by *Culicoides* midges [Du Toit, 1944; Verwoerd, 2009]. Later on, the disease spread rapidly throughout Africa and subsequently to many countries beyond the African continent. BT was described in other breeds of sheep and in Europe, America and Asia, probably due to transport of infected animals and vector expansion. Currently, it seems that cattle have replaced antelope as a maintenance host of the virus [Gerdes, 2004].

Aetiology

BTV is the prototype of the genus *Orbivirus* within the family *Reoviridae* [Mertens *et al*., 2004], which includes other viruses responsible of important animal diseases as African horse sickness (AHS), epizootic haemorrhagic disease (EHD) or equine encephalosis. BTV is a non enveloped virus with a genome of approximately 19.200 base pairs composed by ten linear segments of double-stranded RNA (dsRNA). The

ten dsRNA segments are packaged within a triple layered icosahedral protein capsid (Figure 1). There are at least eleven proteins (seven structural and four non-structural) in BTV. The outer shell is composed of two structural proteins, VP2 and VP5, where VP2 is the major determinant of BTV serotype, with a minor role for VP5 [Mertens *et al*., 1989]. The intermediate layer consists of the major immunodominant VP7 structural protein. The subcore is composed by the VP3 protein, which houses the viral genome segments and three minor proteins involved in transcription and replication which are VP1, VP4 and VP6. The four non-structural proteins (NS1, NS2, NS3/NS3A and NS4) are probably implicated in the control of replication, maturation and export from the infected cell [Ratinier *et al*., 2011; Roy *et al*., 2009; Schwartz-Cornil *et al*., 2008].

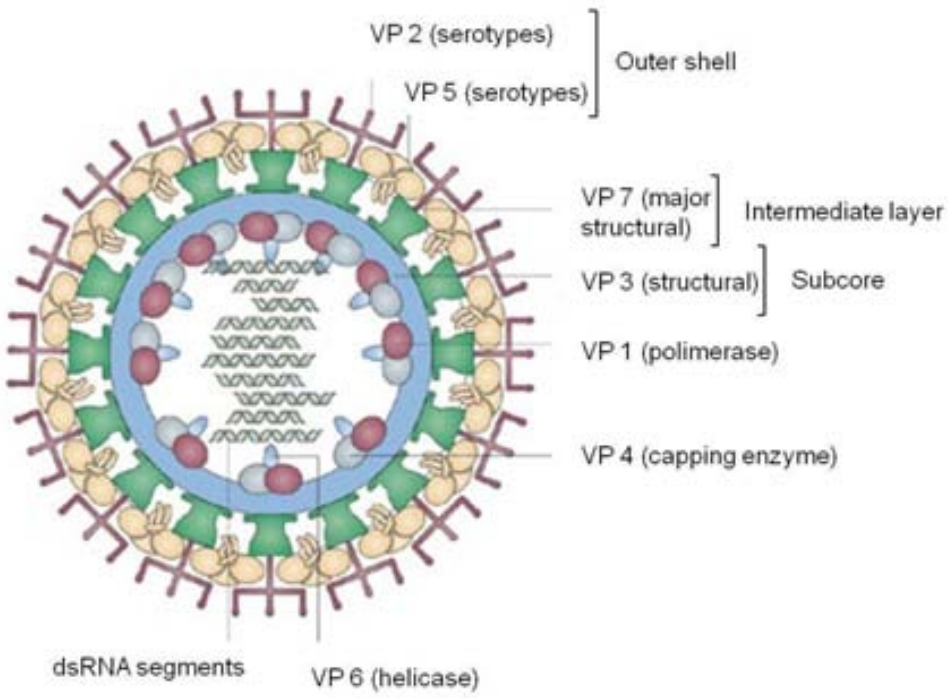


Figure 1. Bluetongue virus morphology [adapted from Roy *et al*., 2009]

1.2. Epidemiology

Since BTV transmission between its vertebrate hosts depends almost exclusively on the bites of haematophagous midges of the genus *Culicoides*, its world distribution is limited to geographical areas where competent vector species are present (Figure 2). Transmission also depends on those periods of the year when climatic conditions are favourable for adult vector activity, and temperatures are warm enough to allow first the virus replication within the vector and then transmission to a susceptible host [Mertens *et al*., 2008]. Therefore, the disease was traditionally considered to be confined to tropical and subtropical areas between latitudes 35ºS and 40ºN [Purse *et al*., 2005].

Bluetongue distribution in Europe

Before 1998

Before 1998, BT was considered an exotic disease in Europe. Its distribution coincided with the known distribution of the Afro-Asiatic species *C. imicola*, and punctual outbreaks were reported in Cyprus, Spain, Portugal, Greece, and Turkey [Mellor & Boorman, 1995]. The limited number of epidemics led European countries to believe that the risk of BT was low [Carpenter *et al*., 2009].

Between 1998 and 2006

From 1998 to 2005, five serotypes (1, 2, 4, 11 and 16) appeared frequently in twelve Mediterranean countries as Italy, Greece, Turkey, France, Spain and in Northern Africa, spreading through 800 kilometres [Purse *et al*., 2008] (Figure 3). Infected livestock movement, wind and expansion of the main vector (*C. imicola*) were the most frequent causes of the outbreaks. From 2005, several serotypes (1, 2, 4, 6, 8, 9, 11 and 16) have been circulating in Europe, some far from the original distribution of *C. imicola*, pointing to the existence of new vector species. Climate change (rising temperature) contributed to the increase and presence of other competent vector populations as *C. obsoletus* and *C. pulicaris* groups [Purse *et al*., 2008; Wilson &

Mellor, 2008; Wilson & Mellor, 2009], which together with the movement of infected animals were considered the main hypothesis.

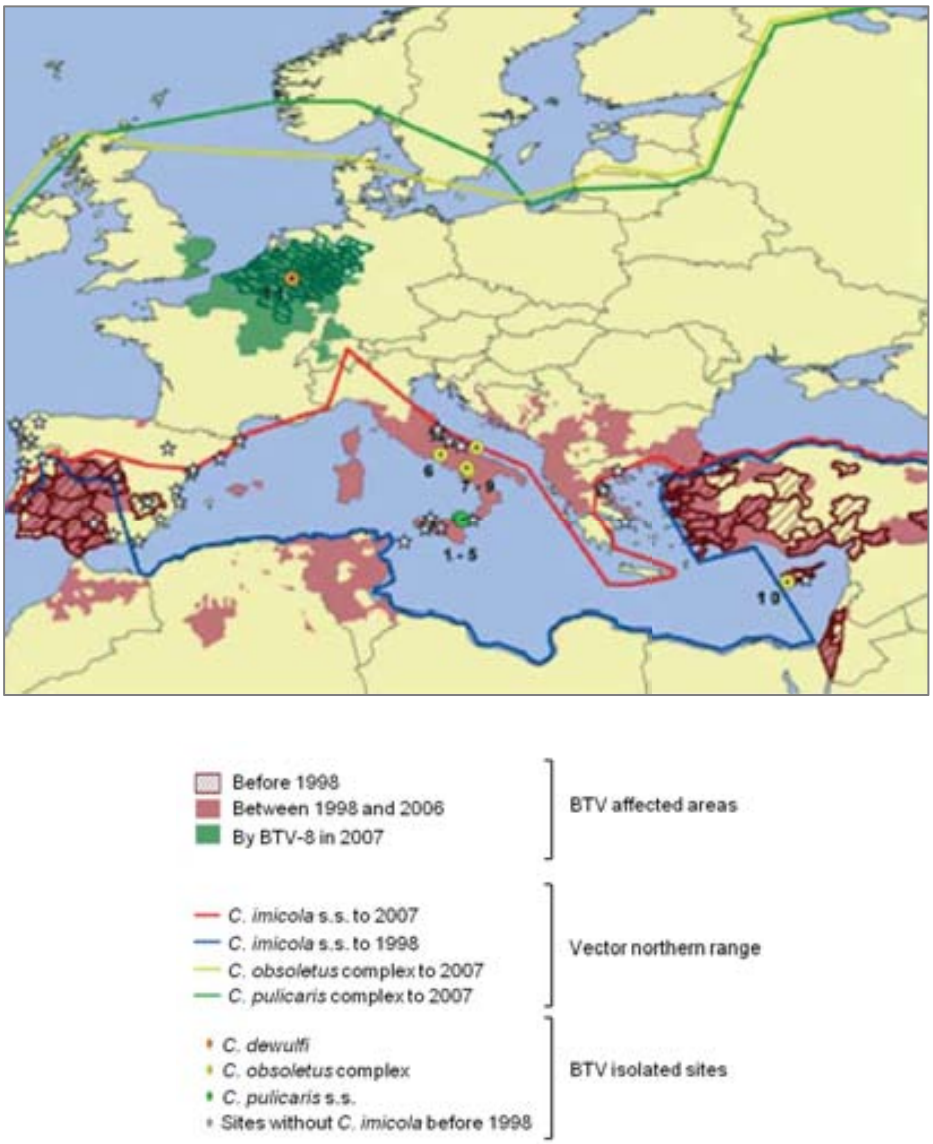


Figure 2. Distribution of bluetongue and competent vectors in Europe before and after 1998 (Southern Europe) and in 2006-2007 (Northern Europe) [adapted from Purse *et al*., 2008].

In 2000, BTV-2 appeared in the Balearic Islands, probably originating from Corsica, and in 2003 BTV-4 was detected in Menorca. In 2004, this serotype was found in Portugal. BTV-4 also reached the south of Spain from Morocco in 2004 [Purse *et al*., 2005] and expanded to the north of the Iberian Peninsula through 2005 and 2006.

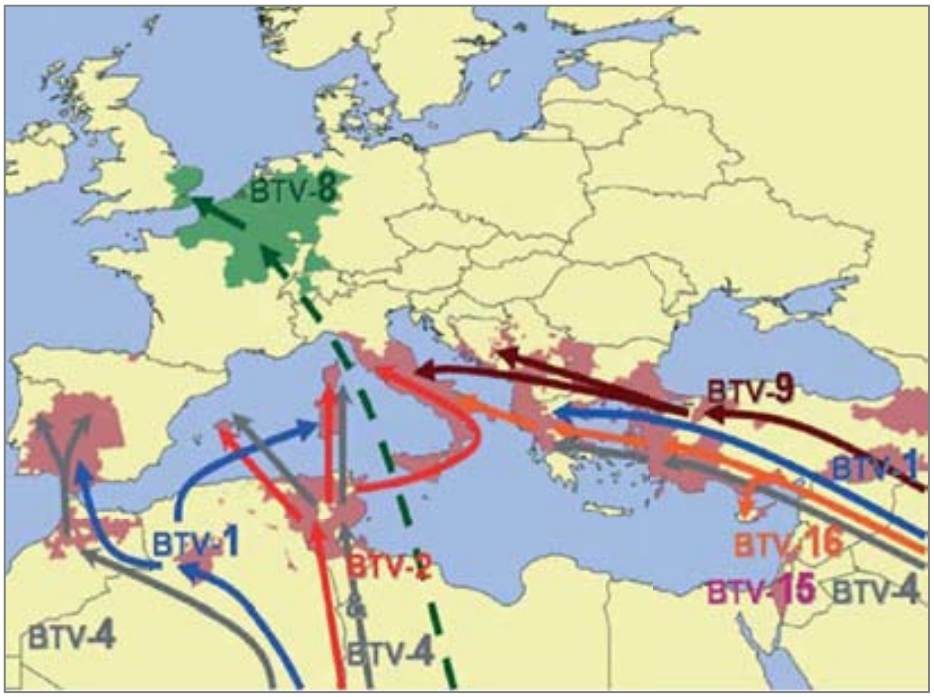


Figure 3. Epidemiology of bluetongue virus in Europe. Incursions of outbreaks that affected Europe with distinct origin routes [adapted from Purse *et al*., 2008].

From 2006 to 2012

In 2006, BTV-8 was detected in the Netherlands before spreading to Germany, Belgium and France. In 2007, BTV-8 infection dramatically extended its European distribution and reached Luxemburg, Denmark, Switzerland, Czech Republic, the United Kingdom and Italy (this last country through import of infected animals). At the beginning of 2008, BTV-8 arrived to Northern Spain. The origin of this outbreak is still unknown and many hypotheses were considered. Simultaneously, during 2007, BTV-1 appeared in Southern Spain and reached the northern regions by the end of the year, most likely through the arrival of BTV-infected *Culicoides* from warm

air masses [García-Lastra *et al*., 2012]. Currently, mostly Mediterranean countries are restricted zones for serotypes such as BTV-1, 2, 4, 8, 9 and 16.

Bluetongue in the Iberian Peninsula

In Spain, the last BTV-4 outbreak was detected in October 2004, and the country was declared officially free of BTV-4 in March 2009 [RASVE, 2009]. However, in October 2010 a new BTV-4 outbreak was detected in Southern Spain (Cádiz), and until December 2010 eight further outbreaks were reported reaching a total of thirteen BTV-4 outrbreaks. In the case of BTV-1, 3520 outbreaks have been detected from 2007 to 2012 [RASVE]. Furthermore, BTV-8 was detected in Northern Spain in January 2008 and also in Southern Spain (Andalusia) in October 2008. A total of 35 BTV-8 outbreaks were reported until March 2009, followed by a period without apparent cases until November 2010, when a new BTV-8 outbreak was confirmed in Andalusia.

1.3. Transmission and pathogenesis

The introduction of BT into an area can stem from four ways. Firstly, through the movement of infected vertebrate hosts (domestic or wild ruminants) or animal germplasm (semen or embryos). Secondly, by the movement of infected vectors carried by living (plants, animals) or inanimate (airplanes, ships) means. The third way is through the active flight of infected vector *Culicoides* and the fourth is through passive transport of infected vectors by the wind [Saegerman *et al*., 2008].

Once introduced, an infection cycle must be established between the ruminant host and the vector [Noad & Roy, 2009]. The virus is transmitted by haematophagous females of approximately thirty species of *Culicoides* midges (Diptera: *Ceratopogonidae*). Vector species differ between regions, as do BTV serotypes and strains [Maclachlan & Osburn, 2006; Tabacknick, 2004]. There are 24 BTV serotypes described, a putative 25th named *Toggenburg Orbivirus* [Chaignat *et al*., 2009; Hoffmann *et al*., 2008] and a 26th detected in Kuwait [Maan *et al*., 2011].

The historical distribution of BTV and its vectors covered a broad band, approximately, between latitudes 35ºS and 40ºN [Mellor, 2000], although in the last years it has extended over 50ºN. Thus, asymptomatic species, and those showing less severe disease, may act as an amplifying host for the subsequent infection of more susceptible species [Noad & Roy, 2009].

After blood feeding from an infected host, BTV passes into the lumen of the *Culicoides* mid-gut. Then, the virus reaches the salivary glands, with or without amplification in other susceptible tissues. Once in salivary glands, the virus multiplies and is released into the saliva, where it is available to infect a second vertebrate host [Mellor, 2004]. When the competent vector bites a susceptible host, the virus reaches lymph nodes, where replication occurs [Barratt-Boyes *et al*., 1995; Pini, 1976]. Afterwards, BTV disseminates to other target tissues such as lung and spleen, where replication takes place again, mainly in phagocytic mononuclear cells, endothelial cells, lymphocytes and other cell types [Barratt-Boyes & Maclachlan, 1994; Darpel *et al*., 2009; Ellis *et al*., 1993; Mahrt & Osburn, 1986; Maclachlan *et al*., 1990]. Finally, BTV associates to platelets and erythrocytes during viraemia and, because of the short lifespan of platelets, virus is largely or exclusively associated with erythrocytes late in the course of BTV infection of ruminants [Maclachlan *et al*., 2009]. BTV association to erythrocytes facilitates both prolonged infection of ruminants and infection of haematophagous insect vectors that feed on viraemic ruminants, and infectious virus can co-circulate for several weeks with high titres of neutralising antibody [Barratt-Boyes & Maclachlan, 1995; Bonneau *et al*., 2002; Brewer & Maclachlan, 1992; Brewer & Maclachlan 1994].

BTV infection is often subclinical or unapparent, but can lead to severe disease with high mortality rates in certain domestic and wild species. Pathogenesis is similar in sheep and cattle and probably in all ruminants [Barratt-Boyes & Maclachlan, 1995; Darpel *et al*., 2007; Maclachlan, 1994; Mahrt & Osburn, 1986; Pini, 1976].

However, there are differences in the severity of the disease between species and breeds after infection and in the infection of the same species with different serotypes [Gard, 1984; Verwoerd & Erasmus, 2004]. Sheep is considered the more susceptible species, with mortality rates between 50-70% in susceptible flocks, but

many factors like breed, serotype, individual differences, nutritional and immunologic status, age or environmental stress (such as high temperatures) can influence the susceptibility [Gard, 1984; Verwoerd & Erasmus, 2004]. Bovine, caprine and other wild ruminants are mostly asymptomatic or have subclinical infections, although BTV serotype 8 (BTV-8) has been reported to cause disease in cattle and camelids [Backx *et al*., 2007; Conraths *et al*., 2009; Darpel *et al*., 2007; Henrich *et al*., 2007].

The onset of the disease in BTV-infected ruminants is typically marked by fever lasting approximately 5-7 days, which correlates with a peak of viraemia. Afterwards, lesions reflect virus-mediated injury to small blood vessels, causing cell injury and necrosis and leading to vascular thrombosis, tissue infraction and haemorrhages. In sheep, principal lesions are congestion, oedema and haemorrhages as a consequence of the vascular injury [Darpel *et al*., 2009; Erasmus, 1975; Maclachlan, 2008; Maclachlan *et al*., 2009; Mahrt & Osburn, 1986; Pini, 1976; Verwoerd & Erasmus, 2004], together with fever, serous to bloody nasal discharge, respiratory difficulty in animals with severe pulmonary oedema, oral erosions and ulcers, lameness with hyperemia of the coronary band and weakness secondary to muscle necrosis. In cattle, BTV-8 can cause severe and extensive ulceration of the muzzle, oral mucosa and teats; rhinitis and muco-haemorrhagic nasal discharge; epiphora and periocular inflammation; and limb oedema [Darpel *et al*., 2007; Elbers *et al*., 2008a; Thirty *et al*., 2006]. In contrast to sheep, infected cattle experience prolonged viraemia [Maclachlan *et al*., 2009].

1.4. Bluetongue in wildlife

Animals, and particularly wild animals, are thought to be the source of more than 70% of all emerging infections [Taylor *et al.*, 2001]. Wildlife diseases are relevant for their effect on animal health and wildlife conservation, as well as the possibility to be zoonoses. The interaction between wild and domestic animals allows the transmission of pathogens that may compromise livestock production. Direct mortality of animals from emerging infections and depopulation policies to protect the safety of international trade and to control the spread of pathogens affect negatively both wild and domestic species [Kuiken *et al.,* 2005]. Susceptible game

species are in general the most important for animal health for their abundance and their phylogenetic proximity to domestic ruminants. In addition, these species are the most accessible for sampling during hunting seasons, and management of wild ruminants with the aim of rearing hunting species is similar to extensive livestock farming. Thus, most wildlife species are susceptible to the same diseases than domestic animals. In the case of ruminants, bluetongue, transmissible spongiform encephalopathy, foot-and-mouth disease, rinderpest, rift valley fever, brucellosis, and tuberculosis, among other infections, are shared between wild and domestic species [Gavier-Widén *et al*., 2012].

Wild ruminants include cervids (family *Cervidae*) and bovids (family *Bovidae*). In the Iberian Peninsula, there are three species of cervids: red deer, fallow deer (*Dama dama*) and roe deer (*Capreolus capreolus*). Especially red deer and roe deer are widely distributed and can reach a density of 10 individuals per km^2 [Lovari *et al*., 2008]. The Iberian bovids include two autochthonous species, the southern chamois (*Rupicapra pyrenaica*) and the Spanish ibex (*Capra pyrenaica*); a feral domestic goat species (*Capra hircus*); and two exotic species introduced for hunting purposes, the European mouflon and the aoudoad or Barbary sheep (*Ammotragus lervia*) [Spanish Wildlife Disease Surveillance Scheme, *Plan Nacional de Vigilancia Sanitaria en Fauna Silvestre*, 2011].

In this thesis, wild ruminant species have been selected for its abundance and wide distribution, as well as for being endemic species, as it is the case of Spanish ibex (Figure 4). Although the term Iberian ibex would be more appropriate and reflects the real distribution (not only in Spain but also in Portugal), the species has been named as Spanish ibex according to the IUCN criteria [IUCN, 2012].

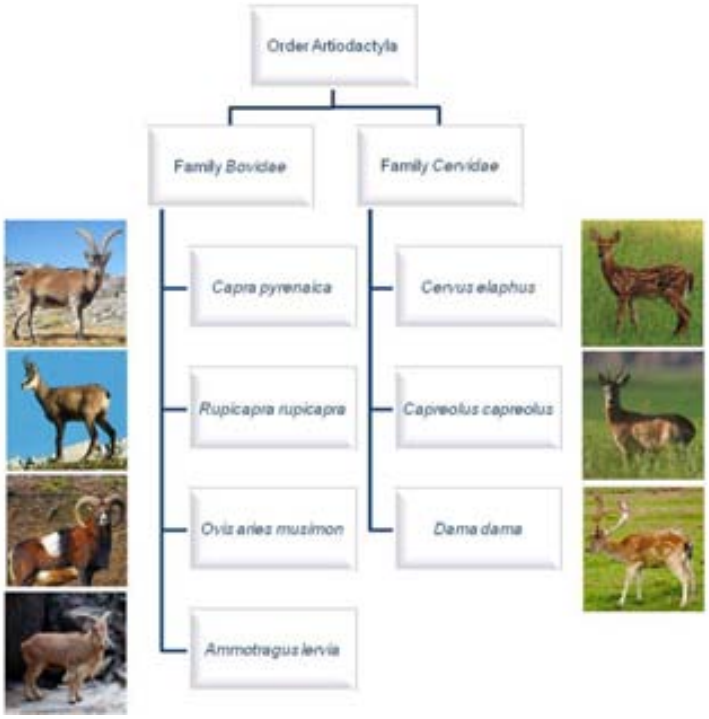


Figure 4. Classification of the wild ruminant species studied in the present thesis.

BT has also been described in wild ruminant species [Verwoerd & Erasmus, 2004]. White-tailed deer (*Odocoileus virginianus*) can develop depression, weakness, fever, loss of fear, anorexia, hyperemic oral mucosa, muco-purulent nasal discharge, crusts in the nares, severe respiratory distress, submandibular oedema, reddening of the muzzle and ears, oedema around the eyes, swollen and cyanotic tongue, excessive salivation, oral ulceration, multifocal haemorrhage in the skin and mucosa, severe bloody diarrhoea, laminitis and consumption coagulopathy, as a consequence of BTV-induced endothelial damage. The only appreciable haematological change described is leucopenia and neutropenia [Howerth & Tyler, 1988; Johnson *et al*., 2006; Vosdingh *et al*., 1968]. Clinical signs reported in the European mouflon (*Ovis aries musimon*), considered a subspecies of domestic sheep, include inflammation of mucous membranes, congestion, swelling and haemorrhages [Fernández-Pacheco *et al*., 2008]. The disease has been described also in free-ranging and captive (zoo) wild ruminants including musk (*Moschus moschiferus*), ox (*Ovibos moschatus*), bison (*Bison bison*), mouflon and yak (*Bos*

grunniens) [Falconi *et al*., 2011; Linden *et al*., 2008; Maclachlan *et al*., 2009; Rodríguez-Sánchez *et al*., 2010b]. Conversely, BTV-infection of less susceptible wild ruminants is asymptomatic or causes only mild clinical signs. In European red deer (*Cervus elaphus*), no clinical signs have been reported, both after natural and experimental BTV-infection [García *et al*., 2009; López-Olvera *et al*., 2010; Rodríguez-Sánchez *et al*., 2010a; Rossi *et al*., 2009; Ruiz-Fons *et al*., 2008]. Moreover, although South American camelids have been considered to be resistant to BT, lethal BTV infection of captive llamas (*Lama glama*) and alpacas (*Vicugna pacos*) has been described in Europe [Henrich *et al.,* 2007; Meyer *et al*., 2009], with acute or severe respiratory distress.

As mentioned above, red deer and Spanish ibex are susceptible to BTV infection, and antibodies against BTV and BTV RNA have been detected in naturally [Fernández-Pacheco *et al*., 2008; García *et al*., 2009; Linden *et al*., 2008; Linden *et al.,* 2010; Rodríguez-Sánchez *et al*., 2010a; Rodríguez-Sánchez *et al*., 2010b; Ruiz-Fons *et al*., 2008] and experimentally infected animals of both species [López-Olvera *et al*., 2010]. As susceptible hosts, they may have a potential role in the transmission and maintenance of BTV [Falconi *et al*., 2011; Falconi *et al*., 2012; García-Bocanegra *et al*., 2011]. A summary of published data regarding BTV in wild ruminants is shown in Table 1.

1.5. Laboratorial diagnostic

The orbiviral species are differentiated by immunological tests detecting conserved viral proteins, and hence are distinguishable by serogrouping tests. Serotypes are identified by neutralisation tests and different strains within a serotype are identified by sequence analysis [OIE, 2009]. Virus identification traditionally requires isolation and amplification of the virus in embryonated hens' eggs, tissue culture or inoculations of susceptible ruminants and the subsequent application of serogroup- and serotype-specific tests [Clavijo *et al*., 2000]. Reverse-transcription polymerase chain reaction (RT-PCR) technology has permitted rapid amplification of BTV RNA in clinical samples, and RT-PCR-based procedures are available [Katz *et al*., 1993; OIE, 2009; Toussaint *et al*., 2007].

Table 1. **Prevalence of BTV specific antibodies and BTV RNA detection in European wild ruminants** [modified from Falconi *et al*., 2011]. Nd = non determined

Host species	Country	Year	Seroprevalence	BTV RNA	Serotype	Reference
Red deer (Cervus *elaphus*)	Spain	2005-2007	21.9%	Nd	1	(1)
		2007	57.6%	16.3-25%	1,4	(2)
		2006-2007	66.3%	Nd	1	(3)
		2006-2010	42.3	2.1% (19)	1,4,8	(4)
		2005-2010	12.9%	Not found	4	(5)
	Belgium	2006	0.9%	Nd	8	(6)
		2006	1.5%	Not found	8	(7)
		2007	40.4%	Nd	8	(6)
		2007	52.3%	Not found	8	(7)
		2008	34.0%	Not found	8	(7)
	France	2008	37.7%	Unknown %	1,8	(8)
Fallow deer (*Dama dama*)	Italy	2004-2005	0.5%	Not found	-	(9)
	Spain	2005-2007	35.4%	Nd	1	(1)
		2006-2007	50.0%	Nd	1	(3)
		2006-2010	32.4%	Not found	1,4,8	(4)
Roe deer (*Capreolus capreolus*)	Spain	2005-2007	5.1%	Nd	1	(1)
		2006-2010	2.0%	Not found	1,4,8	(4)
	Belgium	2006	2.6%	Not found	8	(7)
		2007	2.8%	Not found	8	(7)
		2008	1.7%	Not found	8	(7)
	France	2008	1.2%	Nd	1,8	(8)
Spanish ibex (C*apra pyrenaica*)	Spain	2006-2007	10.8%	Nd	1	(3)
		2009	5.8%	Nd	-	(10)
Ibex (*Capra ibex*)	France	2008	1.6%	Nd	1,8	(8)
European mouflon (*Ovis aries musimon*)	Spain	2005-2007	13.2%	Nd	1	(1)
		2006-2010	27.7%	1 positive	1,4,8	(4)
		2007	-	2/2	4	(11)
Northern chamois (*Rupicapra rupicapra*)	France	2008	1.1%	Nd	1,8	(8)
Aoudad (*Ammotragus lervia*)	Spain	2005-2007	25%	Nd	1	(1)
Goitered gazelle (*Gazella subgutturosa*)	Turkey	2005	40.2%	Nd	-	(12)

(1) Ruiz-Fons *et al*., 2008; (2) Rodríguez-Sánchez *et al*., 2010a; (3) García *et al*., 2009; (4) García-Bocanegra *et al*., 2011; (5) Falconi *et al*., 2012; (6) Linden *et al*., 2008; (7) Linden *et al*., 2010; (8) Rossi *et al*., 2009; (9) De Curtis *et al*., 2007; (10) Santiago-Moreno *et al*., 2011; (11) Rodríguez-Sánchez *et al*., 2010b; (12) Gür, 2008.

Ruminants infected with BTV develop a high titre antibody response to several viral proteins. This serological response appears some 7–14 days after BTV infection and is generally long-lasting. Antibodies directed against the core protein VP7 may be detected with serogroup-reactive assays such as agar gel immunodiffusion and competitive enzyme-linked immunosorbent assay (c-ELISA). On the other hand, serotype-specific neutralising antibodies directed against VP2 can be detected by serum neutralisation test (SNT). Procedures to determine the serotype-specificity of antibodies in sera are more complex and time-consuming, because they assess whether the sera inhibit the infectivity of panels of known virus serotypes in neutralisation tests [OIE, 2009]. The same procedures are used both for domestic or wild ruminants (Table 2). Samples commonly used for diagnostic tests are blood (from living animals) or spleen, liver, red bone marrow, heart blood or lymph nodes (from freshly dead animals) [OIE, 2009].

1.6. Immune response to BTV infection

In domestic ruminants, viraemia is usually detected at 2-4 days post-infection (dpi) and peaks around 7-9 dpi, decreasing afterwards, and seroconversion occurs around 7 dpi coinciding with the viraemia peak. Both humoral and cellular immune effectors mechanisms react against BTV infection and disease [Schwartz-Cornil *et al*., 2008]. BTV specific antibodies can confer protection in a serotype specific manner, suggesting an *in vivo* role for antibody-mediated viral neutralisation [Jeggo *et al*., 1984]. VP2 and VP5 are the only BTV proteins shown to induce neutralising antibodies [Lobato *et al*., 1997, Roy *et al*., 1990], VP2 being the major protein involved in serotype specificity. Neutralising antibodies generally protect only against homologous virus, although serial infections of sheep with two serotypes can protect the animals against challenge with a third serotype [Jeggo *et al*., 1984].

Depending on the serotypes, the antibodies can protect against the infection by a limited number of other serotypes, associated to similarities of sequences in VP2 [Maan *et al*., 2007; Umeshappa *et al*., 2010]. However, the level of neutralising antibodies does not always correlate with the degree of protection [Jeggo *et al*.,

1984], and protection in the absence of detectable levels of neutralising antibodies has also been reported [Stott *et al*., 1979].

Table 2. Procedures for BTV identification and antibody detection [adapted from OIE, 2009]

Identification of the agent		
a. Virus isolation	• In embryonated hens' eggs • In cell culture • In sheep	
b. Immunological methods	Serogrouping of viruses	• Immunofluorescence • Antigen capture ELISA • Immunospot tests
	Serotyping by virus neutralisation	• Plaque reduction • Plaque inhibition • Microtitre neutralisation • Fluorescence inhibition test
c. Reverse-transcription polymerase chain reaction (RT-PCR)	• Extraction of viral RNA • RT-PCR • Electrophoretic analysis of RT-PCR product	
d. Real-time RT-PCR tests		
Serological tests		
a. Complement fixation		
b. Agar gel immunodiffusion		
c. Competitive ELISA		
d. Indirect ELISA		

1.7. Bluetongue control

Both sanitary and medical prophylaxes are used to control BT. Sanitary prophylaxis includes animal movement and vector control, while medical prophylaxis is based on vaccination. Pathogen surveillance varies greatly among countries, and it is less intensive in wildlife (when it exists) than in domestic animals [Kuiken *et al*., 2005].

Vaccines may be used for different purposes or strategies, depending on the epidemiological situation of the affected area and the strategy desired. The main purposes of BT vaccination strategies are to prevent clinical disease, to limit the regional extension of BTV infection by reducing the spread of the virus, to allow regional or country eradication of the disease based on the reduction of BTV circulation, and to authorize the safe movement of susceptible animals between affected and free zones [Savini *et al*., 2008]. Currently, both attenuated and inactivated vaccines are available, which confer serotype-specific protection, probably due to the key role of the outer protein VP2 in the B and T cell mediated protective immunity [Schwartz-Cornil *et al*., 2008]. Attenuated vaccines are not used currently in Europe, due to the possibility to infect unvaccinated animals and reassortment with field strains. Several monovalent or bivalent commercial inactivated vaccines are available, and probably are the most effective tool to control BT. However, in endemic areas where multiple BTV serotypes may be present, efficient vaccines against several serotypes may be necessary. Furthermore, vaccines against BTV need to be safe and should allow differentiating between vaccinated and infected animals (DIVA vaccines). Due to these three requirements (multi-serotypes, safety and DIVA properties), vaccination against BTV is a complex and controversial issue [Schwartz-Cornil *et al.,* 2008]. There is a need to develop vaccines and strategies that allow rapid protection for emerging BTV strains, and to identify animals vaccinated against one serotype but infected and viraemic for another serotype.

To reduce direct losses due to disease and indirect losses due to trade restriction caused by BTV circulation, European authorities have undertaken vaccination campaigns according to their individual national policies, the geographic distribution of the incurring BTV serotype(s), and the availability of appropriate vaccines [Savini *et al*., 2010]. Therefore, in areas like Europe where only limited

numbers of BTV serotypes are currently present, biosecurity will remain of paramount importance in preventing future outbreaks [Noad & Roy, 2009]. To effectively control BTV, vaccination campaigns in Europe should achieve at least 80% coverage of susceptible ruminants using inactivated vaccines, a threshold that suggested would all but halt spread of the disease [Enserink, 2008]. Wild ruminants are known to be susceptible hosts but they are not included in the control strategies, which would make vaccination fail to reach this target. Further studies are needed in order to understand their role in the vector-host cycle among wild and domestic ruminants and their potential as a source for BTV re-emerging, as well as their response to BTV vaccination.

2. OBJECTIVES

2.1. General objective

BTV-1 and BTV-8 outbreaks have affected Spain and other European countries from 2006 to 2008, and Spain is currently considered a restricted zone for BTV-1, BTV-4 and BTV-8. However, the role of wild ruminants as potential reservoirs of BTV in the Iberian Peninsula remains to be determined.

The general objective of the present thesis is to evaluate the implication of wild ruminants in the epidemiology of BT in the Iberian Peninsula and to determine the protection conferred by vaccination in these species.

2.2. Specific objectives

1. To study the epidemiology of BTV-1, BTV-4 and BTV-8 in the wild ruminants of the Iberian Peninsula [Chapters 1 and 2]

2. To evaluate the susceptibility to BTV-1 and BTV-8 infection in red deer (*Cervus elaphus*) and Spanish ibex (*Capra pyrenaica*) [Chapters 3 and 4]

3. To evaluate the protection conferred by two commercial inactivated vaccines against BTV-1 and BTV-8 in red deer and Spanish ibex [Chapters 3 and 4]

4. To determine the duration of specific BTV-1 and BTV-8 antibodies after vaccination in Spanish ibex [Chapter 5]

I. EPIDEMIOLOGY

3. Chapter 1

Epidemiological surveillance of bluetongue virus in wild ruminants from 2006 to 2011 in the Iberian Peninsula

Submitted

3.1. Abstract

Wild and domestic ruminants are susceptible to Bluetongue virus (BTV) infection, which is transmitted by biting midges of the genus *Culicoides* and is the causal agent of Bluetongue (BT). Three BTV serotypes (BTV-1, BTV-4 and BTV-8) have been detected in Spain since 2004. Control strategies have been applied to livestock, but BTV still persists in wild ruminant populations [García *et al*., 2009; García-Bocanegra *et al*., 2011; Ruiz-Fons *et al*., 2008]. The aim of the present study is to describe the epidemiology of BTV in free-ranging wild ruminants in the Iberian Peninsula.

A total of 2011 sera samples from red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), southern chamois (*Rupicapra pyrenaica*), Spanish ibex (*Capra pyrenaica*), European mouflon (*Ovis aries musimon*) and aoudad (*Ammotragus lervia*) collected between 2006 and 2011 were tested by ELISA. All positive ELISA samples were analysed by serum neutralisation test (SNT) in order to detect specific antibodies against BTV-1, BTV-4 and BTV-8. Spleen samples from 320 seropositive animals were additionally analysed by real time RT-PCR (RT-qPCR) to detect the presence of BTV RNA.

Nine hundred and ninety-one (49.3%; $CI_{95\%:}$ 47.1-51.4) sera were positive by ELISA, while 292 out of 698 (41.8%; $CI_{95\%:}$ 38.2-45.5) were also positive by means of SNT. The remaining ELISA positive samples could not be analysed due to cytotoxicity of sera. Seropositivity to BTV-1 was found in 182 (26.3%; $CI_{95\%:}$ 23.2-29.7) sera samples, 168 (24.7%; $CI_{95\%:}$ 21.6-28.1) were positive to BTV-4 and 4 (0.6%; $CI_{95\%:}$ 0.2-1.5) to BTV-8. BTV RNA was detected in 32 out of 320 spleen samples. All positive samples were from red deer (28) and fallow deer (4).

Although BT outbreaks in livestock have decreased substantially in the last years after vaccination campaigns, our results indicate that wild ruminants have been exposed to BTV. The detection of BTV RNA is consistent with the hypothesis of virus circulation and BTV maintenance within Iberian free-ranging wild ruminant populations.

3.2. Introduction

Bluetongue (BT) is a vector-borne infectious disease that has expanded its geographical range in Europe [Enserink, 2008; Eschbaumer *et al*., 2009; European Commission, 2009; Purse *et al*., 2005; Rodríguez-Sánchez *et al*., 2008]. The causal agent, bluetongue virus (BTV) is transmitted by the bites of *Culicoides* midges. Vector and host density, as well as environmental factors are implicated in the distribution of BT. The disease is considered endemic in wild ruminants in large parts of Africa and North America [Gerdes, 2004; Stallknecht *et al*., 1996]. European wild ruminants are susceptible to BTV infection and able to participate in the maintenance and transmission of the virus [García-Bocanegra *et al*., 2011; Linden *et al*., 2010; Ruiz-Fons *et al*., 2008]. However, the role of European wild ruminants in BTV transmission and maintenance is still under debate [Durand *et al*., 2010; Falconi *et al*., 2011].

BTV serotype 1 (BTV-1) appeared in Southern Spain in 2007, probably from infected *Culicoides* carried by the wind from North Africa. BTV-8 was present in Europe since 2006 and reached Northern Spain in 2008. Moreover, BTV-4 was present from 2004 in the south and was detected in livestock until the end of 2007. Spain was declared free from BTV-4 in 2009, but currently the country is considered a restricted zone for BTV-1, BTV-4 and BTV-8 [RASVE].

Several species of wild ruminants have been previously investigated to elucidate their potential influence on BTV control, as well as the occurrence of clinical disease. BTV specific antibodies have been detected both in free-ranging and farmed wild ruminants in several European countries [Conraths *et al*., 2009; De Curtis *et al*., 2007; Fernández-Pacheco *et al*., 2008; García *et al*., 2009; Rodríguez-Sánchez *et al*., 2010a, 2010b; Rossi *et al*., 2009; Ruiz-Fons *et al*., 2008] and BTV RNA has been detected in wild free-ranging ruminants [García-Bocanegra *et al*., 2011; Linden *et al*., 2010], suggesting BTV circulation in these species.

In order to understand the role of wildlife, the aim of this study is to determine the prevalence of BT in wild ruminants in the Iberian Peninsula.

3.3. Materials and Methods

3.3.1. Samples

A total of 2011 sera samples and 320 spleen samples from 1398 red deer (*Cervus elaphus*), 230 fallow deer (*Dama dama*), 129 roe deer (*Capreolus capreolus*), 166 southern chamois (*Rupicapra pyrenaica*), 55 Spanish ibex (*Capra pyrenaica*), 30 European mouflon (*Ovis aries musimon*) and 3 aoudad (*Ammotragus lervia*) were collected from 2006 to 2011. Blood samples were collected into sterile tubes without anticoagulant either by jugular venipuncture from live animals or from the heart or thoracic cavity of legally harvested animals during the hunting season. Sera were obtained after centrifugation at 300 x G for 15 min and stored at -20ºC until analysis. Spleen samples were kept at -80ºC until analysis.

3.3.2. Study area

Samples were collected in the Iberian Peninsula from fourteen different sampling areas (Asturias, Cantabria, Galicia, País Vasco, Valle del Ebro, Meseta Norte Oriental, Meseta Norte Occidental, Sistema Ibérico, Sistema Central, Montes de Toledo, Valle del Guadiana, Sierra Morena Occidental, Sierra Morena Oriental, and Portugal) from bio-regions 1 to 4, according to the Spanish Wildlife Disease Surveillance Scheme, and Portugal (Figure 5) [Spanish Wildlife Disease Surveillance Scheme (*Plan Nacional de Vigilancia Sanitaria en Fauna Silvestre*), 2011].

Figure 5. Distribution of the bio-regions in the Iberian Peninsula. (1) Atlantic, (2) Northern Plateau, (3) South-Central, (4) Interior Mountains, (5) South and East coast, and (6) Portugal. [Adapted from the Spanish Wildlife Disease Surveillance Scheme, (*Plan Nacional de Vigilancia Sanitaria en Fauna Silvestre*), 2011]

3.3.3. Serological analyses

All sera samples were tested for the presence of BTV-specific antibodies against the major core protein VP7 using a commercial double-antigen ELISA assay (Ingezim BTV DR12.BTV.KO Ingenasa, Spain), according to maunfacturer's instructions.

ELISA positive sera (698 out of 991) were further analysed by serum neutralisation test (SNT) to detect BTV-1, BTV-4 and BTV-8 specific neutralising antibodies, as previously reported [OIE, 2009]. The remaining sera (31.3%) could not be analysed by SNT due to cytotoxicity. Briefly, heat inactivated sera (56ºC for 30 minutes) were diluted from 1:2 to 1:4096 in microplates (Costar® Cat. Nº 3915, Cultek, Madrid, Spain) using MEM Earle (Eagle's minimum essential medium with Earle salts) and mixed with 100 $TCID_{50\%}$ of each reference strain (BTV-1, BTV-4 and BTV-8). Mixtures were incubated for one hour at 37ºC, and 100 µL of a VERO E6 cell suspension in MEM supplemented with 15% foetal bovine serum (FBS; PAA Laboratories GmbH, Austria), 300 µg/l-glutamine/mL, 300 U penicillin/mL and 300 µg streptomycin/mL, were added to a final concentration of $1.5x10^4$/well. The mixture was further incubated for six days at 37ºC, plate readings for cytopathic effect (CPE) were done at four and six days. Developing CPE was compared with control wells containing 100 $TCID_{50\%}$ of virus and negative control wells (without virus). Only samples that showed neutralisation (absence of CPE) at dilutions ≥1:4 were considered positive.

3.3.4. BTV RNA detection

Two hundred and fifty-five spleen samples from ELISA positive red deer and 65 spleen samples for the remaining species were analysed to detect BTV RNA. All spleen samples were analysed by RT-qPCR and confirmed by RT-PCR. Previously, total RNA was extracted using the Biosprint 96® kit (Qiagen). RT-qPCR was performed using the primers and the specific probe for segment 5 of BTV as previously described [Toussaint *et al*., 2007]. Amplification of BTV was carried out using an AgPath-IDTM One-Step RT-PCR kit (Applied Biosystems) in 7500 Fast equipment using 2 µL of eluted RNA in a total volume of 20 µL. according to the National BTV Reference Laboratory in Algete (Madrid), reactions were carried out using an amplification program consisting of an initial denaturing step at 95°C for 5

minutes and the following cycling conditions: 48ºC for 10 minutes, 95ºC for 10 minutes and 40 cycles at 97°C for 3 seconds and 61°C for 30 seconds. RT-PCR was performed according a procedure previously described [Agüero *et al*., 2002; OIE, 2009]. Primers amplified a region of segment 5 (NS1) [Katz *et al*., 1993].

3.3.5. Statistical analysis

Associations between serological results and independent variables such as species, year and sampling area were analysed by means of a Pearson's chi-square test. When observations per category were less than six, Fisher's exact test was used. Differences between variables were analysed by Tukey tests. Differences were considered statistically significant when *P*-value<0.05. Statistical analyses were performed using SPSS 15.0 (Statistical Package for Social Sciences (SPSS) Inc., Chicago, IL, USA).

3.4. Results

A total of 991 (49.3%) out of the 2011 samples analysed by ELISA were positive to BTV. Seropositivity was detected in all periods, in all the species and in all sampled areas (Table 3). Statistically significant differences were observed among species. Red deer, roe deer, European mouflon and southern chamois showed significantly higher seroprevalence by ELISA than fallow deer and Spanish ibex (*P*<0.05).

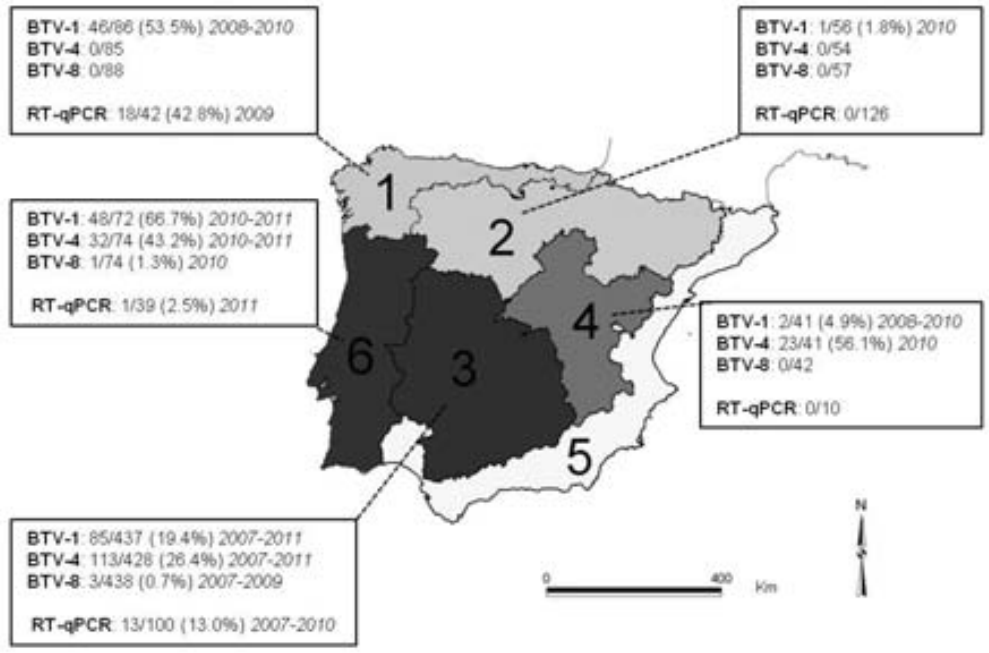
BTV seroprevalence by SNT in the Iberian Peninsula was 41.8% ($CI_{95\%}$: 38.2-45.5). BTV-1 was present in bio-regions 1 to 4 and Portugal (182 out of 692, 26.3%, $CI_{95\%}$: 23.2-29.7). BTV-4 was present in regions 3, 4 and Portugal (168 out of 680, 24.7%, $CI_{95\%}$: 21.6-28.1). BTV-8 was detected only in four red deer from bio-region 3 and Portugal (4 out of 699, 0.6%, $CI_{95\%}$: 0.2-1.5) (Figure 6A).

Table 3. Number of BTV positive sera (ELISA) by species and sampling period. Dashes (-) indicate no samples available.

Species	2006		2007		2008		2009		2010		2011		Total	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Red deer	43	**32.3**	186	**48.9**	160	**56.2**	526	**55.3**	381	**58.3**	102	**53.9**	1398	**54.6**
Roe deer	-	**-**	22	**36.4**	33	**48.5**	43	**51.2**	6	**83.3**	25	**48.0**	129	**48.8**
Fallow deer	39	**0**	39	**43.6**	34	**20.6**	45	**40.0**	73	**12.3**	-	**-**	230	**22.2**
Spanish ibex	-	**-**	13	**23.1**	19	**0**	3	**33.3**	20	**0**	-	**-**	55	**7.3**
European mouflon	-	**-**	-	**-**	-	**-**	11	**72.7**	8	**87.5**	11	**18.2**	30	**56.7**
Southern chamois	36	**75.0**	40	**42.5**	53	**54.7**	19	**36.8**	17	**64.7**	1	**0**	166	**54.8**
Aoudad	-	-	-	-	-	-	3	**66.7**	-	-	-	**-**	3	**66.7**
Total	118	**34.7**	300	**45.3**	299	**47.5**	650	**53.7**	505	**50.3**	139	**49.6**	2011	**49.3**

BTV RNA was detected in 32 (10%) out of the 320 spleen samples analysed. Red deer resulted positive for 28 out of 257 samples and fallow deer for 4 out of 40 samples, whereas all the samples from other species were negative (*Ct* > 40) to RT-qPCR. Atlantic was the bio-region with a higher detection of BTV RNA ($P<0.05$), all positive samples belonging to the Cantabria area (18 out of 32) (Figure 6A). Five out of the 18 positive samples showed positivity to BTV-1 by SNT. The serotype of the remaining thirteen positive samples from Cantabria could not be determined by SNT due to cytotoxicity of the sera. In South-Central bio-region, all positive samples (13 out of 100) were detected in southern areas (Sierra Morena). Nine out of the 13 samples were positive to BTV-1 by SNT, one was positive both to BTV-1 and BTV-4 and the remaining three positive samples could not be determined by SNT. The BTV RNA positive sample found in Portugal could not be correlated to SNT results due also to cytotoxicity of the serum. Once BTV-1 and BTV-4 were detected in 2007, these two serotypes have been detected in wild ruminants until the end of the study (2011) (Figure 6B).

A.



B.

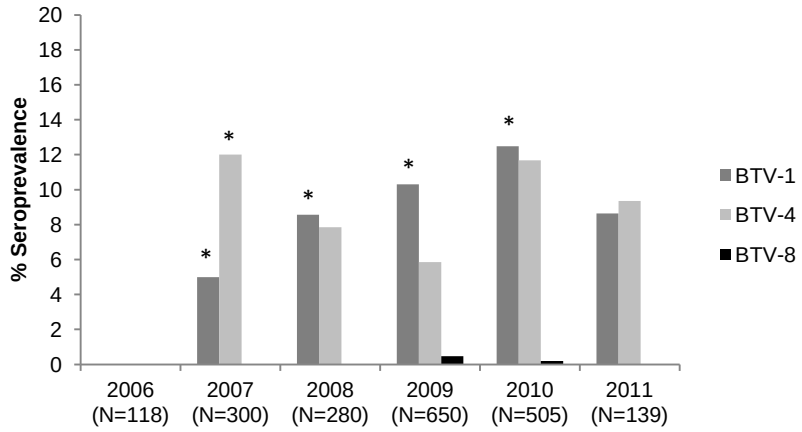


Figure 6. BTV seropositive samples by SNT and BTV RNA positive samples detected by RT-qPCR (A) Spatial distribution by bio-region. Light grey = presence of BTV-1; medium grey = presence of BTV-1 and BTV-4; dark grey = presence of BTV-1, BTV-4 and BTV-8. **(B) Temporal distrution by sampling period**. Asterisks (*) indicate the presence of positive BTV RNA samples by sampling period.

3.5. Discussion

The results indicate that in spite of vaccination campaigns applied to livestock, BTV-1, BTV-4 and BTV-8 continued to be present in wild ruminant populations in the Iberian Peninsula, as demonstrated by seroprevalence and BTV RNA detection until 2011. Although BTV antibodies in cattle can last until three years after vaccination [Oura *et al*., 2012], the decrease of protection after vaccination programs and BTV RNA circulation in non vaccinated ruminants can lead to reinfection of non protected livestock.

Overall, distribution of BTV in wild ruminants corresponded with serotype detection in domestic ruminants. BTV-1 was detected mainly in Atlantic (1) and South-Central (3) bio-regions and in Portugal, which are areas where the same serotype was detected in livestock [RASVE, 2012]. As well, BTV-4 was found in Interior mountains (4) and South-Central (3) bio-regions and Portugal too, correlating with data reported in domestic ruminants since BTV-4 outbreaks where firstly detected in the southern regions of the Iberian Peninsula [RASVE, 2012]. However, BTV-8 was less frequently detected and with lower antibody titres than BTV-1 and BTV-4, which is also in accordance with limited circulation of BTV-8 reported in livestock in these areas. The possibility to correlate samples from the same animal, comparing the results of BTV RNA detection with the results of SNT, suggests that most of the positive samples detected by RT-qPCR are from BTV-1 infected animals.

Although no statistically significant differences were found between red deer, roe deer, mouflon and southern chamois, red deer is the most abundant and widely distributed species, which probably suggest tahat is the one more implicated in the transmission and maintenance of BTV and could be used as sentinel species for BT in the Iberian Peninsula. This hypothesis has been also suggested in previous publications [Falconi *et al*., 2012; García *et al*., 2009; Linden *et al*., 2008; Linden *et al*., 2010; Rodríguez-Sánchez *et al*., 2010a; Rossi *et al*., 2009; Ruiz-Fons *et al*., 2008].

In conclusion, the results confirm that wild ruminant populations from the Iberian Peninsula were exposed to BTV-1, BTV4 and BTV-8. Red deer is the species that seems to play a major role in the epidemiology of BT. The spatial and temporal results suggest that BTV-1 and BTV-4 circulation is endemic but not homogeneous in wild ruminants in the Iberian Peninsula, whereas the low seroprevalence of BTV-8 is consistent with the limited circulation reported in livestock.

3.6. Acknowledgements

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4. Chapter 2

Epidemiological surveillance of bluetongue virus serotypes 1, 4 and 8 in Spanish ibex (*Capra pyrenaica*) in Southern Spain

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4.1. Abstract

A cross-sectional study was carried out to assess the prevalence and circulation of bluetongue virus (BTV) in Spanish ibex (*Capra pyrenaica hispanica*). A total of 770 sera samples, 380 blood samples and 34 spleen samples were collected between 2006 and 2009 in Andalusia (Southern Spain), a region and time period with a wide circulation of BTV in livestock.

Thirty-one out of 770 (4.0%; $CI_{95\%}$: 2.6-5.4) sera samples analysed by ELISA showed antibodies against BTV. Twenty-four out of 31 seropositive samples were tested against BTV serotypes 1, 4 and 8 by serum neutralisation test (SNT). Neutralising antibodies against BTV-1 and BTV-4 were detected in seven and ten animals, respectively, four of them showed neutralising antibodies to both serotypes. The animals seropositive to BTV-4 were sampled between 2006 and 2008, while BTV-1 circulation was confirmed in ibexes sampled between 2007 and 2009. None of the ibexes presented neutralising antibodies against BTV-8.

Statistically significant differences were found among regions and years, which is in coincidence with what occurred in domestic ruminants. There were no statistically significant differences between sexes, age classes and habitats (captivity *vs* free-living). BTV RNA was not found in any of the 380 blood samples analysed. However, BTV-1 RNA was detected from spleen in one Spanish ibex from Málaga province in August 2008. This finding evidences the presence of BTV-1 in Spanish ibex in a municipality where BT outbreaks were not detected in domestic ruminants during that period.

Results of the present study show that Spanish ibexes were exposed and responded serologically to both BTV-1 and BTV-4. The low seroprevalence obtained suggests that Spanish ibex is not a relevant species in the dissemination of BT. However, the detection of BTV-1 RNA and the presence of seropositive ibexes in areas where BT outbreaks were not detected in livestock, could not exclude a significant role in the epidemiology of BTV in certain areas.

4.2. Introduction

Bluetongue (BT) is a reportable disease of socioeconomic impact in the international trade of ruminants. To date, 24 distinct BT virus (BTV) serotypes have been identified; four of which have been found in Spain in the last decade. BTV serotype 2 (BTV-2) was detected in 2000 in the Balearic Islands [Mellor & Wittmann, 2002]. In 2003, BTV-4 emerged in Menorca and a second incursion of BTV-4 from Morocco was registered in Southern Spain in October 2004 [OIE, 2010]. In July 2007, a new BT outbreak caused by BTV-1 affected the southern regions of Spain. Furthermore, in January 2008, BTV-8 appeared in Northern Spain and arrived to Southern Spain in October 2008. Currently, BTV-1 and BTV-8 are endemic in Spain [Anonymous, 2010] and the country is considered a restriction zone for these serotypes.

In Spain, BTV circulation has been reported in red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), European mouflon (*Ovis aries musimon*), aoudad (*Ammotragus lervia*) and Spanish ibex (*Capra pyrenaica hispanica*) from central and southern regions [García *et al*., 2009; Rodríguez-Sánchez *et al*., 2010a; Ruiz-Fons *et al*., 2008]. In addition, the first BTV isolation in clinically affected mouflons has been recently reported in Southern Spain [Fernández-Pacheco *et al*., 2008, Rodríguez-Sánchez *et al*., 2010b]. Although the role played by wild ruminant species in the epidemiology of BT in Europe is still unknown, the importance of those species as potential reservoirs of BTV has been suggested, especially in regions where livestock and wild ruminants share the same habitat [Linden *et al*., 2010; López-Olvera *et al*., 2010; Rodríguez-Sánchez *et al*., 2010a].

The Spanish ibex is the only native wild caprine in Spain. Their populations are currently found throughout the southern and eastern regions of the country [Pérez *et al*., 2002]. In the past few decades, Spanish ibex has been affected by contagious diseases, such as sarcoptic mange, which caused a drastic reduction in many populations [León-Vizcaíno *et al*., 1999]. Habitat fragmentation, illegal hunting, loss of genetic diversity, local overabundance, disequilibrium in the population sex ratio and age structure have also contributed to a significant population decline [González-Candela *et al*., 2006]. Andalusia (Southern Spain) is the region with the largest number of Spanish ibexes, with an estimated census of 30,000 individuals

currently inhabiting 34 main populations (Figure 7). Their populations share pastures seasonally with other domestic ruminants [Pérez *et al*., 2002].

To get further insights on BT in Spanish ibex, the aims of the present study were: (i) to evaluate the presence and circulation of BTV in Spanish ibex between 2006 and 2009 and (ii) to assess their potential role on BT epidemiology.

Figure 7. Estimated distribution of the Spanish ibex in Andalusia. Data from Regional Ministry of Environment of the Government of Andalusia.

4.3. Materials and methods

4.3.1. Sampling

Sera samples from 770 Spanish ibexes were collected in five different provinces of Andalusia, Southern Spain, between 2006 and 2009 (Figure 8). Blood or pleural fluid (n = 241) and spleen (n = 38) samples from hunted free-ranging Spanish ibexes were obtained from heart or thoracic cavity during the hunting season (October to February). In addition, blood samples (n = 529) from captive animals captured alive by gamekeepers using box-traps were taken by jugular venipuncture. Samples were collected into sterile tubes without anticoagulant and later centrifuged at 400 g for 15 minutes. Besides, 380 blood samples (271 from captive animals and 109 from free-ranging ibexes) were placed in sterile tubes containing EDTA for RT-qPCR analysis. Serum or fluid exudates, blood and spleen samples were stored at -20ºC until analysis.

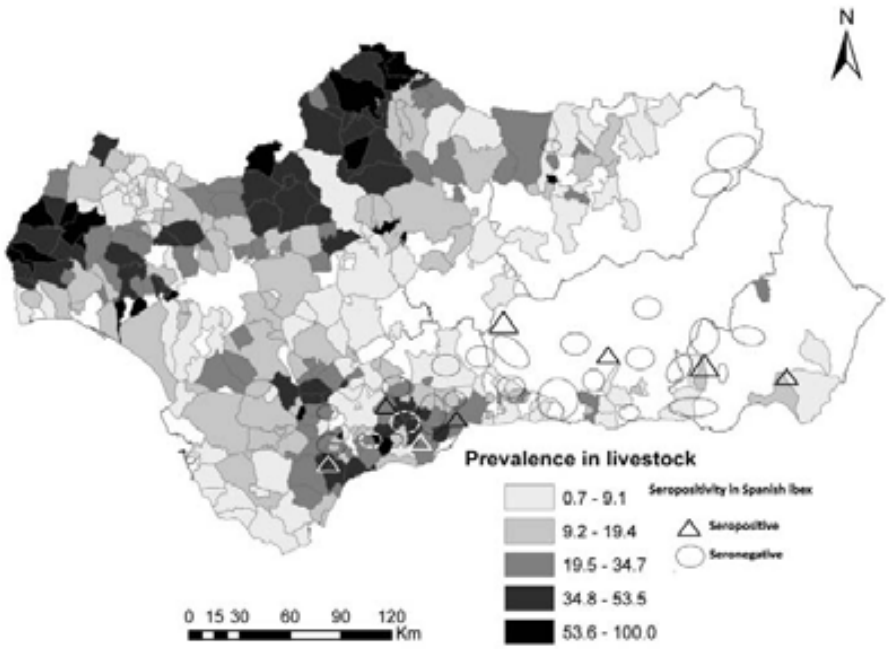

Figure 8. Sampling locations in Andalusia. The gradient of grey indicates the farm prevalence of BTV in domestic ruminants. Dots and triangles indicate sampled areas with absence and presence of antibodies against BTV in Spanish ibex, respectively.

Age of the ibexes was determined by horn segment counts [Fandos, 1995]. The animals were classified into three age groups: yearlings (< 2 year old), juveniles (between 2 and 4 years old) and adults (> 4 years old). Unfortunately, the ages of 308 animals and the sexes of 327 could not be obtained.

Data on BTV outbreaks in domestic animals in Andalusia were obtained from the Spanish Ministry of Agriculture, Fisheries and Food [RASVE, 2010]. The livestock census was provided by the Autonomous Government of Andalusia.

4.3.2. Serological analyses

The presence of antibodies against BTV was determined using a commercial double-antigen enzyme linked assay (ELISA; INGEZIM BTV DR 12.BTV.K0, INGENASA, Madrid, Spain) according to manufacturer's recommendations. ELISA positive sera were then tested by means of serum neutralisation test (SNT) to detect serotype specific antibodies as described previously [OIE, 2009]. Serum samples were inactivated at 56ºC for 30 min prior to analysis. Sera were diluted (1:2 – 1:256) in MEM (Eagle's minimum essential medium) and mixed with 100 $TCID_{50\%}$ (50% tissue culture infective doses) of each reference strain, BTV-1, BTV-4 and BTV-8. Plates were incubated for 1 h 30 min at 37ºC. Finally, 100 µL of a VERO E6 cell suspension ($1.5x10^4$/well) was added in cell growth media (MEM supplemented with 15% fetal calf serum, 300 µg L-glutamine/mL, 300 U penicillin/mL and 300 µg streptomycin/mL). The mixture was further incubated for 6–7 days at 37ºC until cytopathic effect (CPE) was developed in control wells containing 100 $TCID_{50\%}$ of virus and no serum. Only samples that showed neutralisation (absence of CPE) at dilutions ≥1:4 were considered positive. Controls for cytotoxicity in the absence of virus were included for each sample at a dilution of 1:2.

4.3.3. Virological analyses

A total of 380 blood samples and 38 spleen samples were sent to the National BTV Reference Laboratory in Algete (Madrid) to assess the presence of BTV RNA. Samples were analysed using a semi-quantitative real time reverse transcriptase-PCR (RT-qPCR) detecting a conserved region within the segment 5 of BTV genome [Toussaint *et al*., 2007]. Serotype specific RT-qPCR was performed on BTV positive

samples, according to the following methods: for BTV-1, Agüero *et al*., 2008; for BTV-4 Agüero, not published; and for BTV-8, Hoffman *et al*., 2009. A virus suspension of BTV-2 isolated in the Balearic Islands (2002) was used as a positive RT-PCR extraction control. On the other hand, field-isolated strains were used as positive controls for PCR amplification: BTV-1 (BTV-1 SPA/2007/01), BTV-4 (BTV-4 isolated in Cádiz in 2004) and BTV-8 (BTV-8 BEL/2006/01).

4.3.4. Statistical analyses

Associations between serological results and independent variables such as location, age, sex, year and habitat were analysed by means of a Pearson's chi-square test. When observations per category were less than six, Fisher´s exact test was used. Differences between variables were analysed by Bonferroni or Tukey tests. Differences were considered statistically significant when $P<0.05$. Statistical analyses were performed using SPSS 15.0 (Statistical Package for Social Sciences (SPSS) Inc., Chicago, IL, USA).

4.4. Results and Discussion

Thirty-one out of 770 (4.0%; $CI_{95\%}$: 2.6-5.4) Spanish ibexes analysed by ELISA showed antibodies against BTV. Twenty-four positive ELISA samples were tested by SNT. Seven seropositive ELISA sera could not be analysed by SNT due to cytotoxicity of the sample. Neutralising antibodies were detected in 13 Spanish ibexes. Three out of 24 analysed samples against BTV-1 were positive. Six ibexes showed neutralising antibodies against BTV-4. In addition, four animals presented antibodies against both serotypes. Unfortunately, eleven of the seropositive ELISA sera were BTV-1 and BTV-8 negative by SNT, but could not be tested against BTV-4 due to the limited volume of the sample. Seropositivity against BTV-8 was not detected in any of the analysed Spanish ibexes.

To our knowledge, this is the first study of BTV serotypes in free-ranging wild ruminant species. Although a limited circulation of BTV in Spanish ibexes was observed, the results confirm that Spanish ibex populations from Southern Spain

were exposed and responded serologically to BTV-1 and BTV-4. The overall seroprevalence obtained was similar to those previously reported in this species [García *et al*., 2009; Santiago-Moreno *et al*., 2011] and lower than those found in other wild ruminant species in Spain [Ruiz-Fons *et al*., 2008; García *et al*., 2009]. Differences among species might be related to the natural resistance of the host, population densities, geographical distribution, sampling period or management factors.

Seroprevalence among locations, age classes, sexes, years and habitats are shown in Table 4. A significantly higher seroprevalence was found in Málaga (6.5%; $CI_{95\%}$: 3.5-9.5) compared to Granada (3.3%; $CI_{95\%}$: 1.5-5.1) (P= 0.004).

Table 4. Frequency of antibodies against BTV in Spanish ibex. *Missing values are omitted.

Category	Value	No. samples*	No. positive (%)
Location			
	Almería	49	2 (4.1)
	Cádiz	5	0 (0.0)
	Granada	365	12 (3.3)
	Jaén	90	0 (0.0)
	Málaga	261	17 (6.5)
Age			
	Juveniles	95	3 (3.2)
	Sub-adults	75	3 (4.0)
	Adults	292	17 (5.8)
Sex			
	Female	256	15 (10.4)
	Male	187	7 (3.7)
Year			
	2006	48	5 (10.4)
	2007	36	6 (16.7)
	2008	285	9 (3.2)
	2009	401	11 (2.7)
Habitat			
	Captivity	528	18 (3.4)
	Free-living	242	13 (5.4)

Essentially, the areas where seropositive Spanish ibexes were found coincided with the municipalities where BTV was detected in domestic ruminants (Figure 8). No seroprevalence was detected in Jaén province, which is in agreement with the absence of BT outbreaks in livestock in this area.

The spatial distribution of BTV-1 over Andalusia evidenced that the risk of infection was not homogeneous over the territory, being higher in the western part of the region [Allepuz *et al*., 2010]. Vector or host density and environmental factors are possibly implicated in the spatial distribution of BTV [Allepuz *et al*., 2010; Calvete *et al*., 2008]. In our study, seropositivity to BTV was found in two municipalities where BTV was not detected in domestic ruminants. Similar results were previously reported in other wild ruminant species [García *et al*., 2009], supporting the idea that surveillance on wild ruminants may be useful to detect the disease in the areas of distribution of these species [Fernández-Pacheco *et al*., 2008; Rodríguez-Sánchez *et al*., 2010a].

Significantly higher seroprevalences were observed during 2006-2007 compared to 2008-2009 ($P<0.001$). Seropositivity to BTV-4 was detected between 2006 and 2008, while neutralising antibodies against BTV-1 were found during 2007-2009 (Table 5). In livestock, BTV-4 was detected for the last time in Andalusia in November 2005 (a total of 316 outbreaks), while the last outbreak of BTV-4 in Spain was reported in November 2006. The country was declared free of BTV-4 in March 2009 by the European Union Standing Committee on the Food Chain and Animal Health [RASVE, 2009]. In our study, two adult ibexes sampled in 2008 presented antibodies against BTV-4, three years after the last outbreak reported in livestock. This finding could indicate a high persistence of antibodies against BTV in Spanish ibex. Moreover, a two-years-old ibex, sampled in 2007, was seropositive to BTV-4. This fact could support the hypothesis of longer virus circulation on this species. However, BTV-4 RNA was not detected in any of the analysed samples.

BTV-1 circulation in Spanish ibexes was detected in 2007, coinciding with the first outbreak reported in livestock. In addition, the higher seroprevalence detected in 2007 in our study compared to the other periods correlates with the large number of outbreaks reported in domestic ruminants during that year (4,436, 92.7% of the total outbreaks detected between 2004 and 2009). The absence of

seropositive ibexes to BTV-8 was not unexpected taking into account that only 22 outbreaks have been reported in livestock in Andalusia [RASVE, 2010]. No significant differences were observed in BTV seroprevalence between ages, sexes and habitat conditions.

Table 5. Distribution of BTV serotypes by years and locations. * BTV RNA was found in an ibex from Malaga in 2008. **The serotype could not be determined in any of the two seropositive samples due to cytotoxicity.

Location	Year			
	2006	2007	2008	2009
Málaga	BTV-4	BTV-4, BTV-1	BTV-4, BTV-1*	Unknown**
Almería	No samples	No samples	BTV-4	BTV-1
Granada	No samples	No samples	BTV-1	BTV-1
Jaén	Negative	Negative	Negative	Negative
Cádiz	No samples	No samples	No samples	Negative

Viral BTV RNA was not detected in any of the 380 blood samples analysed, including samples from eleven seropositive ibexes, which indicates limited circulation of BTV in Spanish ibex populations in the studied area. However, the presence of BTV-1 RNA (*Ct*: 35.5) in the spleen of one free-living Spanish ibex confirms the susceptibility of this species to BTV infection. The infected animal was a juvenile male from Málaga province born in spring 2007. This ibex was sampled in August 2008 from Ojén, Málaga province and no clinical signs compatible with BT were observed. One BTV-1 outbreak was reported in livestock in the same municipality in October 2007 and only ten BTV-1 outbreaks were detected in livestock during 2008, all of them in October 2008 [RASVE, 2010]. To the authors' knowledge, this is the first reported detection of BTV RNA from a Spanish ibex. The blood sample was RT-qPCR negative and the results for both ELISA and SNT showed absence of antibodies against BTV in this individual. Individual resistance without viremia and restricted local multiplication of BTV or an initial infection could explain these results.

4.5. Conclusions

The results of the present study confirm that Spanish ibexes were exposed to both BTV-1 and BTV-4 and that BTV circulation occurred in wild populations from Southern Spain. The low seroprevalence obtained suggests that Spanish ibex is not a relevant species in the dissemination of BT. However, the detection of BTV-1 RNA and the presence of seropositive ibexes in areas where BT outbreaks were not detected in livestock, could not exclude a significant role in the epidemiology of BTV in certain areas. Further research would be needed to clarify the real role that Spanish ibexes play on BTV epidemiology.

4.6. Acknowledgements

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II. VACCINATION AND EXPERIMENTAL INFECTION

5. Chapter 3

Evaluation of the efficacy of commercial vaccines against bluetongue virus serotypes 1 and 8 in experimentally infected red deer (*Cervus elaphus*)

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5.1. Abstract

Red deer (*Cervus elaphus*) is a widespread and abundant species susceptible to bluetongue virus (BTV) infection. Inclusion of red deer vaccination among BTV control measures should be considered. Four out of twelve BTV antibody negative deer were vaccinated against serotype 1 (BTV-1), and four against serotype 8 (BTV-8). The remaining four deer acted as unvaccinated controls. Forty-two days after vaccination (dpv), all deer were inoculated with a low cell passage of the corresponding BTV strains. Serological and virological responses were analysed from vaccination until 28 days after inoculation (dpi).

The vaccinated deer reached statistically significant ($P<0.05$) higher specific antibody levels than the non vaccinated deer from 34 (BTV-8) and 42 (BTV-1) dpv, maintaining stable neutralising antibodies until 28 dpi. The non vaccinated deer remained seronegative until challenge, showing neutralising antibodies from 7 dpi. BTV RNA was detected in the blood of the non vaccinated deer from 2 to 28 dpi, whereas no BTV RNA was found in the vaccinated deer. BTV was isolated from the blood of non vaccinated deer from 7 dpi to 28 dpi (BTV-1) and from 9 to 11 dpi (BTV-8). BTV RNA could be identified by RT-PCR at 28 dpi in spleen and lymph nodes, but BTV could not be isolated from these samples. BT-compatible clinical signs were unapparent and no gross lesions were found at necropsy.

The results obtained in the present study confirm that monovalent BTV-1 and BTV-8 vaccines are safe and effective to prevent BTV infection in red deer. This finding indicates that vaccination programs on farmed or translocated red deer could be a useful tool to control BTV.

5.2. Introduction

Bluetongue (BT) is an arthropod-borne disease caused by bluetongue virus (BTV), an Orbivirus transmitted by *Culicoides* midges [Mellor and Wittmann, 2002; Mertens *et al*., 2004]. Currently, 24 BTV serotypes have been recognized worldwide, with an additional probable 25th serotype, *Toggenburg Orbivirus*, recently identified [Chaignat *et al*., 2009; Hofmann *et al*., 2008]. Since 1998, BTV serotypes 1, 2, 4, 6, 8, 9, 11 and 16 have been circulating through Europe causing the most severe outbreak of BT on record [Maclachlan & Guthrie, 2010]. Among these serotypes, BTV-1, which appeared in southern regions of Spain in 2007, and BTV-8, introduced for the first time in central Europe in 2006, have been the most prevalent serotypes in Europe since 2008 [Allepuz *et al*., 2010; Rodríguez-Sánchez *et al*., 2008; Saegerman *et al*., 2008; Schwartz-Cornil *et al*., 2008]. BTV is currently expanding northwards, also associated to climate change and the consequent expansion of *Culicoides* vectors distribution [Breard *et al*., 2007; Enserink, 2008; Eschbaumer *et al*., 2010; Mellor & Wittmann, 2002; Purse *et al*., 2008].

The most clinically susceptible host is domestic sheep, while other domestic ruminants are considered as asymptomatic reservoirs [MacLachlan, 1994], although they may also show clinical signs [Allepuz *et al*., 2010; Elbers *et al*., 2008a; Elbers *et al*., 2008b]. BT has also been described in wild ruminants such as white-tailed deer (*Odocoileus virginianus*), European mouflon (*Ovis aries musimon*), musk (*Moschus moschiferus*), ox (*Ovibos moschatus*), bison (*Bison bison*), and yak (*Bos grunniens*) [Falconi *et al*., 2011; Fernández-Pacheco *et al*., 2008; Linden *et al*., 2008; Maclachlan *et al*., 2009], and BTV antibodies and RNA have been reported in wild European red deer (*Cervus elaphus*) [Falconi *et al*., 2011; García *et al*., 2009; Linden *et al*., 2008; Linden *et al*., 2010; Rodríguez-Sánchez *et al*., 2010a; Rodríguez-Sánchez *et al*., 2010b; Ruiz-Fons *et al*., 2008]. Antibodies against BTV have been recently found in wild ruminants in areas where no outbreaks had been previously detected in domestic animals [Lorca-Oró *et al*., 2011], indicating a potential for transmission of BTV from wild ruminants to domestic livestock once vaccine protection is over in domestic ruminants.

Measures to control or eradicate BTV include vaccination, movement restrictions and surveillance, which have demonstrated to be effective to control the disease in livestock [Bhanuprakash *et al*., 2009; Noad & Roy, 2009; Savini *et al*., 2008]. These control measures rely on minimum vaccine coverage of 80% of the susceptible population [Ferrari *et al*., 2005], including red deer, which populations could interfere with this minimum required goal.

Red deer is a widespread and abundant species [Acevedo *et al*., 2008; Lovari *et al*., 2009] susceptible to BTV-1 and BTV-8 infection [López-Olvera *et al*., 2010]. Therefore, BTV vaccination in this species should be evaluated and considered among BTV control measures. The objective of this study is to investigate the protection induced by BTV-1 and BTV-8 commercial inactivated vaccines in red deer.

5.3. Materials and methods

5.3.1. Deer vaccination

Twelve one-year-old red deer females from a private deer farm (Los Llanos, Albacete, Spain) were tested negative for BTV antibodies and genome. The deer were ear tagged, inoculated with 1.5 mL of Ivermectin (Ivomec®, Merial Laboratories), bled by jugular venipuncture and randomly assigned to three experimental groups according to the vaccine received (day 0). Eight out of the twelve deer were intramuscularly (IM) vaccinated with 4 mL of inactivated vaccines against BTV-1 (Syvazul 1 for bovine 08002P, four deer) or BTV-8 (Syvazul 8 for bovine, 08016P, four deer) on the lateral region of the neck. Twenty-one days after vaccination (dpv), the vaccinated deer received a second dose with the same amount of vaccine, according to manufacturer's instructions (Laboratorios SYVA, León, Spain). The remaining four non vaccinated deer underwent the same handling and were blood sampled at the same time as the vaccinated deer.

All twelve deer were housed together in a 50 square meters pen from day 0 until 36 dpv. On 36 dpv, they were transported to the Biosafety Level 3 (BSL3)

facilities at the Centre de Recerca en Sanitat Animal (CReSA, Bellaterra, Spain). Each group of vaccinated deer was housed in a box with their respective non vaccinated two controls, accounting for a total of six deer per box.

Handling procedures and sampling frequency were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish laws (R.D. 223/1988, R.D.1021/2005), and current guidelines for ethical use of animals in research (2006). The present study was approved by the Ethical and Animal Welfare Committee of the Universitat Autònoma de Barcelona (Spain).

5.3.2. Experimental infection

Viral inocula consisted of infected VERO E6 clone culture supernatants of BTV-1/ALG/2006 strain with $10^{6.5}$ $TCID_{50}$/mL and BTV-8/BEL/2006 strain with $10^{6.6}$ $TCID_{50}$/mL.

By 42 dpv, six deer (the four BTV-1 vaccinated deer and two non vaccinated controls) were inoculated in the jugular vein with 2 mL of the BTV-1 suspension. The other six deer (the four BTV-8 vaccinated deer and the remaining two controls) were also inoculated in the jugular vein with 2 mL of the BTV-8 suspension. At 0, 2, 4, 7, 9, 11, 14, 17, 21, 24 and 28 dpi, blood samples (with and without EDTA) were collected by jugular puncture, and clinical signs and rectal temperature were measured. Sera was extracted from whole blood tubes after centrifugation (600 x G for 15 minutes) and stored at -20ºC. EDTA blood samples were stored at 4ºC until analysis.

By 28 dpi all deer were euthanized with xylazine (Xilagesic 20%, Laboratorios Calier, 1 mg/kg) and an overdose of barbiturate (intravenous infusion of pentobarbital at 100 mg/kg). At necropsy, standard sample collection was performed, including spleen and prescapular lymph nodes for BTV RNA detection, BTV isolation, and histopathological studies.

5.3.3. Serological analyses

Sera from 0, 21 and 34 dpv and 0, 2, 4, 7, 9, 11, 14, 17, 21, 24, 28 dpi were analysed for the presence of BTV-specific antibodies. Antibody levels against the

BTV major core protein VP7 were determined using a commercial double-antigen ELISA assay (Ingezim BTV DR12.BTV.KO Ingenasa, Spain), according to manufacturer's instructions.

Serotype specific antibodies were detected by means of serum neutralisation test (SNT) as described previously [OIE, 2009]. Serum samples were inactivated at 56ºC for 30 minutes prior to analysis. Sera were diluted 1:2 to 1:4096 in microplates (Costar® Cat. Nº 3915, Cultek, Madrid, Spain) using MEM Earle (Eagle's minimum essential medium with Earle salts) and mixed with 100 $TCID_{50\%}$ of each reference strain (BTV-1 and BTV-8). Mixtures were incubated for one hour at 37ºC, and 100 µL of a VERO E6 cell suspension in MEM supplemented with 15% fetal bovine serum (FBS; PAA Laboratories GmbH, Austria), 300 µg/l-glutamine/mL, 300 U penicillin/mL and 300 µg streptomycin/mL, were added to a final concentration of $1.5x10^4$/well. The mixture was further incubated for 6 days at 37ºC, plate readings for cytopathic effect (CPE) were done at 4 and 6 days. Developing CPE was compared with control wells containing 100 $TCID_{50\%}$ of virus and negative control wells (without virus). Only samples that showed neutralisation (absence of CPE) at dilutions ≥1:4 were considered positive.

5.3.4. Virological analyses

Total RNA was extracted from EDTA blood on 0, 2, 4, 7, 9, 11, 14, 17, 21, 24 and 28 dpi, and spleen and lymph node samples (28 dpi) using Nucleospin® Viral RNA isolation kit (Macherey-Nagel GmbH & Co, Cultek, Madrid, Spain). RT-PCR was performed according a procedure previously described [Agüero et al., 2002; OIE, 2009]. Primers amplified a region of segment 5 (NS1) as described by Katz *et al*. (1993). PCR products were visualized by electrophoresis on agarose gel stained with ethidium bromide. EDTA blood samples were evaluated by RT-qPCR. The primers and the specific probe for segment 5 of BTV were described by Toussaint *et al*. (2007). Amplification of BTV was carried out using an AgPath-IDTM One-Step RT-PCR kit (Applied Biosystems) in Fast7500 equipment using 2 µL of eluted RNA in a total volume of 20 µL. According to the National BTV Reference Laboratory in Algete (Madrid), reactions were carried out using an amplification program consisting of an initial denaturing step at 95°C for 5 min and the following cycling

conditions: 1 cycle at 48ºC for 10 min, 1 cycle at 95ºC for 10 min and 40 cycles at 97°C for 3 s, 61°C for 30 s.

BTV isolation from EDTA blood, spleen or lymph node samples was performed by inoculating 500 µL of lysed EDTA blood samples, spleen or lymph node supernatants onto six well plates of confluent VERO cells. After incubation of 90 minutes at 37ºC, the inoculum was removed and replaced with fresh MEM. Cells were incubated at 37ºC for five days. A second cell passage was done to amplify virus replication and enable final CPE reading as previously described [Clavijo *et al*., 2000].

5.3.5. Haematological values

Red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), haemoglobin concentration (HGB), haematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined by a semi-automated haematologic counter (ABX ABC Vet Hematology, Horiba, Kyoto, Japan). 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).

5.3.6. Statistical analyses

A repeated measures analysis of the variance (ANOVA) was performed to detect statistical differences regarding specific BTV antibodies tested by ELISA (% S/P ratio) and SNT (log2 transformation), body temperatures and haematological values using the PROC MIXED COVTEST procedure of SAS 9.1 (SAS Institute Inc., Cary, NC, USA). The main factor was treatment (vaccinated or non-vaccinated) and the repeated factor was the experimental day. *P*-values lower than 0.05 were considered statistically significant.

5.4. Results

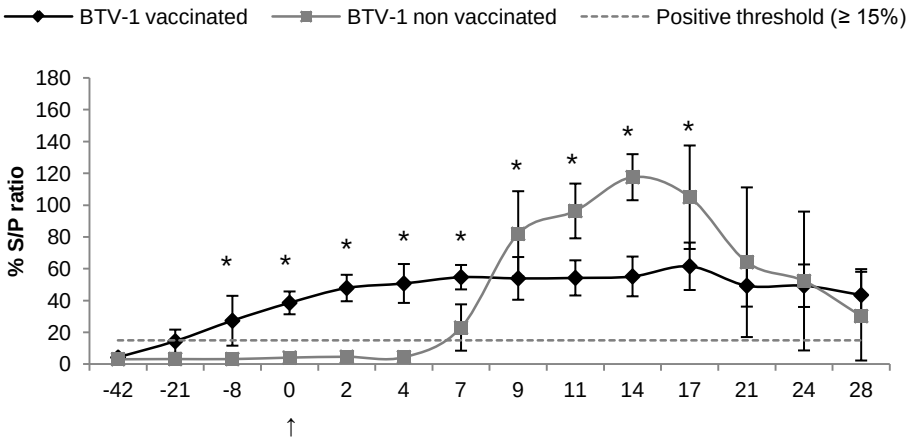
5.4.1. Serology

None of the deer presented BTV specific antibodies at the start of the study. Circulating antibodies were detected by ELISA after the first vaccination dose (21 dpv) in both the BTV-1 and the BTV-8 immunized groups, and the second vaccination dose boosted the immune response, causing a statistically significant increase of the antibody titres by 34 dpv (Figure 9). The vaccinated deer showed stable antibody titres from 34 dpv to the end of the study.

After BTV inoculation (42 dpv), BTV antibodies were detected in the non vaccinated challenged deer from 7 dpi to the end of the experiment (28 dpi), increasing from 7 to 9 dpi, and BTV-8 antibodies increased in non vaccinated deer until 14 (BTV-1) and 17 (BTV-9) dpi, when they started to decrease. Statistically significant differences (P<0.05) in antibody levels were found between groups (vaccinated *vs*. non vaccinated) both before and after challenge.

Neutralising antibodies appeared after the first vaccination (21 dpv) in all the BTV-1 immunized deer and two of the BTV-8 immunized deer. The other two BTV-8 immunized deer developed detectable neutralising antibodies only after the second vaccination dose (Figure 10). At 34 dpv both vaccinated groups showed stable neutralising antibodies levels. The non vaccinated deer remained seronegative until challenge and they seroconverted between 7 and 14 dpi. The vaccinated deer had significantly higher levels of neutralising antibodies than the non vaccinated deer both before (34 and 40 dpv) and after (7 and 14 dpi for BTV-1 and 7 dpi for BTV-8) challenge. From 14 dpi to the end of the study (28 dpi) no differences between vaccinated and non vaccinated groups were detected.

A.



B.

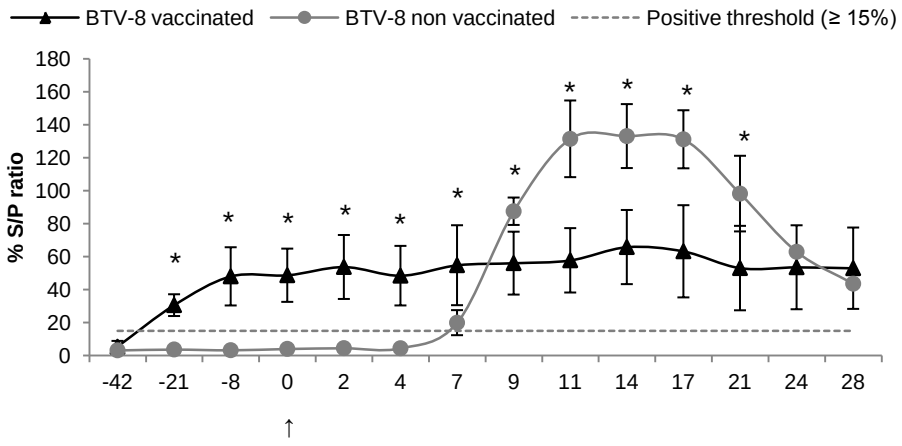
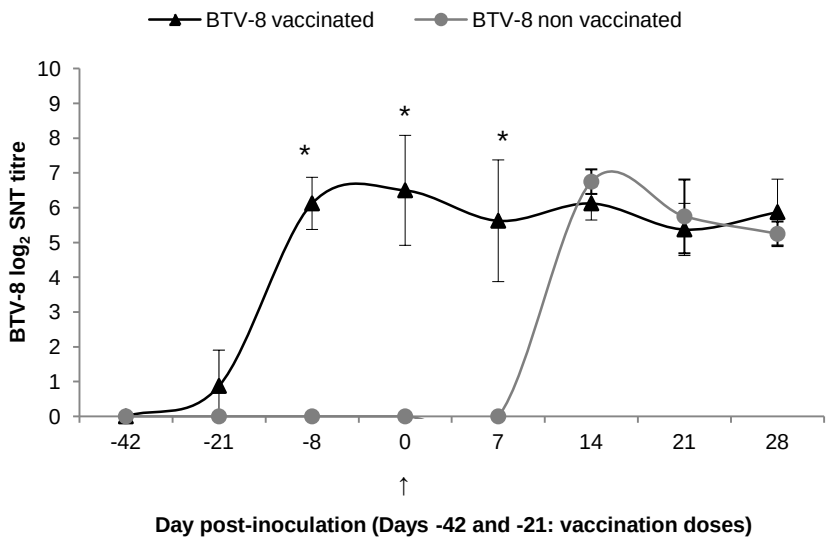


Figure 9. Mean and standard deviation of specific BTV-antibody response measured by ELISA test in vaccinated and non vaccinated deer challenged with BTV-1 (A) and BTV-8 (B). The deer were challenged at day 0 (short arrow,↑) . Asterisks (*) indicate statistically significant differences (P<0.05) between vaccinated and non vaccinated deer.

A.



B.

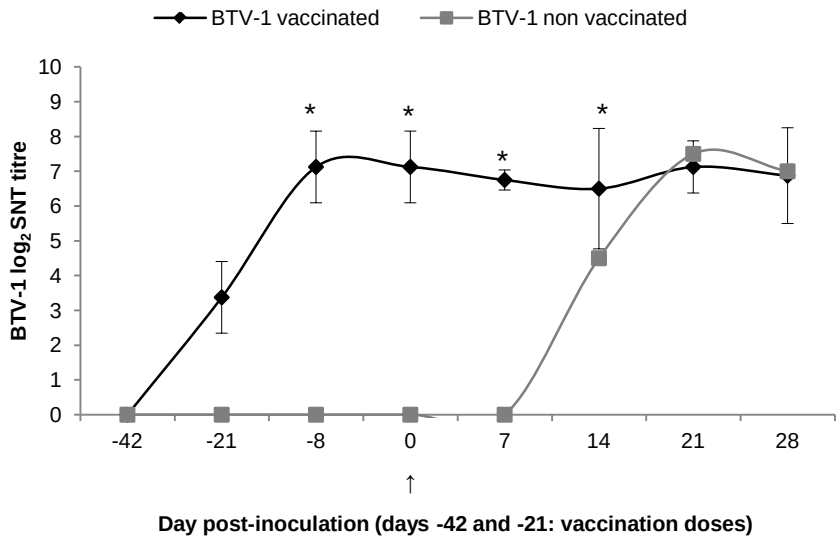


Figure 10. BTV-neutralising antibodies measured by a serum neutralisation test (SNT) in vaccinated and non vaccinated deer challenged with BTV-1 (A) and BTV-8 (B). The deer were challenged at day 0 (short arrow,↑). Asterisks (*) indicate statistically significant differences (P<0.05) between vaccinated and non vaccinated deer.

5.4.2. Viraemia

BTV RNA was detected by RT-PCR in all the non vaccinated deer on 2, 4, 7, 9, 11, 14, 17, 21, 24 and 28 dpi, except for one of the BTV-8 challenged non vaccinated deer on 2 and 4 dpi, which also showed low threshold cycle (*Ct*) values by RT-qPCR. *Ct* values from RT-qPCR are shown in Figure 11. No BTV RNA was found in the vaccinated deer.

BTV-1 was successfully isolated from 7 to 14 dpi in one of the non vaccinated BTV-1 inoculated deer and from 7 to 28 dpi in the other non vaccinated BTV-1 inoculated deer. Conversely, BTV-8 was isolated only at 9 and 11 dpi from one of the non vaccinated BTV-8 inoculated deer, the other BTV-8 non vaccinated deer (the one showing low *Ct* values) remaining negative throughout the whole study period.BTV RNA was detected in spleen and lymph node samples in all non vaccinated challenged deer at 28 dpi, but BTV isolation was not successful.

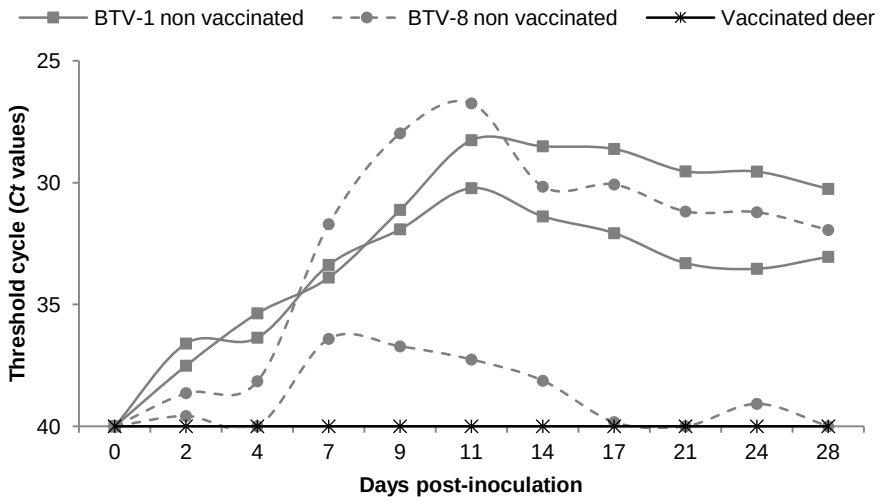


Figure 11. Threshold cycle (*Ct*) values of real-time RT-PCR in vaccinated and non vaccinated deer challenged with BTV-1 and BTV-8. Values for the non vaccinated deer are presented separately. Samples from the vaccinated deer were negative. Negative results are shown as a *Ct* of 40.

5.4.3. Clinical signs and lesions

Clinical signs compatible with BT were unapparent during the experiment in both the non vaccinated and vaccinated deer. Body temperature was constant throughout the study, in both studied groups. No statistically significant differences in body temperature were found between vaccinated and non vaccinated deer. No gross lesions compatible with BT were found after challenge or at necropsy.

5.4.4. Haematological values

Lymphocyte count decreased significantly immediately after inoculation in all groups. This decrease was significantly higher ($P<0.05$) in the BTV-8 non vaccinated deer as compared to the BTV-8 vaccinated deer on 4, 7, 9 and 11 dpi (Figure 12). No statistically significant differences were found between both BTV-1-challenged groups. The decrease in lymphocyte count was the only clinical sign observed in all the experimentally infected deer, and it is opposite to the trend previously reported in

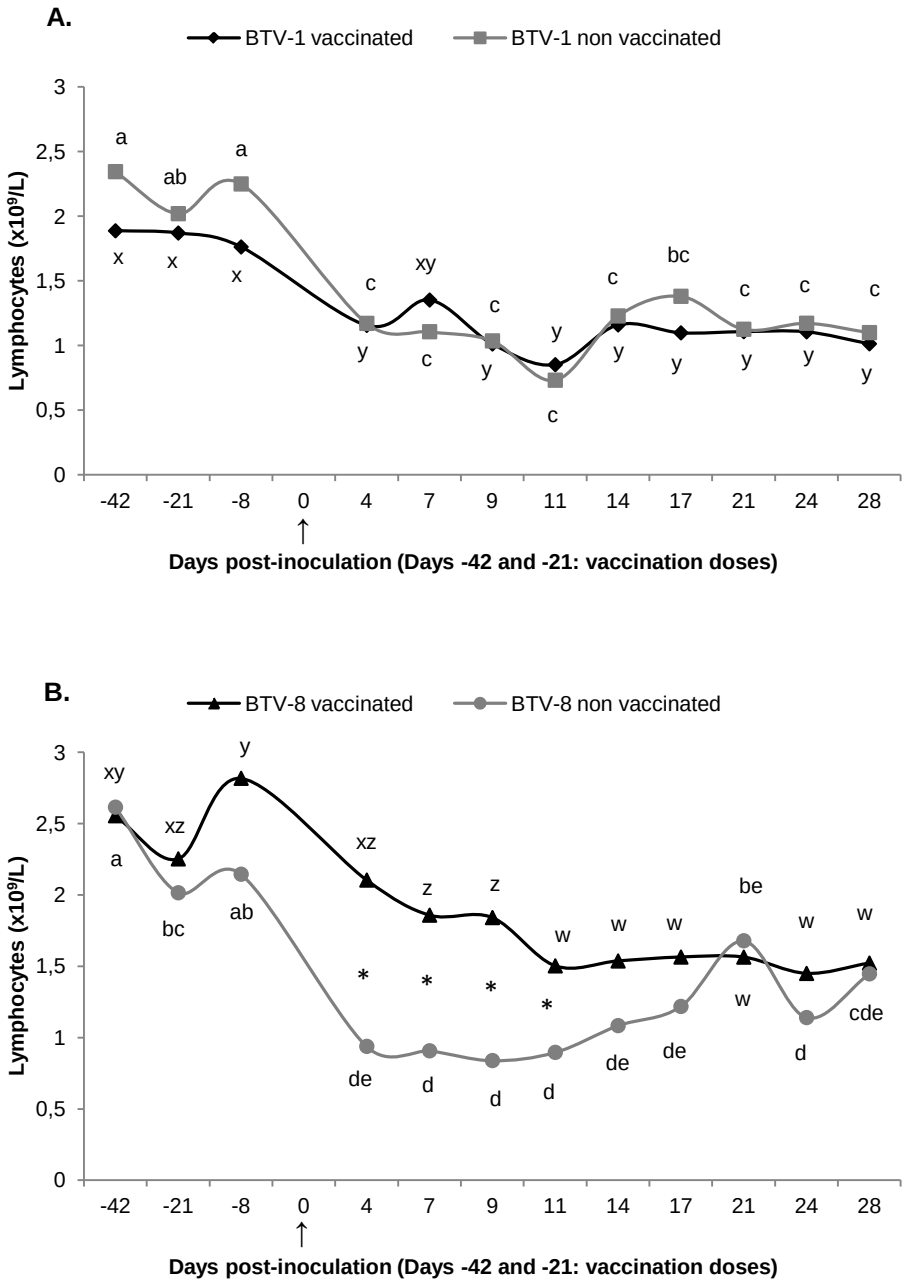


Figure 12. Mean lymphocyte count (x 10^9/L) of BTV-1 (A) and BTV-8 (B) challenged red deer. The deer were challenged at day 0 (short arrow, ↑). Asterisks (*) indicate statistically significant differences (*P*<0.05) between vaccinated and non vaccinated deer. Means with different superscript are significantly different from each other in the same group.

5.5. Discussion

The results obtained both by ELISA and SNT suggest that only one dose of vaccine could provide protection against BT in red deer and may be a useful strategy in susceptible wild ruminants. The second vaccination boosted immunity to reach stable protective antibody levels comparable to those previously reported in domestic ruminants [Calistri *et al*., 2010; Eschbaumer *et al*., 2009; Hamers *et al*., 2009; Umeshappa *et al*., 2010; Wackerlin *et al*., 2010].

BTV RNA detection, both by RT-PCR and RT-qPCR, and BTV isolation further confirm the already demonstrated susceptibility of red deer to asymptomatic long-lasting (up to 112 days) BTV infection [López-Olvera *et al*., 2010 ; Rodríguez-Sánchez *et al*., 2010a], with viremia comparable in intensity and duration to domestic ruminants. Low transient BTV RNA detection in red deer infected with BTV-8, as the one observed in one of the non vaccinated deer of this study, has already been reported [López-Olvera *et al*., 2010].

BTV-8 experimentally infected North American elk (*Cervus elaphus canadensis*) [Murray & Trainer, 1970]. Differences in leukocyte infection pattern depending on host species have already been reported [Karstad & Trainer, 1967]. The fact that this decrease was significantly higher in the BTV-8 vaccinated deer further confirms the protective effect of the vaccine against BTV infection. The absence of differences between both BTV-1 inoculated groups could be due to differences in the pathogenesis of each BTV serotype, and therefore differences in the vaccine effect.

Several studies have shown the potential role of wild ruminants, including red deer, in the epidemiology of BTV [García *et al*., 2009; Linden *et al*., 2008; Linden *et al*., 2010; López-Olvera *et al*., 2010 ; Lorca-Oró *et al*., 2011 ; Rodríguez-Sánchez *et al*., 2010a; Ruiz-Fons *et al*., 2008]. Since BTV vaccination programs are only addressed to domestic ruminants [European Commission, 2011], red deer may suppose a source of re-infection for livestock once vaccination campaigns are over and herd immunity decreases [Falconi *et al*., 2011]. Therefore, red deer should be also considered in BTV surveillance and control programs, including vaccination of susceptible wild ruminants, particularly those raised in game farms, zoo facilities, or

undergoing handling procedures in private hunting estates. Further studies on the efficacy of protection of different vaccination routes and protocols would help to elucidate the best way to achieve this objective.

5.6. Conclusions

The results of the present study indicate that the administration of commercial monovalent BTV-1 and BTV-8 vaccines is safe and protective in red deer. To our knowledge, this is the first time that vaccination followed by experimental infection with BTV is carried out in red deer. It remains to be determined the duration of protection provided by vaccination in this species and other wild ruminants, as well as the role played by wild ruminant species that may be involved in the epidemiology of BT.

5.7. Acknowledgements

This work was supported by the project FAU2008-00019-C03-01, from the Instituto Nacional de Investigación y Tecnología Agroalimentaria (INIA). Vaccines were provided by Syva Laboratories.

6. Chapter 4

Protection of Spanish ibex (*Capra pyrenaica*) against bluetongue virus serotypes 1 and 8 in a subclinical experimental infection

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6.1. Abstract

Many wild ruminants such as Spanish ibex (*Capra pyrenaica*) are susceptible to Bluetongue virus (BTV) infection, which causes disease mainly in domestic sheep and cattle. Outbreaks involving either BTV serotypes 1 (BTV-1) and 8 (BTV-8) are currently challenging Europe. Inclusion of wildlife vaccination among BTV control measures should be considered in certain species. In the present study, four out of fifteen seronegative Spanish ibexes were immunized with a single dose of inactivated vaccine against BTV-1, four against BTV-8 and seven ibexes were non vaccinated controls. Seven ibexes (four vaccinated and three controls) were inoculated with each BTV serotype. Antibody and IFN-gamma responses were evaluated until 28 days after inoculation (dpi). The vaccinated ibexes showed significant (P<0.05) neutralising antibody levels after vaccination compared to non vaccinated ibexes. The non vaccinated ibexes remained seronegative until challenge and showed neutralising antibodies from 7 dpi. BTV RNA was detected in the blood of non vaccinated ibexes from 2 to the end of the study (28 dpi) and in target tissue samples obtained at necropsy (8 and 28 dpi). BTV-1 was successfully isolated on cell culture from blood and target tissues of non vaccinated ibexes. Clinical signs were unapparent and no gross lesions were found at necropsy. Our results show for the first time that Spanish ibex is susceptible and asymptomatic to BTV infection and also that a single dose of vaccine prevents viraemia against BTV-1 and BTV-8 replication.

6.2. Introduction

Domestic and wild ruminants are thought to be susceptible to BTV infection, which causes BT, a disease that has a high economic impact on animal health. BTV belongs to the genus *Orbivirus* (family *Reoviridae*) and is transmitted by blood-feeding midges of the genus Culicoides (*Diptera, Ceratopogonidae*) [Mellor & Wittmann, 2002; Mertens *et al*., 2004]. There are at least 24 different BTV serotypes, and two putative new serotypes, the 25th named *Toggenburg Orbivirus* [Chaignat *et al.,* 2009; Hofmann *et al*., 2008] and a 26th [Maan *et al*., 2011], coinciding with the distribution of competent vectors in all continents except Antarctica. BT is considered an emerging and re-emerging disease in Europe. Since 1998, at least eight serotypes (BTV-1, -2, -4, -6, -8, -9, -11 and -16) have been detected in Europe, where BT has expanded its geographical range northwards [Enserink, 2008; Eschbaumer *et al*., 2009; Purse *et al*., 2008; Rodríguez-Sánchez *et al*., 2008].

The spreading of BTV-8 through Europe since its introduction in 2006 caused severe disease, mainly in cattle, but also in sheep, and heavy financial losses in animal industry. Previously, BTV-1 infections caused epizootics in Southern Europe. BTV-1 and -8 were detected in livestock in Spain in 2007 and 2008, respectively. For safety reasons, immunization against BTV-1 was carried out together with a mass vaccination campaign against BTV-8 using inactivated vaccines to control the expansion of these serotypes in the affected countries of Europe. The target of the vaccination program was to achieve at least 80% coverage of susceptible ruminants [Enserink, 2008; Hateley, 2009; Rodríguez-Sánchez *et al*., 2008].

The origin of BT is probably African, and wild ruminants are the natural hosts of BTV, although it is thought that cattle have replaced antelope as BTV maintenance host [Gerdes, 2004]. Information on the role of wild ruminants in the maintenance and spread of BTV is still limited. Several studies have been performed in wild ruminants from North America, where a range of species are frequently infected with BTV [Stallknecht *et al*., 2004]. However, studies on the susceptibility of native wild ruminant species are scarce in Europe. From 2006 to 2010, antibodies

against BTV-1, -4, and -8 have been found in red deer, fallow deer, mouflon, roe deer, aoudad and Spanish ibex in Spain [Falconi *et al*., 2011; García *et al*., 2009; García-Bocanegra *et al*., 2011; Lorca-Oró *et al*., 2011; Rodríguez-Sánchez *et al*., 2008; Rodríguez-Sánchez *et al*., 2010a; Ruiz-Fons *et al*., 2008]. Although BTV infection is often subclinical or unapparent in some wild ruminants, bighorn sheep and mouflon can develop fatal clinical disease, as do closely related domestic sheep [Fernández-Pacheco *et al*., 2008; Robinson *et al*., 1967; Rodríguez-Sánchez *et al*., 2008]. Experimental infection of pronghorn antelope (*Antilocapra americana*), American bison and African buffalo (*Syncerus caffer*) also produced clinical disease [Howerth *et al*., 2001; Tessaro & Clavijo, 2001], whereas blesbock (*Damaliscus pygarus*) [Bender *et al*., 2003] and mountain gazelle (*Gazella gazella*) [Barzilai & Tadmor, 1972] did not show clinical signs after natural or experimental infection. Recent studies observed susceptibility to experimental infection with BTV-8 in red deer [López-Olvera *et al*., 2010]. Red deer vaccination against BTV-1 and BTV-8 has proved to be safe and effective to prevent viraemia in experimentally inoculated deer [Lorca-Oró *et al*., 2012a].

Spanish ibex is an endemic species from Spain, with populations widespread throughout the southern and eastern regions of the country [Pérez *et al*., 2002]. This wild mountain ungulate has a great value for its conservation as it has been listed as threatened and currently of least concern in the IUCN Red List of Threatened Species [IUCN, 2011]. In the last decades, contagious diseases such as sarcoptic mange, habitat fragmentation, illegal hunting, loss of genetic diversity, local overabundance and disequilibrium in the population sex ratio and age structure have also contributed to a significant decline of its populations [González-Candela *et al*., 2006; Granados *et al*., 2001; León-Vizcaíno *et al*., 1999; Pérez, 2001]. Spanish ibex frequently share the same habitat with domestic ruminants, especially in summer months when exploiting the summer high mountain pastures [Pérez *et al*., 2002]. Allochthonous wild ungulate species, such as aoudad, fallow deer and mouflon, also suppose a threat to Spanish ibex and increase the potential risk of shared diseases transmission [Acevedo *et al*., 2007; Fandos & Reig, 1992].

The aim of the present study is to evaluate the efficacy of commercial inactivated BTV vaccines in Spanish ibex, a potential BTV susceptible species.

6.3. Materials and Methods

6.3.1. Ethics statement

Animals included in the present study were ibex from the Captive Breeding Center of Sierra Nevada (Granada, Southern Spain). Permits for vaccination and transport were approved by the Consejería de Medio Ambiente – Junta de Andalucía (Registration number: 1626). Handling procedures and sampling frequency were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish laws (R.D. 223/1988, R.D.1021/2005), and current guidelines for ethical use of animals in research (2006). The present study was approved by the Ethical and Animal Welfare Committee of the Universitat Autònoma de Barcelona (Permit Number: 4485).

6.3.2. Vaccination

Fifteen Spanish ibexes (four females and eleven males) one to three years old were distributed in three groups. Four out of the fifteen ibexes were subcutaneously vaccinated with a single dose of 2 mL of BTV-1 inactivated vaccine (Syvazul 1, 10003P; Laboratorios SYVA, León, Spain) on the dorsal region of the neck. Other four ibexes underwent the same handling with BTV-8 (Syvazul 8,10005P, Laboratorios SYVA, León; Spain). The remaining seven ibexes were kept as non vaccinated controls. All ibexes used in the present study were seronegative by ELISA and SNT and RT-PCR negative before BTV vaccination.

6.3.3. Experimental infection

Twenty-seven days after the vaccination (dpv), the fifteen ibexes were transported to the Biosafety level 3 (BSL3) facilities of the Centre de Recerca en Sanitat Animal (CReSA, Bellaterra, Spain). The four BTV-1 vaccinated and three non vaccinated ibexes were housed in one box (box 1), whereas the four BTV-8 vaccinated ibexes were housed in another box (box 2) with four non vaccinated ibexes. After an adaptation period of five days, all the ibexes except one non vaccinated ibex in box 2, were challenged against BTV-1 (box 1) or BTV-8 (box 2) serotypes with 2 mL of

BTV viral suspension in the jugular vein. Viral inocula consisted of infected VERO E6 culture supernatants of BTV-1/ALG/2006/E6 strain (six passages) with $10^{6.5}$ $TCID_{50}$/mL and BTV-8/BEL/2006/E6 strain (five passages) with $10^{6.6}$ $TCID_{50}$/mL. The viruses were given one passage on embryonated chicken eggs, one passage on baby hamster kidney cells and three (BTV-8) or four (BTV-1) on VERO cells.

Blood samples (with and without EDTA) were collected by jugular puncture, and clinical signs and rectal temperature were measured at days 0, 2, 4, 7, 9, 11, 14, 17, 21, 24 and 28 post-inoculation (dpi). Heparinized blood was collected at 0, 7, 14, 21 and 28 dpi to obtain peripheral blood mononuclear cells (PBMCs). Body weights were measured at 0 dpv (-33 dpi) and at necropsy (8 or 28 dpi). Sera was extracted from whole blood tubes after centrifugation (300 x G for 15 minutes) and stored at -20ºC. EDTA blood was stored at 4ºC until analysis.

At 8 dpi, three non vaccinated ibexes (one inoculated with BTV-1, one with BTV-8 and the one non inoculated ibex) were anesthetized with xylazine (Xilagesic 20%, Laboratorios Calier, 1 mg/kg) and euthanized with an overdose of barbiturate (intravenous infusion of pentobarbital at 100 mg/kg) to study BTV lesions at viraemia peak period. By 28 dpi the remaining twelve ibexes were euthanized using the same protocol. At necropsy, ordinary sampling was performed, including tissue collection (spleen, lung, liver, kidney, bowel, skin, tongue, lip, skin, nasal and oral mucosae, palate, pulmonary artery, heart, epididymis, testicle, urinary bladder, ileum, ileocaecal valve, and mediastinal, mesenteric, axillary and iliac lymph nodes) for BTV RNA detection, BTV isolation and histopathological studies.

6.3.4. Serology

Sera before vaccination (-33 dpi) and at -5, 0, 2, 4, 7, 9, 11, 14, 17, 21, 24 and 28 dpi were analysed for the presence of specific antibodies against the BTV major core protein VP7, using a commercial double-antigen ELISA assay (Ingezim BTV DR12.BTV.KO Ingenasa, Spain).

Serotype specific antibodies were detected by means of SNT as described previously [OIE, 2009]. Briefly, serum samples were inactivated at 56 ºC for 30 minutes prior to analysis. Sera were diluted 1:2 to 1:4096 in microplates (Costar®

Cat. Nº 3915, Cultek, Madrid, Spain) using MEM Earle (Eagle's minimum essential medium with Earle salts) and mixed with 100 $TCID_{50\%}$ of each reference strain (BTV-1 and BTV-8). Samples were tested against both BTV-1 and BTV-8 to determine a possible cross-neutralisation of BTV serotypes. Mixtures were incubated for one hour at 37 ºC, and 100 µL of a VERO E6 cell suspension in MEM supplemented with 15% foetal bovine serum (FBS; PAA Laboratories GmbH, Austria), 300 µg/l-glutamine/mL, 300 U penicillin/mL and 300 µg streptomycin/mL, were added to a final concentration of $1.5x10^4$/well. The mixture was further incubated for 6 days at 37ºC, plate readings for CPE were done at 4 and 6 days. Developing CPE was compared with control wells containing 100 $TCID_{50\%}$ of virus and negative control wells (without virus). Only samples that showed neutralisation (absence of CPE) at dilutions ≥1:4 were considered positive to avoid false positive results from unspecific reactions of sera.

6.3.5. BTV detection and isolation

Total RNA was extracted from EDTA blood at 0, 2, 4, 7, 9, 11, 14, 17, 21, 24 and 28 dpi and tissue samples from necropsy (8 and 28 dpi) using the Nucleospin® Viral RNA Isolation kit (Macherey –Nagel GmbH & Co, Cultek, Madrid, Spain). All samples were analysed by RT-PCR and confirmed by RT-qPCR. RT-PCR was performed according a procedure previously described [Agüero *et al*., 2002; OIE, 2009]. Primers amplified a region of segment 5 (NS1) as previously described [Katz *et al*., 1993]. PCR products were visualized by electrophoresis on agarose gel stained with ethidium bromide. RT-qPCR was performed using the primers and the specific probe for segment 5 of BTV described by Toussaint *et al*. (2007). Amplification of BTV was carried out using an AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems) in 7500Fast equipment using 2 µL of eluted RNA in a total volume of 20 µL. According to the National BTV Reference Laboratory in Algete (Madrid), reactions were carried out using an amplification program consisting of an initial denaturing step at 95°C for 5 minutes and the following cycling conditions: 1 cycle at 48ºC for 10 minutes, 1 cycle at 95ºC for 10 minutes and 40 cycles at 97°C for 3 seconds and 61°C for 30 seconds. By including serial dilutions (over six orders of magnitude) of a known titrated virus in each RT-qPCR test, estimated titres of each sample could be calculated. The estimated titres could be expressed in the

form of an equation of linear regression matching the relation of virus titre against *Ct* values (coefficient of regression: $R^2 \geq 0.99$).

BTV isolation was performed from blood and tissue samples by inoculating 500 µL of lysed EDTA blood or tissue supernatants, respectively, onto six well plates of confluent VERO cells. After 90 minutes of incubation at 37ºC, the inoculum was removed and replaced with fresh MEM. Cells were incubated at 37ºC for five days. A second cell passage was done to amplify virus replication and enable final CPE reading as previously described [Clavijo *et al*., 2000].

6.3.6. Interferon-gamma response in PBMCs

PBMCs from 0, 7, 14, 21 and 28 dpi were isolated being layered on a density gradient (Histopaque d = 1.077; Sigma-Aldrich, Spain) and centrifuged at 350 x G for 30 minutes. Trypan blue stain was used to assess cell viability. Cells were re-suspended in RPMI medium (Invitrogen, Spain). Frequencies of BTV-specific interferon-gamma (IFN-γ) secreting cells (SC) in PBMCs were analysed by an Enzyme linked inmuno spot assay (ELISPOT) using commercial monoclonal antibodies (mAbs) (Bovine IFN-γ AM05875PU-N and AM05867BT-N, Acris, AntibodyBcn, Spain). Briefly, ELISA plates (Costar 3590, Corning, USA) were coated overnight at 4ºC with 5 µg/mL of IFN-γ capture antibody (AM05875PU-N) diluted in carbonate–bicarbonate buffer (pH 9.6). Plates were then washed and blocked for 1 hour at 37°C with 150 µL of PBS with 1% of bovine serum albumin. After removal of the blocking solution, 2.5×10^5 live PBMC were dispensed per well in triplicates and stimulated with phytohaemagglutinin (PHA) (10 µg/mL) (Sigma-Aldrich, Spain) and BTV-1 or BTV-8 strains at 0.04 of multiplicity of infection (moi). The BTV strains were the same used previously at challenge. Non stimulated cells (only RPMI) were kept as background controls. After 20 hours of incubation at 37 °C in a 5% CO_2 atmosphere, cells were removed, and the biotinylated detection antibody (AM05867BT-N) was added at 2.5 µg/mL (50 µL) and incubated for 1 hour at 37°C. The reaction was revealed by sequential incubation of plates with streptavidin-peroxidase at 0.5 µg/mL for 1 hour and insoluble 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich, Spain). To calculate the BTV-specific frequencies of IFN-γ-SC, counts of spots in non stimulated wells were subtracted

from counts in virus-stimulated wells. Frequencies of IFN-γ-SC were expressed as responding cells in 10^6 PBMCs.

6.3.7. Haematology

Erythrocytic parameters (RBC, HGB, HTC, MCV, MCH and MCHC), WBC and PLT were determined by a semi-automated haematologic counter (Horiba ABX ABC Vet Hematology Analysers, Scil Vet abc, Divasa-Farmavic, Spain). Differential leukocyte count was performed by identifying 200 leukocytes on blood smears stained with a commercial Diff-Quick-like stain (Quimica Clínica Aplicada, Spain).

6.3.8. Statistical analyses

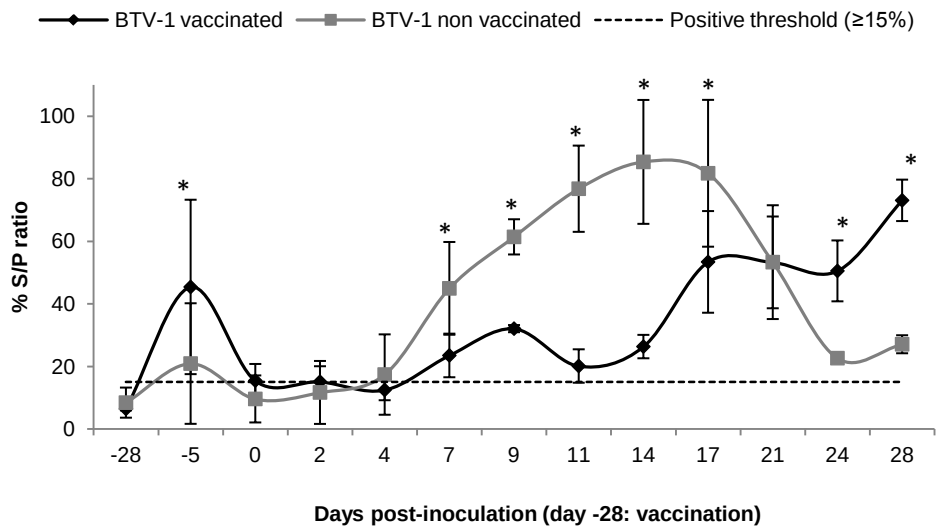
A repeated measures analysis of the variance was performed to detect statistical differences regarding specific BTV antibodies (tested by ELISA and SNT), body temperatures, IFN-γ-SC and haematological parameters, using the PROC MIXED COVTEST procedure of SAS 9.1. (SAS Institute Inc., Cary, NC, USA). The main factor was vaccine (vaccinated or non-vaccinated) and the repeated factor was DPV (day post vaccination). Differences were considered statistically significant when $P<0.05$.

6.4. Results

6.4.1. Antibody response to vaccination and infection

Non vaccinated ibexes were seronegative until challenge. BTV-specific antibodies measured by ELISA increased significantly ($P<0.05$) by 23 days after vaccination (-5 dpi) in the vaccinated ibexes, which showed protective antibody levels along the challenge. Conversely, BTV antibodies increased from 4-7 dpi in the non vaccinated ibexes, reaching its maximum at 17 dpi for BTV-1, and at 9 dpi for BTV-8, which showed a shorter and faster dynamics than BTV-1. Mean and standard deviation of percentage values of VP7 ELISA assays before and after BTV challenge are shown in Figure 13.

A.



B.

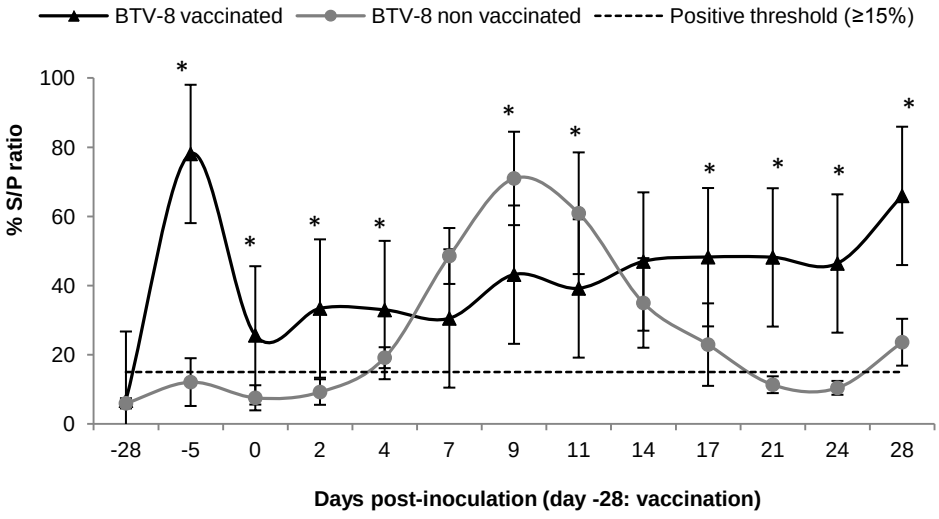
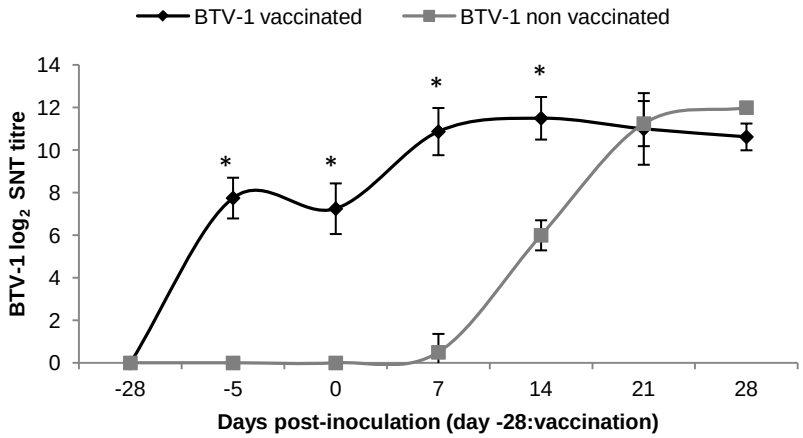


Figure 13. ELISA antibody response after BTV vaccination and experimental infection. Evolution of mean percentages (O.D. sample/O.D. positive control x100) with standard deviation of VP7 antibodies for each group of vaccinated and non vaccinated ibexes challenged with BTV-1 (A) and BTV-8 serotypes (B).

Neutralising antibodies followed a similar pattern in both the BTV-1 and BTV-8 inoculated groups, either vaccinated or non vaccinated. The vaccinated ibexes showed statistically significant higher antibody titres (P<0.05) by SNT than the corresponding non vaccinated groups from -5 to 14 dpi. Non vaccinated ibexes started to show neutralising antibodies from 7 dpi in both inoculated groups, reaching similar values to the vaccinated groups by 21 dpi (Figure 14).

A.



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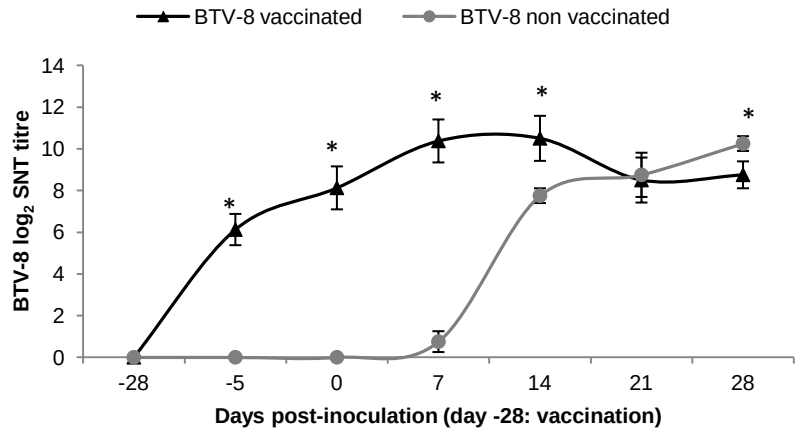


Figure 14. Neutralising antibody response after BTV vaccination and experimental infection. Evolution of mean BTV-1 (A) and BTV-8 (B) neutralising antibody titres (with standard deviation) for vaccinated and non vaccinated ibexes.

6.4.2. BTV RNA detection and isolation

No BTV RNA was detected in any blood sample of the vaccinated ibexes during the experimental period. BTV-1 non vaccinated inoculated ibexes were RT-PCR positive from 4 (two out of three) or 7 (one out of three) dpi until 28 dpi. For the BTV-8 inoculated ibexes, one non vaccinated ibex was RT-PCR positive from 7 to 17 dpi, a second one from 9 to 14 dpi and the one euthanized at 8 dpi remained negative. Results of RT-qPCR are shown as *Ct* values and estimated titres in Figure 15.

BTV was successfully isolated in VERO cells only from blood samples of the two BTV-1 non vaccinated inoculated ibexes at 7 and 9 dpi. Blood samples from the BTV-8 non vaccinated and all the vaccinated ibexes were negative to virus isolation throughout the experimental period.

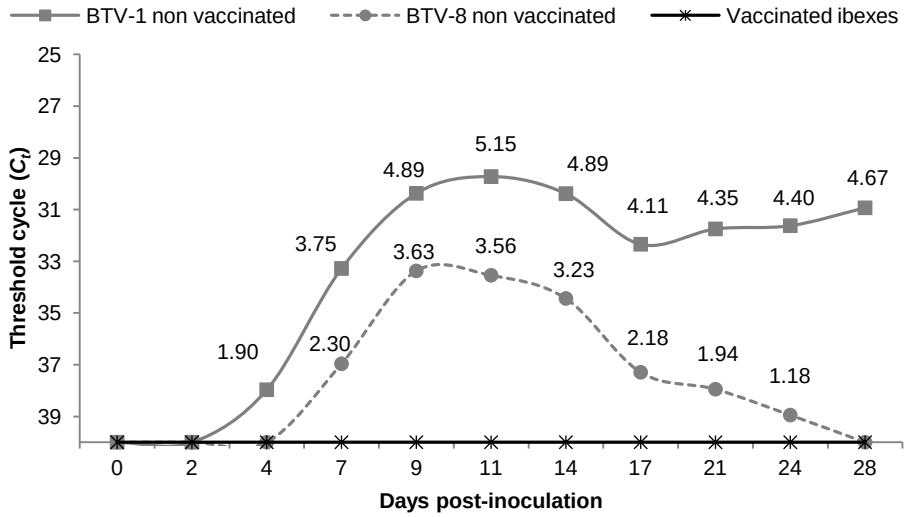


Figure 15. Detection of viraemia after BTV inoculation. Threshold cycle (*Ct*) values of real-time RT-PCR from blood samples of vaccinated and non vaccinated ibexes challenged with BTV-1 and BTV-8. Negative results are shown as a *Ct* of 40. Superscripts indicate the estimated BTV titres ($TCID_{50}$/mL).

RT-qPCR results from the tissue samples of the three ibexes euthanized at 8 dpi are shown in Table 6. BTV was isolated in the BTV-1 non vaccinated inoculated ibex from spleen, lymph nodes (prescapular, axillary, ileal, gastric and submandibular), diaphragmatic lung lobe, nasal mucosae and pulmonary artery. BTV was not isolated from any sample of the BTV-8 inoculated ibex. At 28 dpi, all the four remaining inoculated and non vaccinated ibexes were RT-qPCR positive for spleen and lymph node samples as shown in Table 7. BTV was not isolated from any sample of any ibex at 28 dpi.

6.4.3. IFN-γ response in PBMCs

No statistically significant differences in IFN-γ response of stimulated PBMCs were found between vaccinated and non vaccinated ibexes. However, ELISPOT assays revealed that, after immunization and infection with BTV, the PBMCs reacted to the stimulation with the homologous strains of each serotype, especially at 14 and 21 dpi, showing an increase in the expression of IFN-γ. Countings of IFN-γ-SC are shown in Figure 16.

6.4.4. Haematology

The BTV-1 inoculated and vaccinated ibexes showed statistically significant higher leukocyte (7, 11, and 14 dpi) and monocyte (7 and 11 dpi) counts than the BTV-1 inoculated non vaccinated ones. Conversely to the BTV-1 inoculated ibexes, lymphocyte (4, 9, 17, and 24 dpi) and monocyte (11 dpi) counts were higher in the BTV-8 inoculated and non vaccinated ibexes than in the BTV-8 inoculated vaccinated ones.

No statistically significant differences between the vaccinated and non vaccinated groups were found for red blood cell count (RBC), platelet count (PLT), haemoglobin concentration (HGB), haematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

Table 6. Threshold cycle (*Ct*) values and estimated titres ($TCID_{50}$/mL) of specific real-time RT-PCR results on tissue samples at 8 dpi. Undetermined results (out of the detection level) are shown as Negative (N). Samples without superscript were coincident with conventional RT-PCR. The tissue samples from the non vaccinated and non inoculated ibex were all negative.

Tissue sample	BTV-1 non vac.		BTV-8 non vac.	
	Ct	$TCID_{50}$/mL	*Ct*	$TCID_{50}$/mL
Spleen	29.93	3.93	33.83	2.78
Prescapular lymph node	28.29	4.41	34.62	2.55
Mediastinic lymph node	31.57	3.45	36.29	2.05
Axillary lymph node	29.72	3.99	35.77	2.21
Ileal lymph node	30.41	3.79	35.69	2.23
Gastric lymph node	29.18	4.15	35.06	2.42
Submandibular lymph node	30.09	3.88	35.22	2.37
Apical lung lobe	31.28	3.53	35.93	2.16
Middle lung lobe	30.09	3.88	34.69	2.53
Diaphragmatic lung lobe	30.41	3.79	34.91	2.46
Liver	28.19	4.44	32.70	3.11
Kidney	30.59	3.74	32.62	3.14
Nasal mucosae	32.02	3.31	36.54 [b]	1.98
Oral mucosae	33.48	2.88	N	
Lip	34.97	2.44	N	
Tongue	33.39	2.91	37.69 [b]	1.64
Axillary skin	N [a]		N	
Palate	34.80	2.49	N	
Pulmonary artery	32.47	3.18	37.94 [b]	1.57
Heart	31.50	3.46	37.02 [b]	1.84
Epididymis	32.00	3.32	37.01 [b]	1.84
Testicle	30.66	3.72	36.34 [b]	2.04
Urinary bladder	31.51	3.46	N	
Ileum	33.39 [b]	2.91	N	
Ileocaecal valve	36.73	1.92	N	

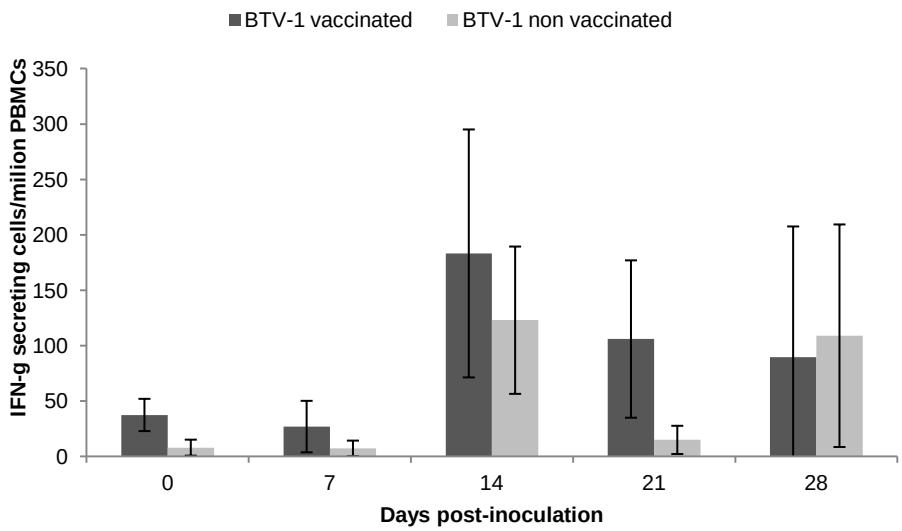
a Sample positive to conventional RT-PCR

b Sample negative to conventional RT-PCR

Table 7. Threshold cycle (*Ct*) values and estimated titres ($TCID_{50}$/mL) of specific real-time RT-PCR results on tissue samples at necropsy at the end of the study (28 dpi). Undetermined results (out of the detection level) are shown as Negative (N).

Treatment	Ibex num.	Tissue sample					
		Spleen		Prescapular lymph node		Mediastinic lymph node	
		Ct	$TCID_{50}$/mL	*Ct*	$TCID_{50}$/mL	*Ct*	$TCID_{50}$/mL
BTV-1 non vac.							
	215	30.41	3.79	26.47	4.95	31.17	3.56
	220	30.60	3.73	28.24	4.43	30.96	3.63
BTV-1 vac.							
	227	N		35.80	2.20	N	
	306	N		N		N	
	310	N		N		N	
	312	N		N		N	
BTV-8 non vac.							
	217	30.52	3.76	31.65	3.42	33.71	2.81
	307	33.03	3.02	34.18	2.68	36.37	2.03
BTV-8 vac.							
	225	N		33.06	3.01	39.24	1.48
	303	N		37.55	1.68	N	
	304	34.70	2.52	29.75	3.98	N	
	305	N		31.05	3.60	N	

A.



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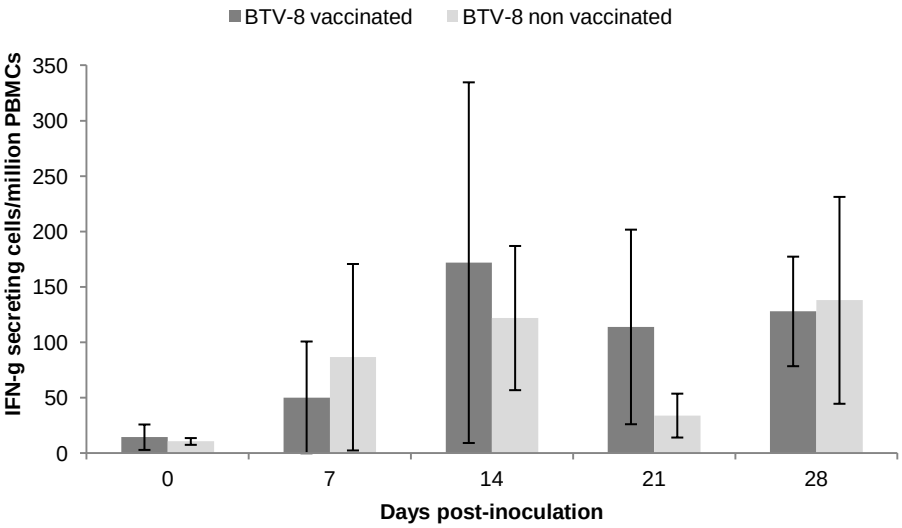

Figure 16. Interferon-gamma spot numbers produced by PBMCs after stimulation with the homologous strains. Mean with standard deviation of IFN-γ expression in 10^6 PBMCs stimulated after challenge with BTV-1 (A) and BTV-8 (B). Counting of spots of the negative control has been substracted from each sample.

6.4.5. Clinical signs and histopathological examination

No clear clinical signs compatible with BTV infection were detected throughout after inoculation (from 0 to 28 dpi). Only the non vaccinated ibexes inoculated with BTV-1 had a punctual increase (P>0.05) in rectal temperature at 7 dpi. No gross lesions were found after challenge, at necropsy, or after histological analysis of target tissues.

6.5. Discussion

BTV RNA detection both by RT-PCR and RT-qPCR, and BTV isolation without specific clinical signs confirm the susceptibility of Spanish ibex to asymptomatic BTV infection. Overall, viraemia dynamics was rather similar to that of other asymptomatic hosts, either domestic (cattle and goat) or wild, namely red deer [Backx *et al*., 2011; Bréard *et al*., 2011; López-Olvera *et al*., 2010; Lorca-Oró *et al*., 2012a; Puentes *et al*., 2008], than to that of clinically susceptible species, also either domestic (sheep) or wild (white-tailed deer and mouflon) [Elis *et al*., 1993; Fernández-Pacheco *et al*., 2008; Murray & Trainer, 1970; Puentes *et al*., 2008; Vosdingh *et al*., 1968]. As demonstrated by ELISA and SNT, vaccination induced protective neutralising antibodies against BTV-1 and BTV-8 in the vaccinated Spanish ibexes, which did not develop viraemia. Therefore, one only dose of vaccination protected Spanish ibex against BTV infection. This enhances the potential usefulness of wild ruminant vaccination as a complementary tool to control BTV transmission and permits a successful immunization with only one handling. After the antibody peak measured by ELISA in the non-vaccinated groups (at 14 dpi in BTV-1 and 9 dpi in BTV-8) antibody levels decreased, even until negative results in the BTV-8 group. These findings are in agreement with the previously observed in domestic ruminants by Echbaumer *et al*. (2011), who demonstrate that double antigen ELISAs are highly sensitive for vaccine induced antibodies but might be less sensitive after infection. Also, in the neutralisation test all non-vaccinated animals seroconverted and remained positive at high levels until the end of the experiment.

To the authors' knowledge, this is the first time that IFN-γ expression is studied in Spanish ibex as a first approach to the cellular immune response to BTV infection. IFN-γ is secreted by natural killer cells, CD4 and CD8 T cells as a response to BTV infection [Hemati *et al*., 2009]. The increase in IFN-γ expression found in the vaccinated ibexes after BTV inoculation agrees with previous results in domestic sheep and cattle [Hund *et al*., 2012; Umeshappa *et al*., 2010]. Monocytes are a preferential target for BTV [Barratt-Boyes *et al*., 1992; Whetter *et al*., 1989]. Higher monocyte counts in the BTV-1 vaccinated ibexes at 7 and 11 dpi could mean that these animals were having a cellular response after the challenge. In spite of reported immune cross reaction between BTV-1 and BTV-8, the differences found in leukocyte, monocyte and lymphocyte trends between the BTV-1 and BTV-8 inoculated Spanish ibexes suggest differences between the strains used in this study (BTV-1/ALG/2006 and BTV-8/BEL/2006, respectively), not only in pathogenesis, but also in vaccine action pathways. Differences in pathogenesis regarding the same virus isolates (BTV-1 and BTV-8) in other wild ruminant species, namely red deer, have been previously suggested [Lorca-Oró *et al*., 2012a]. The more disseminated tissue distribution of BTV-1 as compared to BTV-8 at 8 dpi (Table 6) seems to further confirm this difference in pathogenesis between both strains. A similar low transient BTV-8 RNA detection after experimental infection has already been reported in red deer [López-Olvera *et al*., 2010; Lorca-Oró *et al*., 2012a]. The positive tissue samples from vaccinated ibexes found at 28 dpi without showing viraemia or seroconversion after challenge, especially for the BTV-8 inoculated ones, could be explained by residual viral particles from the inoculum at those tissues. Furthermore, BTV detection in tissues at 8 dpi in our study are in agreement with those previously reported [Sánchez-Cordón *et al*., 2010], which demonstrated both for domestic sheep and goat, that spleen, lymph nodes and lungs are target organs. Moreover, BTV was also detected in the gut-associated lymphoid tissue and liver of goats, as we also found by RT-PCR, RT-qPCR and virus isolation. While domestic sheep and goat showed histopahologic lesions compatible with BT, Spanish ibex did not.

This study demonstrates that: (1) Spanish ibex can be infected with BTV-1 and BTV-8 but is not affected clinically; (2) One single dose of monovalent vaccine prevents BTV viraemia of both BTV-1 and BTV-8. It also suggests that pathogenesis

and host immune response may vary among the different BTV strains and that Spanish ibex can contribute to the maintaining of BTV confirmed by viraemia detected until 28 dpi and probably for longer periods. To the authors' knowledge, this is the first study involving two BTV serotypes immunization and experimental infection in Spanish ibex, which may be useful for possible strategies to control BTV transmission from and among wild ruminants.

6.6. Acknowledgments

The authors would like to thank Syva Laboratories for providing the vaccines and challenge viruses. The authors are also very grateful to the rangers and staff of the National and Natural Park of Sierra Nevada and Agencia de Medio Ambiente y Agua of the Junta de Andalucía working on the Spanish Ibex Management Program**.**

7. Chapter 5

Longitudinal serological analysis in Spanish ibex (*Capra pyrenaica*) vaccinated against bluetongue virus serotypes 1 and 8

Submitted

7.1. Abstract

Bluetongue virus (BTV) is the causal agent of Bluetongue (BT), a disease of wild and domestic ruminants. Control strategies have been applied to livestock in Europe, but BTV still persists in wild ruminants. Thirty out of 104 Spanish ibexes (*Capra pyrenaica*) were subcutaneously vaccinated against BTV serotype 1 and 32 against BTV serotype 8, the remaining 44 ibexes acting as non vaccinated controls.

All ibexes were seronegative before vaccination and RT-qPCR negative both before vaccination and at the end of the study. ELISA and serum neutralisation test were performed on serum samples at 0, 1, 4, 12 and 18 months post-vaccination (mpv). Non vaccinated ibexes remained seronegative, while BTV-1 and BTV-8 vaccinated groups seroconverted from 1 mpv.

In conclusion, one dose of vaccine induced BTV specific neutralising antibodies in Spanish ibex for at least 18 mpv.

7.2. Introduction

Bluetongue disease (BT) is an emerging and re-emerging disease in Europe caused by bluetongue virus (BTV), which is the prototype agent of the genus *Orbivirus* (family *Reoviridae*). BTV is transmitted by the bites of female *Culicoides* midges mainly to domestic and wild ruminants and camelids [Maclachlan *et al*., 2009; Mellor & Wittmann, 2002; Mertens *et al*., 2004]. There are at least 24 different BTV serotypes, and two more serotypes proposed as 25th or *Toggenburg Orbivirus* [Chaignat *et al*., 2009; Hofmann *et al*., 2008] and 26th [Maan *et al*., 2009]. In the last decade, BT has expanded its geographical range northwards [Enserink, 2008; Eschbaumer *et al*., 2009; Purse *et al*., 2008; Rodríguez-Sánchez *et al*., 2008].

Since 2006 with the entry of BTV-8 in Northern and central Europe, epidemiological survey programs, transport restrictions of livestock and a large-scale vaccination program were implemented in the affected countries. For safety reasons, only inactivated monovalent vaccines were used against BTV-8 [Enserink, 2008]. BTV-1 appeared in Southern Spain in 2007 and expanded northwards until the south of France [OIE, 2007]. Shortly after the announcement of the presence of BTV-1 in the North of Spain at the end of 2007, BTV-8 was also detected in a neighbouring area [OIE, 2008]. Vaccination against both serotypes was effective to slow down BT expansion but Spain is still considered a restriction zone for these two serotypes, as well as for BTV-4 in the southern regions [RASVE, 2009]. The role of wild ruminants in BT epidemiology has been recently shown in Spain [Falconi *et al*., 2012; García-Bocanegra *et al*., 2011].

Wild ruminants are mainly asymptomatic hosts and may contribute to virus maintenance. Therefore, they should be considered in the control strategies of BT programs. Spanish ibex (*Capra pyrenaica*) is a wild caprine endemic to the Iberian Peninsula [Pérez *et al*., 2002]. This wild mountain ungulate has a great value for its conservation and has been listed as threatened and currently of least concern in the IUCN Red List of Threatened Species. Antibodies against BTV [García *et al*., 2009; Lorca-Oró *et al*., 2011] and BTV-1 RNA [Arenas-Montes *et al*., 2010; Lorca-Oró *et al*., 2011] have been previously detected in captive and wild free-ranging Spanish ibex, and experimental infection with BTV-1 and BTV-8 induced BTV replication with

RNA detection up to 28 days after BTV inoculation [Lorca-Oró *et al*., 2012b]. These facts further suggest the potential of this species as BT reservoir in the Iberian Peninsula. Although one vaccination dose has proved to induce neutralising antibodies after challenge one month post-immunization [Lorca-Oró *et al*., 2012b], the persistence of such antibodies, which is relevant for BT control, remain to be elucidated.

The aim of the present study is to evaluate the duration of protection after vaccination against BTV serotypes 1 and 8 in a potentially susceptible species such as Spanish ibex.

7.3. Materials and methods

7.3.1. Ethics statement

Animals included in the present study were ibexes from the Captive Breeding Center of Sierra Nevada (Granada, Southern Spain). Permits for vaccination and transport were approved by the Consejería de Medio Ambiente – Junta de Andalucía (Registration number: 1626). Handling procedures were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish laws (R.D. 223/1988, R.D.1021/2005), and current guidelines for ethical use of animals in research (2006). The present study was approved by the Ethical and Animal Welfare Committee of the Universitat Autònoma de Barcelona (Permit Number: 4485).

7.3.2. Vaccination

A total of 104 Spanish ibexes ranging from zero to fourteen years old were included in this study. Thirty ibexes were subcutaneously vaccinated with a single dose of 2mL of inactivated BTV-1 vaccine and 32 with 2 mL of BTV-8 (Syvazul 1, batch 10003P and Syvazul 8, batch 10005P, Laboratorios SYVA, León, Spain). From the remaining 44 ibexes, 16 were left as non vaccinated controls and the remaining 28 were born during the study, also acting as controls. The ibexes were captured with a corral trap, immobilized through a capture chute, and released until next sampling.

Blood samples without anticoagulant were collected by jugular puncture before vaccination and at 4, 12 and 18 months after vaccination (mpv) for serology. Blood samples were also collected from fifteen vaccinated and control ibexes one mpv, before experimental infection with BTV [Lorca-Oró *et al*., 2012b]. Blood samples with EDTA were collected by jugular puncture before vaccination and at 18 mpv for BTV detection. The number of sampled ibexes for each group and date is shown in Table 8. Serum was extracted from whole blood tubes after centrifugation (300 x G for 15 minutes) and stored at -20ºC. EDTA blood was stored at 4ºC until analysis.

Table 8. Number of ibexes per group in each sampling period.

Treatment	Months post vaccination				
	0	1	4	12	18
BTV-1 vaccinated	30	4	22	20	13
BTV-8 vaccinated	32	4	24	19	12
Non vaccinated	16	7	18	13	24
Total N	78	15	54	52	49

7.3.3. Serology and BTV detection

Sera were analysed for the presence of specific antibodies against the BTV major core protein VP7, using a commercial double-antigen ELISA assay (Ingezim BTV DR12.BTV.KO Ingenasa, Spain). Serotype specific antibodies were detected by means of serum neutralisation test (SNT) as described previously [Lorca-Oró *et al*., 2012b; OIE, 2009]. Only samples that showed neutralisation (absence of cytopathic effect) at dilutions ≥1:4 were considered positive.

Real-time quantitative RT-PCR (RT-qPCR) was performed as previously described [Lorca-Oró *et al*., 2012b; Toussaint *et al*., 2007].

7.3.4. Statistical analysis

An analysis of the variance (ANOVA) was performed to detect statistical differences regarding specific BTV antibodies (tested by ELISA and SNT) using the software NCSS [Number Cruncher Statistical Systems; Hintze, 2004]. The main factor was treatment (BTV-1 or BTV-8 vaccinated and non vaccinated) and the repeated factor was sampling period (0, 1, 4, 12, 18 mpv). Differences were considered statistically significant when $P<0.05$.

7.4. Results

All the ibexes included in the present study were negative to BTV specific ELISA, SNT and RT-qPCR negative to BTV before vaccination. BTV-specific antibodies measured by ELISA increased significantly after vaccination, remaining stable from 1 to 18 mpv. Both BTV-1 and BTV-8 vaccinated ibexes showed significantly ($P<0.05$) higher detectable antibody levels compared to non vaccinated ibexes throughout the study (Figure 17). Neutralising antibody titres were also statistically higher ($P<0.05$) in the vaccinated ibexes as compared to the non vaccinated ones, while no statistically significant differences were found between the BTV-1 and BTV-8 vaccinated groups. Non vaccinated ibexes remained seronegative (ELISA positive threshold >15%) and did not show neutralising antibodies during the study period (Figure 18). BTV RNA was not detected by RT-qPCR in any blood sample both at 0 and 18 mpv.

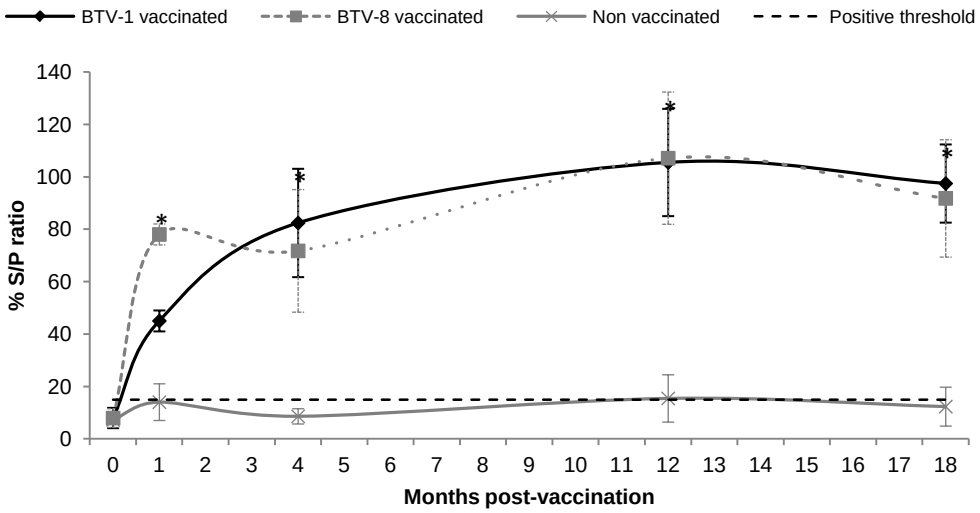

Figure 17. ELISA antibody response after BTV-1 and BTV-8 vaccination. Evolution of mean percentages (O.D. sample/O.D. positve control x100) with standard deviation of VP7 antibodies for each group of vaccinated and non vaccinated ibexes. Asterisks (*) mean statistical differences between vaccinated (both BTV-1 and BTV-8) and non vaccinated ibexes.

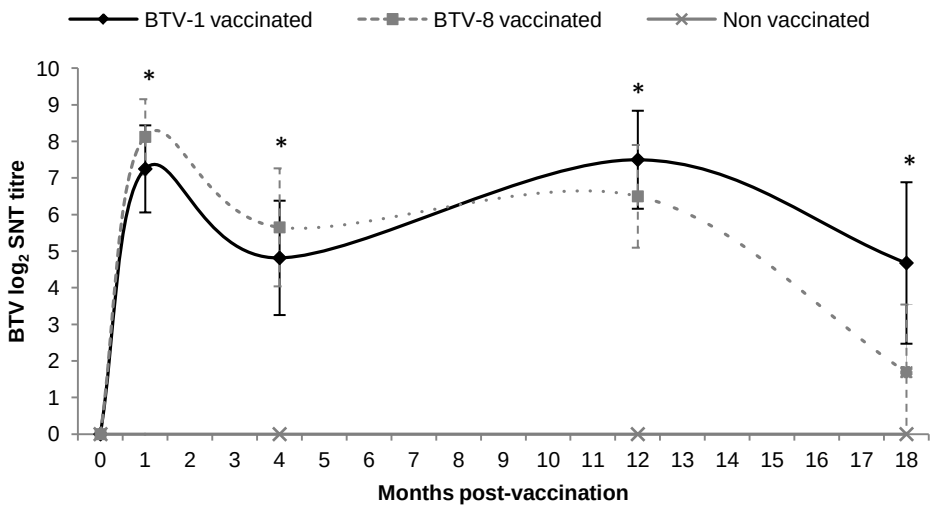

Figure 18. Neutralising antibody response after BTV vaccination. Evolution of mean (with standard deviation) of BTV-1 and BTV-8 neutralising antibody titres for vaccinated and non vaccinated ibexes.

7.5. Discussion

The potential implication of wild ruminants in the maintenance and transmission of BTV has already been suggested [Falconi *et al*., 2011; García *et al*., 2009; Ruiz-Fons *et al*., 2008]. Spanish ibex is a relevant wild ungulate for its conservation and as a game species, and it could participate in the epidemiology of BT due to its susceptibility to BTV infection [García *et al*., 2009; Lorca-Oró *et al*., 2011; Lorca-Oró *et al*., 2012b; Pérez *et al*., 2002]. This study provides novel information on the persistence of both total (ELISA) and neutralising (SNT) antibodies induced by a single dose of monovalent commercial inactivated vaccine against BTV-1 or BTV-8 in Spanish ibex. Both serotypes are currently present in Spain and other European countries. Neutralising antibodies are known to play a key role protecting from disease and viraemia, and protective immunity is generally associated with the presence of type-specific neutralising antibodies [Savini *et al*., 2008].

Vaccination is still the most effective tool to control BT, prevent BTV infection and allow animal translocation with no risk of virus transmission between susceptible ruminants. Inactivated vaccines against BT have been successfully used in domestic ruminants [Bartram *et al*., 2011; Bréard *et al*., 2011; Eschbaumer *et al*., 2009; Oura *et al.*, 2009; Wäckerlin *et al*., 2010]. Repeated application of immunization with inactivated vaccines is generally considered necessary for the induction of long-term protection [Savini *et al*., 2009; Schwartz-Cornil *et al*., 2008]. However, a single dose of commercial inactivated vaccines protected domestic sheep from BTV-8 and BTV-2 experimental infection ten and twelve months after vaccination, respectively [Hamers *et al*., 2009a; Hamers *et al*., 2009b; Oura *et al*., 2009; Wäckerlin *et al*., 2010], but failed to prevent BTV-4 viraemia in cattle seven months after vaccination [Savini *et al*., 2008]. Although BTV challenge was not performed in the vaccinated ibexes at 18 mpv, the levels of neutralising antibodies in the vaccinated groups was similar to protective levels in Spanish ibexes challenged with the homologous serotypes one mpv [Lorca-Oró *et al*., 2012b]. Even though two doses of vaccine could have reduced the heterogeneity of antibody levels in our study, a single-shot is a more realistic approach for vaccinating wildlife.

Mass vaccination of wild ruminants would be logistically challenging in free-ranging wildlife, but useful in semi-free populations (breeding centres, reserves,

zoos or hunting states). In this particular case, as a susceptible but asymptomatic species, vaccination could be useful especially to avoid BTV transmission between wildlife and livestock.

In conclusion, the results suggest that one single dose of available commercial inactivated vaccines against BTV-1 or BTV-8 may be used as a strategy to control BT in handled Spanish ibexes and, therefore, may also avoid the transmission to both wild and domestic ruminants.

7.6. Acknowledgements

The authors are very grateful to the rangers and staff of the National and Natural Park of Sierra Nevada and Agencia de Medio Ambiente y Agua of the Junta de Andalucía working on the Spanish Ibex Management Program. The authors would also like to thank Syva Laboratories for providing the vaccines used in the study.

8. General Discussion

Bluetongue is an infectious disease that can reappear for different reasons including new arrival of competent vectors, movement of infected animals or maintaining the virus in susceptible hosts. Most BT research has been focused in domestic species, and although dynamics of BT are currently (quite) well-known, there are still gaps in understanding key factors as the role wild ruminants play in the epidemiology of BT.

To further characterize the implication of wild ruminant species in the epidemiology of BT and to approach to the control of BTV infection in these populations, the present thesis was formulated. In this general discussion, a summary of the main findings is reviewed.

Epidemiological studies of bluetongue in wild ruminants (Section I)

Several studies have previously pointed towards the importance of wild ruminants in the transmission and maintenance of BTV. However, large-scale studies to elucidate the role of wild ruminants are still limited. The epidemiological studies carried out in this thesis (Chapters 1 and 2), based in serologic and molecular tests, provide new data regarding the temporal and spatial variations of BT in the different species from the Iberian Peninsula. The results evidence that wild ruminants have been exposed and responded serologically to three BTV serotypes (BTV-1, BTV-4 and BTV-8) also detected in domestic ruminants in the same areas. Moreover, BTV circulation, especially BTV-1, has been demonstrated by the detection of BTV RNA. Also, our results suggest that BTV-1 and BTV-4 have remained longer in wild ruminant populations than BTV-8, which is detected less frequently in livestock too. All the species studied in this thesis presented specific antibodies against BTV, although only red deer, fallow deer (Chapter 1) and Spanish ibex (Chapter 2) were positive to BTV RNA. Previous publications have also found such corresponence between wild ruminants BTV seropositivity and domestic livestock outbreaks, although there have been exceptions indicating circulation of BTV in wild ruminants in areas where no outbreaks had been detected in livestock [Falconi *et al*., 2011; García *et al.* 2009; García-Bocanegra *et al*., 2011; Rodríguez-Sánchez *et al*., 2010a; Ruiz-Fons *et al*., 2008].

As suggested by Ruiz-Fons *et al*. (2008), BT has a complex epidemiologic scenario, where there are many suscpetible hosts implicated, with the expansion of the main vector (*C. imicola*) and the presence of new competent vectors (especially *C. obsoletus* and *C. pulicaris* groups). Red deer has a wider distribution in Europe, lives in large groups and moves more than other wild species also abundant as roe deer. Thus, red deer might be more exposed to insects/pathogens than other wild species [Linden *et al*., 2010]. Moreover, red deer is able to maintain BTV for long periods as it has been demonstrated in experimental infections [López-Olvera *et al*., 2010]. Among the different wild species analysed, European red deer is the one that plays a major role in the epidemiology of BT [Falconi *et al*., 2011; Rodríguez-Sánchez *et al*., 2010a]. In the case of Spanish ibex, from a global point of view, our results suggest that this species seems not relevant in the epidemiology of BT. However, as an emblematic species for its conservation and its hunting interest and for its proximity to domestic goat, we also considered it important to study. Regarding the seroprevalence found, other species as fallow deer, roe deer or mouflon can also be relevant, depending in the population density, the chance of having contact with domestics and, as it is the case of mouflons, the sensitivity to the disease. The present results demonstrate that BTV persists in wild ruminants after vaccination campaigns in livestock, but it remains to be elucidated which vector species are shared between wild and domestic ruminants and the possibility to confirm the cycle of BTV transmission from wild to domestic ruminants.

Response to experimental BTV infection (Section II)

The results of the experimental infections (Chapters 3 and 4) confirm the susceptibility of red deer and Spanish ibex to asymptomatic BTV infections. Absence of clinical signs after experimental infections has also been reported in blesbock and mountain gazelle, whereas other species suffered clinical disease after experimental (pronghorn antelope, American bison, and African buffalo) or natural (European mouflon) infections [Barzilai & Tadmor, 1972; Bender *et al*., 2003; Fernández-Pacheco *et al*., 2008; Howerth *et al*., 2001; Tessaro & Clavijo, 2001]. Viraemia dynamics were also similar to that of other asymptomatic hosts, either domestic or wild, including cattle, which is considered a reservoir for BTV [Backx *et*

al., 2011; Bréard *et al*., 2011; Gerdes, 2004; López-Olvera *et al*., 2010]. Therefore, the results evidence that red deer and Spanish ibex may also act as potential BTV reservoirs.

In both experimental infections, BTV-8 presented fluctuant viraemia and was more difficult to isolate than BTV-1, suggesting differences between strains. Despite the absence of clinical signs or lesions in our experimental inoculations in these two species, it is important to notice that the same BTV strains have also been used in experimental infections of domestic ruminants, causing clinical signs and lesions in sheep. These results indicate differences in the susceptibility to BTV infection between hosts.

Response to vaccination (Section II)

In reference to BTV immunization (Chapters 3, 4 and 5), the main challenge regarding strategies to control BT is the lack of vaccines able to confer cross-protection against several serotypes. The monovalent inactivated vaccines used have been safe and protective in both red deer and Spanish ibex. Two vaccine doses were administered in red deer, while Spanish ibex received a single dose, which is a more realistic approach in wild ruminants. In both experimental infections (Chapters 3 and 4), vaccinated animals developed protective antibody levels comparable to those previously reported in domestic ruminants [Calistri *et al*., 2010; Eschbaumer *et al*., 2009; Hamers *et al*., 2009b; Umeshappa *et al*., 2010; Wäckerlin *et al*., 2010]. Moreover, in the case of Spanish ibex, antibodies were detected up to 18 months after a single vaccination (Chapter 5). The antibody levels achieved through the 18 mpv are comparable to those protective after challenge one mpv [Lorca-Oró *et al*., 2012b (Chapter 4)]. The scope of these three studies was not to propose mass vaccination of wild ruminants, but to provide useful information on humoural responses after vaccination especially appropriate for semi-free populations as breeding centres, game farms, zoos or hunting states. These populations are often treated as domestic ruminants, in breeding centres or game farms, sometimes with high population densities and with animal translocations. Thus, these animals are exposed to the same pathogens than domestics but the

control measures are less strict or absent. With that objective, we consider that vaccination can be necessary in certain areas and periods of BT risk, especially to avoid BTV transmission from and among wild ruminants.

Overall, the present thesis provides new insights regarding BT in wild ruminant populations from the Iberian Peninsula. Furthermore, we believe that these studies may be useful in other countries with risk of BT and even for the study of other arboviral diseases involving wildlife. It remains to be ascertained whether a competent vector is able to complete the cycle (both biologically and environmentally) from domestic to wild ruminant hosts (and *viceversa*), which would confirm BTV transmission between wildlife and livestock.

9. Conclusions

1. The seroprevalence found in wild ruminants present in the Iberian Peninsula indicate that these species have been exposed to bluetongue virus (BTV), especially BTV-1 and BTV-4, but less to BTV-8, during and after vaccination campaigns in domestic livestock.

2. Wild ruminants have a potential role as BTV reservoirs in the Iberian Peninsula, and should be included in BTV surveillance programs.

3. Among wild ruminants, red deer seems to play a major role in the epidemiology of BT, probably due to its abundance and wide distribution.

4. Red deer and Spanish ibex are susceptible to BTV-1 and BTV-8 infection, which is asymptomatic, at least with the strains used in the experimental infections.

5. The inactivated vaccines against BTV-1 and BTV-8 used in the studies are safe and protective in red deer and Spanish ibex.

6. A single BTV vaccine dose is protective in Spanish ibex, which might be a more realistic strategy in wild species.

7. BTV vaccination induces long-term (18 months) specific antibodies in Spanish ibex.

10. REFERENCES

A

Acevedo P, Cassinello J, Hortal J, Gortázar C (2007) Invasive exotic aoudad (*Ammotragus lervia*) as a major threat to native Iberian ibex (*Capra pyrenaica*): a habitat suitability model approach. *Diversity and Distributions* 13: 587–597.

Acevedo P, Ruiz-Fons F, Vicente J, Reyes-García AR, Alzaga V, Gortázar C (2008) Estimating red deer abundance in a wide range of management situations in Mediterranean habitats. *Journal of Zoology* 276: 37-47.

Agüero M, Arias M, Romero LJ, Zamora MJ, Sánchez-Vizcaíno JM (2002) Molecular differentiation between NS1 gene of a field strain Bluetongue virus serotype 2 (BTV-2) and NS1 gene of an attenuated BTV-2 vaccine. *Veterinary Microbiology* 86: 337-341.

Agüero M, Buitrago D, Gómez-Tejedor C (2008) False-positive results obtained when bluetongue virus serotype 1 Algeria 2006 was analysed with a reverse transcription-PCR protocol for detection of epizootic hemorrhagic disease virus. *Journal of Clinical Microbiology* 46: 3173-3174.

Allepuz A, García-Bocanegra I, Napp S, Casal J, Arenas A, Saez M, González MA (2010) Monitoring bluetongue disease (BTV-1) epidemic in southern Spain during 2007. *Preventive Veterinary Medicine* 96: 263-271.

Anonymous (2010) Base de datos de Comercio Exterior: http://aduanas.camaras.org/.

Arenas-Montés A, Borge C, Carbonero A, Perea A, González MA, **Cadenas R, Zorrilla I, Arenas A, García-Bocanegra I** (2010) Maintenance du sérotype 1 du virus de la Fièvre Catarrhale Ovine chez les ruminants sauvages dans le sud de l'Espagne. *Proceedings of the 28èmes Rencontres du Groupe d'Êtudes sur l'Ecopathologie de la Faune Sauvage de Montagne (G.E.E.F.S.M.)*. Rocchetta Nervina (Italia), June 10th-13th, 2010, p. 21.

Association for the study of animal behaviour (ASAB) (2006) Guidelines for the treatment of animals in behavioural research and teaching. *Animal Behaviour* 71: 245–253.

B

Backx A, Heutink CG, Van Rooij EMA, Van Rijn PA (2007) Clinical signs of bluetongue virus serotype 8 infection in sheep and goats. *Veterinary Record* 161: 591-593.

Barratt-Boyes SM, Rossitto PV, Stott JL, MacLachlan NJ (1992) Flow cytometric analysis from in vitro bluetongue virus infection of bovine blood mononuclear cells. *Journal of General Virology* 73: 1953-1960.

Barratt-Boyes SM, Maclachlan NJ (1994) Dynamics of viral spread in bluetongue virus infected calves. *Veterinary Microbiology* 40: 361-371.

Barratt-Boyes SM, Maclachlan NJ (1995) Pathogenesis of bluetongue virus infection of cattle. *Journal of the American Veterinary Medical Association* 206: 1322-1329.

Barratt-Boyes SM, Rossitto PV, Taylor BC, Ellis JA, Maclachlan NJ (1995) Response of the regional lymphnode to bluetongue virus infection in calves. *Veterinary Immunology and Immunopathology* 45: 73-84.

Bartram DJ, Heasman L, Batten CA, Oura CAL, Plana-Durán J, Yuen HM, Wylie ADM (2011) Neutralising antibody responses in cattle and sheep following booster vaccination with two commercial inactivated bluetongue virus serotype 8 vaccines. *Veterinary Journal* 188: 193–196.

Barzilai TE, Tadmor A (1972) Experimental infection of the Mountain gazelle (*Gazella gazella*) with bluetongue virus. *Refuah Veterinarith* 29:45-50.

Bender LC, Hong L, Bruce Thomson C, Horrow PC, Valdez R (2003) Infectious disease of gemsbok in New Mexico. *Journal of Wildlife Diseases* 39: 722-778.

Bhanuprakash V, Indrani BK, Hosamani M, Balamurugan V, Singh RK (2009) Bluetongue vaccines: the past, present and future. *Expert Review of Vaccines* 8: 191-204.

Bonneau KR, DeMaula CD, Mullens BA, Maclachlan NJ (2002) Duration of viremia infectious to *Culicoides sonorensis* in bluetongue virus-infected cattle and sheep. *Veterinary Microbiology* 88: 115-125.

Bréard E, Belbis G, Hamers C, Moulin V, Lilin T, Moreau F, Millemann Y, Montange C, Sailleau C, Durand B, Desprat A, Viarouge C, Hoffmann B, de Smit H, Goutebroze S, Hudelet P, Zientara S (2011) Evaluation of humoral response and protective efficacy of two inactivated vaccines against bluetongue virus after vaccination of goats. *Vaccine* 29: 2495-2502.

Bréard E, Sailleau C, Nomikou K, Hamblin C, Mertens PPC, Mellor PS, El Harrak M Zientara S (2007) Molecular epidemiology of bluetongue virus serotype 4 isolated in the Mediterranean Basin between 1979 and 2004. *Virus Research* 125: 191-197.

Brewer AW, Maclachlan NJ (1992) Ultrastructural characterization of the interaction of bluetongue virus with bovine erythrocytes in vitro. *Veterinary Pathology* 29: 356-359.

Brewer AW, Maclachlan NJ (1994) The pathogenesis of bluetongue virus infection of bovine blood cells in vitro: ultrastructural characterization. *Archives of Virology* 136: 287-298.

C

Calistri P, Giovannini A, Savini G, Bonfanti L, Cordioli P, Lelli R, Caporale V (2010) Antibody response in cattle vaccinated against bluetongue serotype 8 in Italy. *Transboundary and Emerging Diseases* 57: 180-184.

Calvete C, Estrada R, Miranda MA, Borrás D, Calvo JH, Lucientes J (2008) Modelling the distributions and spatial coincidence of bluetongue vectors *Culicoides imicola* and the *Culicoides obsoletus* group throughout the Iberian peninsula. *Medical and Veterinary Entomology* 22: 124-134.

Carpenter S, Wilson A, Mellor PS (2009) *Culicoides* and the emergence of bluetongue in northern Europe. *Trends in Microbiology* 17:172-178.

Chaignat V, Worwa G, Scherrer N, Hilbe M, Ehrensperger F, Batten C, Cortyen M, Hofmann M, Thuer B (2009) *Toggenburg Orbivirus*, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. *Veterinary Microbiology* 138: 11-19.

Clavijo A, Heckert RA, Dulac GC, Afshar A (2000) Isolation and identification of bluetongue virus. *Journal of Virological Methods* 87: 13-23.

Conraths FJ, Gethmann JM, Staubach C, Mettenleiter TC, Beer M, Hoffmann B (2009) Epidemiology of bluetongue virus serotype 8, Germany. *Emerging Infectious Diseases* 5: 433-435.

D

Darpel KE, Batten CA, Veronesi E, Shaw AE, Anthony S, Bachanek-Bankowska K, Kgosana L, bin-Tarif A, Carpenter S, Müller-Doblies UU, Takamatsu HH, Mellor PS, Mertens PP, Oura CA (2007) Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. *Veterinary Record* 161: 253-261.

Darpel KE, Monaghan P, Anthony SJ, Takamatsu H, Mertens PP (2009) Bluetongue virus in the mammalian host and the induced immune response. *In: Bluetongue*, P Mellor, M Baylis and P Mertens, Eds, Elsevier, London, pp. 265-284.

De Curtis M, Bartolini C, Canonico C, Duranti A, Leoni F, Mancini P, Moscatelli F, Rocchegiani E, Gavaudan S (2007) Serological monitoring of bluetongue virus in wild ruminants of the Pesaro-Urbino district (Italy) – Monitoraggio sierologico del virus della bluetongue in ruminanti selvatici del distretto di Pesaro Urbino *Webzine Sanità Pubblica Veterinaria*: Numero 40, Febbraio 2007. http://www.spvet.it/ (accessed 02.05.2010).

Durand B, Zanella G, Biteau-Coroller F, Locatelli C, Baurier F, Simon C, Le Dréan E, Delaval J, Prengère E, Beauté V, Guis H (2010) Anatomy of bluetongue virus serotype 8 epizootic wave, France, 2007–2008. *Emerging Infectious Diseases* 16:1861–1868.

Du Toit RM (1944) The transmission of bluetongue and horsesickness by *Culicoides*. *Onderstepoort Journal of Veterinary Science and Animal Industry* 19: 7–16.

E

Elbers AR, Backx A, Meroc E, Gerbier G, Staubach C, Hendrickx G, van der Spek A, Mintiens K (2008a) Field observations during the bluetongue serotype 8 epidemic in 2006. I. Detection of first outbreaks and clinical signs in sheep and cattle in Belgium, France and the Netherlands. *Preventive Veterinary Medicine* 87: 21-30.

Elbers ARW, Backx A, Mintiens K, Gerbier G, Staubach C, Hendrickx G, van der Spek A (2008b) Field observations during the bluetongue serotype 8 epidemic in 2006. II. Morbidity and mortality rate, case fatality and clinical recovery in sheep and cattle in the Netherlands. *Preventive Veterinary Medicine* 87: 31-40.

Ellis JA, Coen ML, Maclachlan NJ, Wilson WC, Williams ES, Leudke AJ (1993) Prevalence of bluetongue virus expression in leukocytes from experimentally infected ruminants. *American Journal of Veterinary Research* 54: 1452–1456.

Enserink M (2008) Animal disease - Exotic disease of farm animals tests Europe's responses. *Science* 319: 710-711.

Erasmus BJ (1975) Bluetongue in sheep and goats. *Australian Veterinary Journal* 51: 165-170.

Eschbaumer M, Hoffmann B, Konig P, Teifke JP, Gethmann JM, Conraths FJ, Probst C, Mettenleiter TC, Beer M (2009) Efficacy of three inactivated vaccines against bluetongue virus serotype 8 in sheep. *Vaccine* 27: 4169-4175.

Eschbaumer M, Wackerlin R, Hoffmann B, Beer M (2010) Re: Detection of bluetongue virus genome after vaccination with an inactivated vaccine. *Vaccine* 28: 881-882.

Eschbaumer M, Schulz C, Wäckerlin R, Gaulyn M, Beer M, Hoffmann B (2011) Limitations of sandwich ELISAs for bluetongue virus antibody detection. *Veterinary Record* 168: 643.

European Commission (2009) Bluetongue. In: Food Safety – From the Farm to the Fork, Animal Health and Welfare (accessed 12.10.09) In: http://ec.europa.eu/food/animal/diseases/controlmeasures/bluetongue_en.htm

European Commission (2011) Bluetongue. In Food Safety - From the Farm to the Fork, Animal Health and Welfare (accessed 18.04.11) In: http://ec.europa.eu/food/animal/diseases/controlmeasures/bluetongue_en.htm

F

Falconi C, López-Olvera JR, Gortázar C (2011) BTV infection in wild ruminants, with emphasis on red deer: A review. *Veterinary Microbiology* 151: 209-219.

Falconi C, López-Olvera JR, Boadella M, Camarena J, Rosell R, **Alcaide V, Vicente J, Sánchez-Vizcaíno JM, Pujols J, Gortázar C** (2012) Evidence for BTV-4 circulation in free-ranging red deer (*Cervus elaphus*) in Cabañeros National Park, Spain. *Veterinary Microbiology* 159: 40-46.

Fandos P, Reig S (1992) Problems associated with mouflon and barbary sheep introductions in Spain. *In: Global Trends in Wildlife Management*, Bobek B, Pierzanowski K, Regelin W, editors. Swiat Press, Krakow-Warzawa, Poland. pp. 139-140.

Fandos P (1995) Factors affecting horn growth in male Spanish ibex (*Capra-pyrenaica*). *Mammalia* 59: 229-235.

Fernández-Pacheco P, Fernández-Pinero J, Agüero M, Jiménez-Clavero MA (2008) Bluetongue virus serotype 1 in wild mouflons in Spain. *Veterinary Record* 162: 659-660.

Ferrari G, De Liberato C, Scavia G, Lorenzetti R, Zini M, Farina F, Magliano A, Cardeti G, Scholl F, Guidoni M, Scicluna MT, Amaddeo D, Scaramozzino P, Autorino GL (2005) Active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy. *Preventive Veterinary Medicine* 68: 103-113.

G

García I, Napp S, Casal J, Perea A, Allepuz A , Alba A, Carbonero A, Arenas A (2009) Bluetongue epidemiology in wild ruminants from Southern Spain. *European Journal of Wildlife Research* 55: 173-178.

García-Bocanegra I, Arenas-Montes A, Lorca-Oró C, Pujols J, González MA, Napp S, Gómez-Guillamón F, Zorrilla I, Miguel ES, Arenas A (2011) Role of wild ruminants in the epidemiology of bluetongue virus serotypes 1, 4 and 8 in Spain. *Veterinary Research* 42:88.

García-Lastra R, Leginagoikoa I, Plazaola JM, Ocabo B, Aduriz G, Nunes T, Juste RA (2012) Bluetongue Virus Serotype 1 Outbreak in the Basque Country (Northern Spain) 2007–2008. Data Support a Primary Vector Windborne Transport. *PLoS One* 7(3): e34421. doi:10.1371/journal.pone.0034421

Gard GP (1984) Studies of bluetongue virulence and pathogenesis in sheep. Department of Primary Industry and Development, Darwin, Australia. *Technical bulletin* No. 103.

Gavier-Widén D, Duff JP, Meredith A (2012) Infectious Diseases of wild mammals and birds in Europe. UK: Blackwell Publishing Ltd.

Gerdes GH (2004) A South African overview of the virus, vectors, survelliance and unique features of bluetongue. *Veterinaria Italiana* 40: 39-42.

González-Candela M, Cubero MJ, Martin-Atance P, León-Vizcaíno L (2006) Potential pathogens carried by Spanish ibex (*Capra pyrenaica hispanica*) in southern Spain. *Journal of Wildlife Diseases* 42: 325-334.

Granados JE, Pérez JM, Márquez FJ, Serrano E, Soriguer RC, Fandos P (2001) La cabra montés (*Capra pyrenaica*, Schinz 1838). *Galemys* 13: 3-37.

Gür S (2008) A serologic investigation of bluetongue virus (BTV) in cattle, sheep and gazella subgutturosa subgutturosa. *Tropical Animal Health and Production* 40: 217–221.

H

Hamers C, Galleau S, Chery R, Blanchet M, Besancon L, Cariou C, Werle-Lapostolle B, Hudelet P, Goutebroze S (2009a) Use of inactivated bluetongue virus serotype 8 vaccine against virulent challenge in sheep and cattle. *Veterinary Record* 165: 369-373.

Hamers C, Rehbein S, Hudelet P, Blanchet M, Lapostolle B, Cariou C, Duboeuf M, Goutebroze S (2009b) Protective duration of immunity of an inactivated bluetongue (BTV) serotype 2 vaccine against a virulent BTV serotype 2 challenge in sheep. *Vaccine* 27: 2789-2793.

Hateley G (2009) Bluetongue in northern Europe: the story so far. *In Practice* 31: 202-209.

Hemati B, Contreras V, Urien C, Bonneau M, Takamatsu H-H, Mertens PPC, Bréard E, Sailleau C, Zientara S, Schwartz-Cornil I (2009) Bluetongue virus targets conventional dendritic cells in skin lymph. *Journal of Virology* 83: 8789-8799.

Henning MW (1956) Animal Diseases in South Africa, 3rd Edit., Central News Agency, South Africa. pp. 809-827.

Henrich M, Reinacher M, Hamann HP (2007) Lethal bluetongue virus infection in an alpaca. *Veterinary Record* 161:764.

Hintze J (2004) NCSS and PASS. Number Cruncher Statistical Systems. Kaysville, Utah. www.NCSS.com

Hofmann MA, Renzullo S, Mader M, Chaignat V, Worwa G, Thuer B (2008) Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. *Emerging Infectious Diseases* 14: 1855-1861.

Hoffmann B, Eschbaumer M, Beer M (2009) Real-time quantitative reverse transcription-PCR assays specifically detecting bluetongue virus serotypes 1, 6, and 8. *Journal of Clinical Microbiology* 47: 2992-2994.

Howerth EW, Greene CE, Prestwood AK (1988) Experimentally induced bluetongue virus infection in whitetailed deer: coagulation, clinical pathologic, and gross pathologic changes. *American Journal of Veterinary Research* 49: 1906-1913.

Howerth EW, Tyler DE (1988) Experimentally induced bluetongue virus infection in white-tailed deer: ultra structural findings. *American Journal of Veterinary Research* 49: 1914–1922.

Howerth EW, Stallknecht DE, Kirkland PD (2001) Bluetongue, epizootic haemorrhagic disease, and other orbivirus-related diseases. In: *Infectious Diseases of Wild Mammals*. Williams ES, Barker IK (Eds) Iowa. State Univ Press, Ames.

Hund A, Gollnick N, Souter-Louis C, Neubauer-Juric A, Lahm H, Büttner M (2012) A two year BTV-8 vaccination follow up: molecular diagnostics and assessment of humoral and cellular immune reactions. *Veterinary Microbiology* 154: 247-256.

I

IUCN Red List of Threatened Species website (2012) Available: www.iucnredlist.org. Version 2011.2.

J

Jeggo MH, Wardley RC, Taylor WP (1984) Role of neutralising antibody in passive immunity to bluetongue infection. *Research in Veterinary Science* 36: 81–86.

Johnson DJ, Ostlund EN, Stallknecht DE, Goekjian VH, Jenkins-Moore M, Harris SC (2006) First report of bluetongue virus serotype 1 isolated from a white-tailed deer in the United States. *Journal of Veterinary Diagnostic Investigation* 18: 398-401.

K

Karstad L, Trainer DO (1967) Histopathology of experimental bluetongue disease of white-tailed deer. *Canadian Veterinary Journal* 8: 247-254.

Katz JB, Alstad AD, Gustafson GA, Moser KM (1993) Sensitive identification of bluetongue virus serogroup by a colorimetric dual oligonucleotide sorbent assay of amplified viral nucleic acid. *Journal of Clinical Microbiology* 31: 3028-3030.

Kuiken T, Leighton FA, Fouchier RAM, LeDuc JW, Peiris JSM, Schudel A, Stöhr K, Osterhaus ADME (2005) Pathogen Surveillance in Animals. *Science* 309:1680-1681.

L

León-Vizcaíno L, Ruiz de Ybáñez MR, Cubero MJ, Ortiz JM, Espinosa J, Pérez L, Simón MA, Alonso F (1999) Sarcoptic mange in Spanish ibex from Spain. *Journal of Wildlife Diseases* 35: 647-659.

Linden A, Mousset B, Gregoire F, Hanrez D, Vandenbussche F, Vandemeulebroucke E, Vanbinst T, Verheyden B, De Clerck K (2008) Bluetongue virus antibodies in wild red deer in southern Belgium. *Veterinary Record* 162: 459-459.

Linden A, Gregoire F, Nahayo A, Hanrez D, Mousset B, Massart L, De Leeuw I, Vandemeulebroucke E, Vandenbussche F, De Clercq K (2010) Bluetongue virus in wild deer, Belgium, 2005-2008. *Emerging Infectious Diseases* 16: 833-836.

Lobato ZI, Coupar BE, Gray CP, Lunt R, Andrew ME (1997) Antibody responses and protective immunity to recombinant vaccinia virusexpressed bluetongue virus antigens. *Veterinary Immunology and Immunopathology* 59: 293–309.

López-Olvera JR, Falconi C, Fernández-Pacheco P, Fernández-Pinero J, Sánchez MA, Palma A, Herruzo I, Vicente J, Jiménez-Clavero MA, Arias M, Sánchez-Vizcaíno JM, Gortázar C (2010) Experimental infection of European red deer (*Cervus elaphus*) with bluetongue virus serotypes 1 and 8. *Veterinary Microbiology* 145: 148-152.

Lorca-Oró C, Pujols J, Arenas A, Gómez-Guillamón F, Zorrilla I, Domingo M, Arenas-Montes A, Ruano MJ, García-Bocanegra I (2011) Epidemiological surveillance of bluetongue virus serotypes 1, 4 and 8 in Spanish ibex (*Capra pyrenaica hispanica*) in southern Spain. *Veterinary Microbiology* 149: 230-235.

Lorca-Oró C, López-Olvera JR, Fernández-Sirera L, Solanes D, Navarro N, García-Bocanegra I, Lavín S, Domingo M, Pujols J (2012a) Evaluation of the efficacy of commercial vaccines against bluetongue virus serotypes 1 and 8 in experimentally infected red deer (*Cervus elaphus*). *Veterinary Microbiology* 154:240-246.

Lorca-Oró C, Pujols J, García-Bocanegra I, Mentaberre G, Granados JE, Solanes D, Fandos P, Galindo I, Domingo M, Lavín S, López-Olvera JR (2012b) Protection of Spanish Ibex (*Capra pyrenaica*) against bluetongue virus serotypes 1 and 8 in a subclinical experimental infection. *PLoS One* 7(5):e36380.

Lovari S, Herrero J, Conroy J, Maran T, Giannatos G, Stubbe M, Aulagnier S, Jdeidi T, Masseti M, Nader I, de Smet K, Cuzin F (2008) *Cervus elaphus*. *In: IUCN 2010*. IUCN Red List of Threatened Species. Version 2010.4. <http://www.iucnredlist.org/apps/redlist/details/41785/0>. Downloaded on 06 May 2011.

M

Maan S, Maan NS, Samuel AR, Rao S, Attoui H, Mertens PP (2007) Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *Journal of General Virology* 88: 621–630.

Maan S, Maan NS, Nomikov K, Veronesi E, Bachanek-Bankowska K, Belaganahalli MN, Attoui H, Mertens PP (2011) Complete genome characterization of a novel 26th bluetongue virus serotype from Kuwait*. PLoS One* 6(10):e26147. doi:10.1371/journal.pone.0026147

Maclachlan NJ, Jagels G, Rossitto PV, Moore PF, Heidner HW (1990) The pathogenesis of experimental bluetongue virus infection of calves. *Veterinary Pathology* 27: 223-229.

Maclachlan NJ (1994) The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comparative Immunology, Microbiology and Infectious Diseases* 17: 197-206.

Maclachlan NJ, Osburn BI (2006) Impact of bluetongue virus infection on the international movement and trade of ruminants. *Journal of the American Veterinary Medical Association* 228: 1346-1349.

Maclachlan NJ, Crafford JE, Vernau W, Gardner IA, Goddard A, Guthrie AJ, Venter EH (2008) Experimental reproduction of severe bluetongue in sheep. *Veterinary Pathology* 45: 310-315.

Maclachlan NJ, Drew CP, Darpel KE, Worwa G (2009) The pathology and pathogenesis of bluetongue. *Journal of Comparative Pathology* 141: 1-16.

Maclachlan NJ, Guthrie AJ (2010) Re-emergence of bluetongue, African horse sickness, and other orbivirus diseases. *Veterinary Research* 41: 35.

Mahrt CR, Osburn BI (1986) Experimental bluetongue virus infection of sheep; effect of vaccination: pathologic, immunofluorescent, and ultrastructural studies. *American Journal of Veterinary Research* 47: 1198-1203.

Martin S, Meek A, Willeberg P (1987) In: *Veterinary Epidemiology*: Principles and Methods. Iowa State University Press. Ames, Iowa.

Mellor PS, Boorman J (1995) The transmission and geographical spread of African horse sickness and bluetongue viruses. *Annals of Tropical Medicine and Parasitology* 89:1-15.

Mellor PS (2000) Replication of arboviruses in insect vectors. *Journal of Comparative Pathology* 123: 231-247.

Mellor PS, Wittmann EJ (2002) Bluetongue virus in the Mediterranean Basin 1998-2001. *Veterinary Journal* 164: 20-37.

Mellor PS (2004) Infection of the vectors and bluetongue epidemiology in Europe. *Veterinaria Italiana* 40 (3): 167-174.

Mertens PP, Burroughs JN, Anderson J (1987) Purification and properties of virus particles, infectious subviral particles, and cores of bluetongue virus serotypes 1 and 4. *Virology* 157: 375-386.

Mertens PPC, Pedley S, Cowley J, Burroughs JN, Corteyn AH, Jeggo MH, Jennings DM, Gorman BM (1989) Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. *Virology* 170: 561-565.

Mertens PPC, Diprose J, Maan S, Singh KP, Attoui H, Samuel AR (2004) Bluetongue virus replication, molecular and structural biology. *Veterinaria Italiana* 40: 426-437.

Mertens PPC, Baylis M, Mellor PS (2008) Bluetongue (Biology of Animal Infections) *Academic Press*.

Meyer G, Lacroux C, Leger S, Top S, Goyeau K, Deplanche M, Lemaire M (2009) Lethal bluetongue virus serotype 1 infection in llamas. *Emerging Infectious Diseases* 15: 608-610.

Murray JO, Trainer DO (1970) Bluetongue virus in North American elk. *Journal of Wildlife Diseases* 6: 144–148.

N

Noad R, Roy P (2009) Bluetongue vaccines. *Vaccine* 27 Suppl 4, D86-89.

O

OIE website (2007) reports 6/11/07 and 23/11/07. Available: http://www.oie.int

OIE website (2009) Bluetongue and Epizootic Haemorrhagic Disease. Chapter 2.1.3.Available: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2008/pdf/2.01.03_BLUETONGUE.pdf.

OIE website (2010) World Animal Health Information Database (WAHID) Interface. http://www.oie.int/wahis/public.php.

Oura CAL, Wood JLN, Sanders AJ, Bin-Tarif A, Henstock M, Edwards L, Floyd T, Simmons H, Batten CA (2009) Seroconversion, neutralising antibodies and protection in bluetongue serotype 8 vaccinated sheep. *Vaccine* 27: 7326–7330.

Oura CAL, Edwards L, Batten CA (2012) Evaluation of the humoral immune response in adult dairy cattle three years after vaccination with a bluetongue serotype 8 inactivated vaccine. *Vaccine* 30:112-115.

P

Pérez JM (2001) Distribución, genética y estatus sanitario de las poblaciones andaluzas de la cabra montés. Universidad de Jaén, Consejería de Medio Ambiente.

Pérez JM, Granados JE, Soriguer RC, Fandos P, Márquez FJ, Crampe JP (2002) Distribution, status and conservation problems of the Spanish Ibex, *Capra pyrenaica* (Mammalia: Artiodactyla). *Mammal Review* 32: 26-39.

Pini A (1976) Study on the pathogenesis of bluetongue: replication of the virus in the organs of infected sheep. *Onderstepoort Journal of Veterinary Research* 43: 159-164.

Puentes E, Fernández-Pinero J, Fernández-Pacheco P, Sánchez MA, Varo I, Salguero J, Pinilla MJ, Villalba R, San Miguel E, Zamora MJ, Arias M, Agüero M, Gómez-Tejedor C, Jiménez-Clavero MA (2008) Inactivated vaccines against bluetongue: protection of cattle and sheep from experimental challenge with bluetongue virus serotypes 1, 4 and 8. In: Epizone 2008 Bluetongue Satellite Symposium. Abstract book, Brescia, Italy. p. 68.

Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PPC, Baylis M (2005) Climate change and the recent emergence of bluetongue in Europe. *Nature Reviews Microbiology* 3:171-181.

Purse BV, Brown HE, Harrup L, Mertens PPC, Rogers DJ (2008) Invasion of bluetongue and other orbivirus infections into Europe: the role of biological

and climatic processes. *Scientific and Technical Review of the Office International des Epizooties* 27: 427-442.

R

RASVE, 2009. Ministerio de Medio Ambiente Medio Rural y Marino (MAPYA). Url: http://rasve.mapa.es/Publica/Noticias/Ficheros/Informelibre serotipo 4 final.pdf.

RASVE, 2010. Ministerio de Medio Ambiente Medio Rural y Marino (MAPYA). Url: http://www.rasve.mapa.es/Publica/Focos/Focos_Consultar.asp.

RASVE, 2012. Ministerio de Medio Ambiente Medio Rural y Marino (MAPYA). Url: http://rasve.magrama.es/RASVE_2008/Publica/Focos/Consulta.aspx

Ratinier M, Caporale M, Golder M, Franzoni G, Allan K, Filipe Nunes S, Armezzani A, Bayoumy A, Rixon F, Shaw A, Palmarini M (2011) Identification and Characterization of a Novel Non-Structural Protein of Bluetongue Virus. *PLOS Pathogens* 7(12): e1002477. doi:10.1371/journal.ppat.1002477

Robinson RM, Hailey TL, Livingston CW, Thomas JW (1967) Bluetongue in the desert bighorn sheep. *Journal of Wildlife Management* 31: 165-168.

Rodríguez-Sánchez B, Iglesias-Martín I, Martínez-Avilés M, Sánchez-Vizcaíno JM (2008) Orbiviruses in the Mediterranean basin: updated epidemiological situation of Bluetongue and new methods for the detection of BTV serotype 4. *Transboundary and Emerging Diseases* 55: 205–214.

Rodríguez-Sánchez B, Gortázar C, Ruiz-Fons F, Sánchez-Vizcaíno JM (2010a) Bluetongue Virus Serotypes 1 and 4 in Red Deer, Spain. *Emerging Infectious Diseases* 16: 518-520.

Rodríguez-Sánchez B, Sánchez-Cordón PJ, Molina V, Risalde MA, de Diego AC, Gómez-Villamandos JC, Sánchez-Vizcaíno JM (2010b) Detection of bluetongue serotype 4 in mouflons (*Ovis aries musimon*) from Spain. *Veterinary Microbiology* 141: 164-167.

Rossi S, Gibert P, Hars J, Mastain O, Couteux P, Barbier S, Zenoni V, Novella C, Gueneau E, Gauthier D, Game Y, Chenouf N, Keck N, Breard E, Zientara S, Moinet M, Ballenghien T, Delecolle JC, Mathieu B, Mathevet P, Bost F (2009) Circulation et impact du virus de la fievre catarrhale ovine (FCO) chez les ruminants sauvages. In: GEEFSM meeting 2009, abstract book.

Roy P, Urakawa T, Van Dijk AA, Erasmus BJ (1990) Recombinant virus vaccine for bluetongue disease in sheep. *Journal of Virology* 64: 1998–2003.

Roy P, Boyce M, Noad R (2009) Prospects for improved bluetongue vaccines. *Nature Reviews Microbiology* 7: 120-128.

Ruiz-Fons F, Reyes-García AR, Alcaide V, Gortázar C (2008) Spatial and temporal evolution of bluetongue virus in wild ruminants, Spain. *Emerging Infectious Diseases* 14: 951-953.

S

Saegerman C, Berkvens D, Mellor PS (2008) Bluetongue epidemiology in the European Union. *Emerging Infectious Diseases* 14: 539-544.

Sánchez-Cordón PJ, Rodríguez-Sánchez B, Risalde MA, Molina V, Pedrera M, Sánchez-Vizcaíno JM, Gómez-Villamandos JC (2010) Immunohistochemical detection of bluetongue virus in fixed tissues. *Journal of Comparative Pathology* 143: 20-28.

Santiago-Moreno J, Carvajal A, Astorga RJ, Coloma MA, Toledano-Díaz A, Gómez-Guillamon F, Salas-Vega R, López-Sebastián A (2011) Potential impact of diseases transmissible by sperm on the establishment of Iberian ibex (*Capra pyrenaica*) genome resource banks. *European Journal of Wildlife Research* 57:211–216.

Savini G, MacLachlan NJ, Sánchez-Vizcaíno JM, Zientara S (2008) Vaccines against bluetongue in Europe. *Comparative Immunology, Microbiology and Infectious Diseases* 31: 101-120.

Savini G, Hamers C, Conte A, Migliaccio P, Bonfini B, Teodori L, Di Ventura M, Hudelet P, Schumacher C, Caporale V (2009) Assessment of efficacy of a bivalent BTV-2 and BTV-4 inactivated vaccine by vaccination and challenge in cattle. *Veterinary Microbiology* 133: 1–8.

Schwartz-Cornil I, Mertens PPC, Contreras V, Hemati B, Pascale F, Breard E, Mellor PS, MacLachlan NJ, Zientara S (2008) Bluetongue virus: virology, pathogenesis and immunity. *Veterinary Research* 39: 46.

Spanish Wildlife Disease Surveillance Scheme (2011) Internal report to the Spanish Ministry of Agriculture, MARM and spatial aggregation of wildlife. *(Plan Nacional de Vigilancia Sanitaria en Fauna Silvestre,* Ministerio de Medio Ambiente y Medio Rural y Marino).

Spreull J (1905) Malarial catarrhal fever (bluetongue) of sheep in South Africa. *Journal of Comparative Pathology and Therapeutics* 18: 321-337.

Stallknecht DE, Howerth EW (2004) Epidemiology of bluetongue and epizootic haemorrhagic disease in wildlife: surveillance methods. *Veterinaria Italiana* 40: 203-307.

Stott JL, Osburn BI, Barber TL, Sawyer MM (1979) Immunological response of sheep to an experimental inactivated BTV vaccine. *Journal of the American Veterinary Medical Association* 175: 611–615.

T

Tabachnick WJ (2004) *Culicoides* and the global epidemiology of bluetongue virus infection. *Veterinaria Italiana* 40:145-150.

Taylor LH, Latham SM, Woolhouse MEJ (2001) Risk factors for human disease emergence. *Philosophical Transactions of the Royal Society of London, Series B* 356:983-989.

Tessaro SV, Clavijo A (2001) Duration of bluetongue viremia in experimentally infected American bison. *Journal of Wildlife Diseases* 37: 722-729.

Thiry E, Saegerman C, Guyot H, Kirten P, Losson B, Rollin F, Bodmer M, Czaplicki G, Toussaint JF, De Clercq K, Dochy JM, Dufey J, Gilleman JL, Messeman K (2006) Bluetongue in northern Europe. *Veterinary Record* 159: 327.

Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K (2007) Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *Journal of Virological Methods* 140: 115-123.

U

Umeshappa CS, Singh KP, Pandey AB, Singh RP, Nanjundappa RH (2010) Cell-mediated immune response and cross-protective efficacy of binary ethylenimine-inactivated bluetongue virus serotype-1 vaccine in sheep. *Vaccine* 28: 2522-2531.

V

Verwoerd DW, Erasmus BJ (2004) In: *Bluetongue. Infectious Diseases of Livestock*, 2nd edn, Vol. 2 (ed. by J. A. W. Coetzer & R. C. Tustin), Oxford University Press, Cape Town pp. 1201–1220.

Verwoerd DW (2009) History of bluetongue research at Onderstepoort. *Onderstepoort Journal of Veterinary Research* 76: 99–102.

Vosdingh RA, Trainer DO, Easterday BC (1968) Experimental bluetongue disease in white-tailed deer. *Canadian Journal of Comparative Medicine and Veterinary Science* 32: 382–387.

W

Wäckerlin R, Eschbaumer M, Konig P, Hoffmann B, Beer M (2010) Evaluation of humoral response and protective efficacy of three inactivated vaccines against bluetongue virus serotype 8 one year after vaccination of sheep and cattle. *Vaccine* 28: 4348-4355.

Whetter LE, MacLachlan NJ, Gebhard DH, Heidner HW, Moore PF (1989) Bluetongue virus infection of bovine monocytes. *Journal of General Virology* 70: 1663-1676.

Wilson A, Mellor P (2008) Bluetongue in Europe: vectors, epidemiology and climate change. *Parasitology Research* 103: S69-S77.

Wilson A, Mellor P (2009) Bluetongue in Europe: past, present and future. *Philosophical Transactions of the Royal Society, Series B* 364: 2669–2681.

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Fins sempre!!!

“Viaggiare sentendosi sempre, nello stesso momento, nell'ignoto e a casa, ma sapendo di non avere, di non possedere una casa. Chi viaggia è sempre un randagio, uno straniero, un ospite; dorme in stanze che prima e dopo di lui albergano sconosciuti, non possiede il guanciale su cui posa il capo né il tetto che lo ripara. E cosí comprende che non si può mai veramente possedere una casa, uno spazio ritagliato nell'infinito dell'universo, ma solo sostarvi, per una notte o per tutta la vita, con rispetto e gratitudine. Non per nulla il viaggio è anzitutto un ritorno e insegna ad abitare piú liberamente la propria casa. Poeticamente abita l'uomo su questa terra, dice un verso di Holderlin, ma solo se sa, come dice un altro verso, che la salvezza cresce là dove cresce il pericolo. Nel viaggio, ignoti fra gente ignota, si impara in senso forte a essere Nessuno, si capisce concretamente di essere Nessuno. Proprio questo permette, in un luogo amato divenuto quasi fisicamente una parte o un prolungamento della propria persona, di dire, echeggiando don Chisciotte: qui io sono chi sono.”

Viaggiare non per arrivare ma per viaggiare, per arrivare più tardi possibile, per non arrivare possibilmente mai.

(Claudio Magris)