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Viral diversity and heterologous protection in the cluster of ruminant alphaherpesviruses related to bovine herpesvirus 1

Diversité virale et protection hétérologue dans le groupe des alphaherpèsvirus de ruminants apparentés à l'herpèsvirus bovin 1

Julien Thiry

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TABLE OF CONTENTS

aa	amino acid
ASCs	antibody secreting cells
ATCC	American type culture collection
BoHV-1	bovine herpesvirus 1
BoHV-4	bovine herpesvirus 4
BoHV-5	bovine herpesvirus 5
BuHV-1	bubaline herpesvirus 1
bp	base pair
C	cytosine
CI	confidence interval
CMC	carboxymethylcellulose
CPE	cytopathic effect
CpHV-1	caprine herpesvirus 1
CvHV-1	cervid herpesvirus 1
CvHV-2	cervid herpesvirus 2
DNA	deoxyribonucleic acid
D-MEM	Dulbecco-minimum essential medium
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ElkHV-1	elk herpesvirus 1
FBS	fœtal bovine serum
FITC	fluorescein isothiocyanate
G	guanine
gX	glycoprotein X
HSV	human simplex virus
HHV-2	human herpesvirus 2
IBR	infectious bovine rhinotracheitis
ICP	infected-cell protein
Ig	immunoglobulin
IPV	infectious pustular vulvovaginitis
IR	internal repeat
Da	dalton
MDBK	Madin Darby bovine kidney
MEM	minimum essential medium
MOI	multiplicity of infection
NALT	nasopharynx-associated lymphoid tissue
NP40	nonidet P-40
ORF	open reading frame
p	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PS	penicilin streptomycin
REA	restriction enzyme analysis
SDS	sodium dodecyl sulfate
SuHV-1	suid herpesvirus 1
T	thymine
TAP	transporter associated with antigen processing
TCID	tissue culture infectious dose
TE	tris EDTA
TR	terminal repeat
UL	unique sequence long
US	unique sequence short
VCAM-1	vascular cell adhesion molecule-1
VZV	varicella zoster virus

CHAPTER ONE

Ruminant alphaherpesviruses related to bovine herpesvirus 1

Julien Thiry, Véronique Keuser, Benoît Muylkens, François Meurens, Sacha Gogev, Alain Vanderplasschen and Etienne Thiry

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Herpesviruses have mainly co-evolved with their hosts for millions of years. Consequently, different related host species may have been infected by various genetically related herpesviruses. Illustrating this concept, several ruminant alphaherpesviruses have been shown to form a cluster of viruses closely related to bovine herpesvirus 1 (BoHV-1): namely bovine herpesvirus 5, bubaline herpesvirus 1, caprine herpesvirus 1, cervid herpesviruses 1 and 2 and elk herpesvirus 1. These viruses share common antigenic properties and the serological relationships between them can be considered as a threat to BoHV-1 eradication programmes. BoHV-1 is a herpesvirus responsible for infectious bovine rhinotracheitis, which is a disease of major economic concern. In this article, the genetic properties of these ruminant alphaherpesviruses are reviewed on a comparative basis and the issue of interspecific recombination is assessed. The pathogenesis of these infections is described with emphasis on the host range and crossing of the host species barrier. Indeed, the non bovine ruminant species susceptible to these ruminant alphaherpesviruses may be potential BoHV-1 reservoirs. The differential diagnosis of these related infections is also discussed. In addition, epidemiological data available are used to assess the potential of cross-infection in ruminant populations. A better knowledge of these ruminant alphaherpesvirus infections is essential to successful control of infectious bovine rhinotracheitis.

Introduction

Members of the family *Herpesviridae* are DNA viruses showing a spectacular evolutionary success. The name, derived from the Greek ερπειν (*herpein*), "to creep", refers to the characteristic lesions caused by two common human herpesviruses: fever blisters caused by herpes simplex virus (HSV), as well as varicella and shingles induced by varicella zoster virus (VZV). This virus family includes nearly two hundred viruses isolated from hosts as diverse as molluscs, fishes, amphibians, reptiles, birds and mammals (Roizman and Pellett, 2001). In nature, most herpesviruses are closely associated with a single host species, and almost all the animal hosts investigated to date support infections by at least one herpesvirus species. Host susceptibility to herpesviruses indicates that the viruses have mainly co-evolved with their hosts, leading to a close adaptation (Davison, 2002).

The family *Herpesviridae* is divided into three subfamilies, called *Alpha-*, *Beta-* and *Gammaherpesvirinae*. The *Alphaherpesvirinae* subfamily contains four genera: *Simplexvirus*, *Varicellovirus*, *Mardiviruses* and *Iltoviruses* (ICTV, 2002). This subfamily includes viruses characterised by a large host range, a short replication cycle and a capacity to induce latent infection mainly, but not exclusively, in neurons (Roizman and Pellett, 2001). Among alphaherpesviruses infecting ruminants, the prototype is bovine herpesvirus 1 (BoHV-1), a pathogen of cattle associated with two major syndromes, called infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV), and a variety of clinical signs, such as conjunctivitis, encephalitis and abortions (Pastoret et al., 1982). IBR is a disease of major economic concern in many parts of the world and especially in Europe, both in countries where this infection has been eradicated and in those where the control of IBR is currently or will be undertaken (Thiry et al., 1999).

Seroepidemiological surveys have been performed in other species of domestic or wild ruminants in order to investigate whether these animals could be potential BoHV-1 reservoirs. Antibodies against BoHV-1 have been detected in many ruminant species (Thiry and Lemaire, 2001). In addition, several ruminant alphaherpesviruses related to BoHV-1 have been isolated and characterised. Bovine herpesvirus 5 (BoHV-5) is responsible for meningo-encephalitis in calves (Johnston et al., 1962). Caprine herpesvirus 1 (CpHV-1) causes systemic disease in young kids and abortion in adult goats (Saito et al., 1974). Cervid herpesvirus 1 (CvHV-1) has been isolated from ocular disease in red deer (*Cervus elaphus*) in Scotland (Inglis et al., 1983). Cervid herpesvirus 2 (CvHV-2) has been isolated from reindeer (*Rangifer tarandus*) in Finland (Ek-Kommonen et al., 1986), and serological evidence of infection with a virus related to BoHV-1 has been reported in caribou (*Rangifer tarandus caribou*) in Canada (El Azhary et al., 1979). Elk herpesvirus 1 (ElkHV-1) has been isolated from elk (*Cervus elaphus*) in North America (Deregt et al., 2000) and bubaline herpesvirus 1 (BuHV-1) infection has been reported in water buffalo (*Bubalus bubalis*) in Australia (St George and Philpott, 1972) (Table 1).

Successful control of IBR depends on the use of efficient, sensitive and specific diagnostic tests. However, since most of the diagnostic tests are based on the detection of antibody in polyclonal sera, their specificity is compromised by the existence of a serological cross-reactivity between BoHV-1 and these other closely related ruminant alphaherpesviruses. This can be illustrated by the original situation observed in Finland. In 1982, 23% of reindeer of this country had antibodies against BoHV-1, while all cattle were seronegative (Ek-Kommonen et al., 1982). A superficial analysis of these data suggested a BoHV-1 infection of reindeer with an absence of transmission to cattle due to an apparent lack of contact between the two ruminant species. However, this simple hypothesis was rapidly rejected. Indeed, from a BoHV-1 seropositive

reindeer, a new virus was isolated and further characterised as CvHV-2. This infection provided a likely explanation for the presence of anti-CvHV-2 antibodies cross-reacting with BoHV-1 in reindeer (Ek-Kommonen et al., 1986). In spite of this epidemiological situation in reindeer, the Finnish cattle population maintained an IBR free status. This example reveals the importance of clarifying the cause of potential serological cross-relationships with BoHV-1.

Table 1 – Ruminant alphaherpesviruses related to BoHV-1.

virus	natural host	disease	geographic distribution
BoHV-1	bovine	infectious bovine rhinotracheitis infectious pustular vulvovaginitis	Europe, America, Asia, Australia
BoHV-5	bovine	bovine encephalitis	Europe, America, Australia
BuHV-1	water buffalo	subclinical genital infection	Europe, Australia
CpHV-1	goat	vulvovaginitis, abortion, neonatal systemic infection	Europe, America, Australia
CvHV-1	red deer	ocular syndrome	Europe
CvHV-2	reindeer	subclinical genital infection	Europe
ElkHV-1	elk	subclinical genital infection	North America

This review focuses on ruminant alphaherpesviruses related to BoHV-1, with particular emphasis on molecular virology, pathogenesis, host range, diagnosis and prevention. Indeed, it is of major interest to obtain greater knowledge about the risks of acute and latent infections of cattle with other ruminant herpesviruses and about the potential presence of BoHV-1 reservoirs among ruminant species other than cattle.

Molecular virology

Morphology and genomic organisation

The inner part of the herpesvirus virion consists of a core containing the viral genome, protected by an icosahedral nucleocapsid of 100 to 110 nm diameter made up of 150 hexamers and 12 pentamers. This structure is surrounded by a proteinaceous layer, defined as the tegument. The latter is surrounded by a lipid bilayer, the envelope, containing a large number of viral glycoproteins, among which glycoproteins gB, gC and gD are the most abundant. The mature virion particle ranges from 120 to 300 nm in diameter (Roizman and Pellet, 2001) (Figure 1).

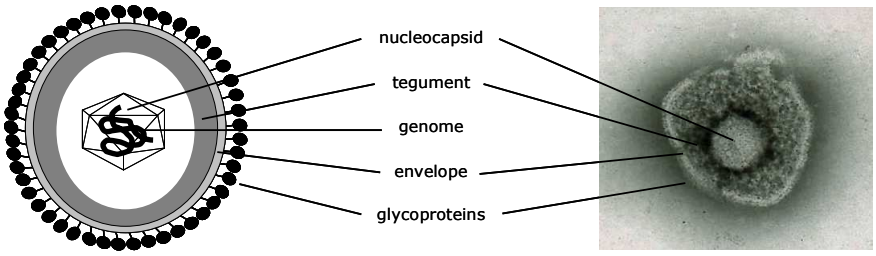

Figure 1 – Morphology of ruminant alphaherpesviruses.

The genome of ruminant alphaherpesviruses consists of a double-stranded linear DNA. Like the other alphaherpesviruses belonging to the *Varicellovirus* genus, it contains an arrangement of one long unique unit (U_L) and one short unique unit (U_S) flanked by two inverted repeat sequences, named internal repeat (IR) and terminal repeat (TR) (Schwyzer and Ackermann, 1996) (Figure 2). These characteristics allow its classification in class D of herpesvirus genomes (Roizman

and Knipe, 2001). In this genomic class, the U_L segment is predominantly fixed in only one orientation, called the prototype orientation, so that equimolar amounts of the two isomers can be predicted in virion DNA due to the inversion of the U_S segment (Roizman and Pellett, 2001). However, low levels (5%) of genomes having the U_L segment in an inverted orientation have been detected in BoHV-1 virion DNA (Schynts et al., 2003).

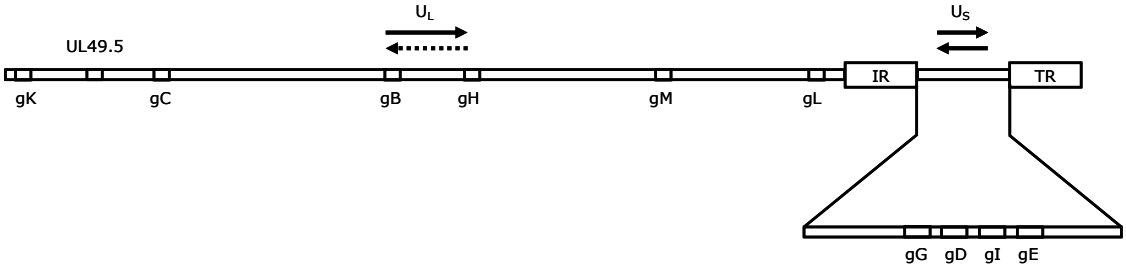

Figure 2 – Genomic organisation of ruminant alphaherpesviruses. The genome consists of a double-stranded linear DNA. It contains an arrangement of one long unique unit (U_L) and one short unique unit (U_S) flanked by two inverted repeat sequences, named internal repeat (IR) and terminal repeat (TR). The genome includes ten genes encoding glycoproteins: six are located in the U_L segment and four in the U_S segment. The U_S segment can have two possible orientations (depicted by the arrows). The U_L segment is predominantly present in one orientation. The broken arrow illustrates that about 5% of the encapsidated genomes show the U_L segment in this inverted orientation.

Alphaherpesvirus genes are expressed in a cascade-like fashion. The first step occurs immediately after release of the virion genome from the capsid to produce immediate-early proteins. The second step occurs after the synthesis of immediate-early proteins, leading to the production of early proteins. The third step, in which late proteins are produced, is delayed until after the synthesis of virion DNA. Finally, early-late protein expression starts before viral DNA replication but does not decrease during late protein expression (Roizman and Knipe, 2001).

Genomic comparison between ruminant alphaherpesviruses

The genomes of BoHV-1 and BoHV-5 have been sequenced (Delhon et al., 2003; Meyer et al., 1997; Schwyzer and Ackermann, 1996). The BoHV-5 genome is 138,390 bp long (72% G+C content), with 2 518 bp more than BoHV-1 (75% G+C content). It includes 72 genes, of which 68 are present as single copies within the unique regions and two are completely located within the repeat region (BICP4 and BICP22) (Delhon et al., 2003). The BoHV-1 genome comprises 67 unique genes and two genes, both duplicated, in the inverted repeats (Schwyzer and Ackermann, 1996). The highest BoHV-5 similarity to BoHV-1 products (≥ 95% amino acid identity) is found in proteins involved in viral DNA replication and processing (UL5, UL15, UL29 and UL39) and in virion proteins (UL14, UL19, UL48 and US6). Among the least conserved (≤75%) are the homologues of immediate-early proteins BICP0, BICP4 and BICP22, these proteins being longer in BoHV-5 than in BoHV-1 (Delhon et al., 2003). It can be hypothesised that the same range of homology is shared by the other related ruminant alphaherpesviruses.

The comparison between other ruminant alphaherpesviruses is based on limited sequence data. The genome sizes are estimated to be 137 kbp for CpHV-1, 141 kbp for CvHV-1 and 145 kbp for CvHV-2 (Engels et al., 1987; Vanderplasschen et al., 1993). Based on the complete gB gene sequence, which is the most conserved between herpesviruses (Griffin, 1991), the percentages of nucleotide sequence identity with BoHV-1 gB are 78.5% for CpHV-1, 84% for CvHV-2, 85.8% for CvHV-1, 87.4% for BoHV-5 and 87.7% for BuHV-1 (Ros and Belak, 2002). The gC and gD genes sequences are fully known for each ruminant alphaherpesviruses, except for BuHV-1 (Delhon et al., 2003; Deregt et al., 2005; Keuser et al., 2006; Schwyzer and Ackermann, 1996; Tempesta et al.,

2004b). On this basis, the percentages of amino acid sequence identity between BoHV-1 and ElkHV-1 are 71.7% (gC) or 72.9% (gD) (Deregt et al., 2005). The G+C contents of the gC (or gD) genes are 79.4% (77.2%) for CvHV-1, 79.2% (76%) for CvHV-2 and 78.7% (78.5%) for ElKHV-1 (Deregt et al., 2005). Comparison of the coding sequence of the CvHV-2 gC region with other ruminant alphaherpesviruses has revealed a conserved central part of the gC gene, while the N-terminal is highly variable with large deletions and insertions (Ros and Belak, 1999; Ros et al., 1999). These results have allowed the classification of these viruses into a consistent group, where BoHV-5 and BuHV-1 are most closely related to BoHV-1, followed by ElkHV-1, CvHV-1, CvHV-2 and, more distantly, by CpHV-1 (Deregt et al., 2005; Ros and Belak, 2002) (Figure 3).

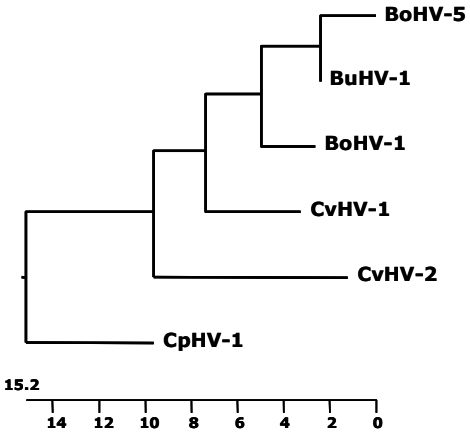

Figure 3 – Phylogenetic relationship between ruminant alphaherpesviruses. Phylogenetic tree inferred by amino acid sequences from the gB regions of ruminant alphaherpesviruses (reproduced from Ros and Belak (2002), with permission of Kluwer Academic Publishers). Based on the gC and gD sequences, ElkHV-1 clusters with CvHV-1 (Deregt et al., 2005).

Viral glycoproteins

Viral glycoproteins of herpesviruses play an important role in the interactions between these viruses and their host-cells. They are involved at several steps of the viral cycle, such as the attachment, penetration, maturation and egress of the virus. Therefore, they constitute an important target for the host immune response. Some glycoproteins also have immunogenic properties, allowing their use as a component of vaccines or diagnostic tests.

Most of the BoHV-1 glycoproteins have already been characterised (Tikoo et al., 1995). The U_L segment includes genes coding for glycoproteins gB (UL27), gC (UL44), gH (UL22), gL (UL1), gK (UL53) and gM (UL10), while four genes corresponding to gG (US4), gD (US6), gI (US6) and gE (US8) are grouped in a tandem organisation in the U_S segment (Schwyzer and Ackermann, 1996) (Figure 1). Several constructions of mutant viruses have shown that gC, gI, gE, gG and gM are non essential glycoproteins (Baranowski et al., 1996; Konig et al., 2002). On the other hand, the deletion of the gene encoding either gB or gD or gH reduces the capacity of in vitro replication (Fehler et al., 1992; Meyer et al., 1998). Glycoproteins L and K are essential in HSV-1 and this is very likely to be the case in the other alphaherpesviruses (Hutchinson et al., 1992; Roop et al., 1993). In the pseudorabies virus, an additional glycoprotein, designated gN (UL49.5), is a glycosylated 10 kDa transmembrane protein and is identified as an immune evasion molecule inhibiting the transporter associated with antigen processing (TAP) driven peptide import into the endoplasmic reticulum. The UL49.5 homologue encoded by BoHV-1 has the same immune evasion function, but is not classified as glycoprotein N (Koppers-Lalic et al., 2005).

Considering the lack of data on glycoproteins, we hypothesise by analogy that the main

elements of structure and function of BoHV-1 glycoproteins are shared by the other ruminant alphaherpesviruses. Monoclonal antibody analysis allows the identification of antigenic differences between BoHV-1 and BoHV-5 (Collins et al., 1993; Egyed et al., 1992; Friedli and Metzler, 1987; Metzler et al., 1986). Glycoprotein C shows more divergence between BoHV-1 and BoHV-5 than gB and gD (Collins et al., 1993; Friedli and Metzler, 1987). BoHV-5 gC, although not essential, regulates neurotropism in some areas of the olfactory pathway and is important for BoHV-5 neurovirulence in rabbits (Chowdhury et al., 2000b). This glycoprotein is also able to transfer the heparin binding phenotype of BoHV-5 to a recombinant BoHV-1 (Liman et al., 2000). The BoHV-5 gE is involved in neural spread and neurovirulence within the central nervous system and cannot be substituted by BoHV-1 gE. However, BoHV-5 gE is not essential for the initial entry into the olfactory pathway (Chowdhury et al., 2000a). Glycoprotein I is not required for the neurovirulence of gE in BoHV-5 (Al-Mubarak and Chowdhury, 2004), but a glycine-rich BoHV-5 gE-specific epitope is essential for expression of the full virulence potential of BoHV-5 (Al-Mubarak et al., 2004). Generally, gE and gI seem to be important for BoHV-5 neuropathogenicity and its ability to reactivate from latency (Hubner et al., 2005). Glycoprotein H is one of the most conserved glycoproteins in herpesviruses (Gompels et al., 1988). BoHV-1 and BoHV-5 gH are both expressed as early-late proteins. Glycoprotein H is essential for both penetration and cell-to-cell spread (Baranowski et al., 1995; Meyer et al., 1998; Meyer et al., 1999). In CpHV-1, gD shows a relatively high homology to BoHV-1 gD amino acid sequences (similarity of 68.8%) and has a molecular mass similar to BoHV-1 gD. CpHV-1 gD contains complex *N*-linked oligosaccharides, but the presence of *O*-glycosylation has not been demonstrated. In contrast with BoHV-1 gD, CpHV-1 gD is seemingly expressed as a late protein (Keuser et al., 2006).

Recombination between ruminant alphaherpesviruses

Recombination is thought to be an important source of genetic variation in herpesviruses. Several studies, performed in vitro and in vivo, have detected recombinant viruses after the co-inoculation of two distinguishable strains of the same herpesvirus species (Thiry et al., 2005; Thiry et al., 2006). After experimental co-inoculation of calves with two parental BoHV-1 mutants, recombination has been identified as a frequent event in vivo. Recombinant viruses have been detected both during primary infection and after reactivation from latency (Schynts et al., 2003). The mechanism involved is mainly homologous recombination. Its role in creating viral diversity needs to be evaluated in BoHV-1 control and eradication programmes. In order to assess the risk of recombination between BoHV-1 gE negative marker vaccine and field strains, different factors influencing the onset of recombinant viruses have been investigated. In natural conditions, superinfection is a more likely event than co-infection. Superinfection with a time interval of 2 to 8 h between two successive infections allows the establishment of a barrier to prevent recombination between BoHV-1 strains (Meurens et al., 2004a). Therefore, only co-infection or delayed infection over a very short interval of time might lead to the production of recombinant viruses.

The efficiency of interspecific recombination has been assessed between ruminant alphaherpesviruses. Indeed, successful homologous recombination requires two closely related parental viruses. Two recombinants between BoHV-1 and BoHV-5 have been isolated, but no recombinant has been detected between BoHV-1 and the less closely related CpHV-1 and CvHV-2 (Meurens et al., 2004b). Nevertheless, even in the absence of mature recombinant viruses, these results do not exclude a recombination process occurring in the cell, because mixed concatemers between BoHV-1 and CvHV-2, and between CvHV-1 and CvHV-2, have been identified in co-infected cells (Meurens, 2004). The close genetic relatedness between ruminant alphaherpesviruses suggests a theoretical risk of recombination between these virus species. This risk must also be evaluated by considering the sites of alphaherpesvirus replication: two

alphaherpesviruses are unlikely to recombine if they replicate in two distant sites, such as the genital and the respiratory mucosae.

Pathogenesis and clinical signs

The pathogenesis of BoHV-1 infection is well described (Engels and Ackermann, 1996; Jones, 2003; Pastoret et al., 1982). After infection and virus replication in mucosa, BoHV-1 spreads both in the extracellular space and also from cell-to-cell. By this latter mechanism, it may enter axons of local nerve cells. Then, by intra-axonal transport, the viruses reach the sensitive neuronal bodies in the regional nervous ganglia, where latency can be established. This pathogenic mechanism is shared by the other ruminant alphaherpesviruses. Although infection can lead to lethal or systemic disease, especially in newborn animals, clinical signs are usually mild, but may vary according to the animal species and the virus species and strain concerned. Alphaherpesviruses, like other herpesviruses, are able to interfere with the host's innate and adaptative immune responses. A growing list of immune evasion mechanisms is described (Vossen et al., 2002), such as, for example, inhibition of TAP proteins (Koppers-Lalic et al., 2005) or Fc receptor activity of viral glycoproteins (Favoreel et al., 2003).

Bovine herpesvirus 5

The pathogenesis of bovine herpesvirus encephalitis is not yet completely elucidated. Comparative experiments of inoculation with BoHV-1 and BoHV-5 have been carried out in cattle (Bagust, 1972; Bagust and Clark, 1972; Belknap et al., 1994; Hall et al., 1966; Meyer et al., 2001; Schudel et al., 1986). After primary infection by intranasal inoculation, BoHV-1 and BoHV-5 replicate at the portal of entry in the respiratory mucosa (Bagust and Clark, 1972). Experimentally infected calves show similar profiles for the length of excretion (ten to sixteen days) and for the amount of excreted viruses. The rate of virus excretion is high, with a peak between the fourth and sixth days post infection (Belknap et al., 1994; Meyer et al., 2001; Thiry et al., 1999). After primary infection, BoHV-5 induces a transient viremia and virus is recovered in secondary organs (Belknap et al., 1994).

The dissemination of BoHV-5 infection probably follows the same three routes as BoHV-1: blood, nervous system and cell-to-cell spread (Bagust and Clark, 1972; Belknap et al., 1994; Hall et al., 1966; Meyer et al., 2000; Pastoret et al., 1982). BoHV-5 induces a replication cycle in cells of the respiratory epithelium and reaches the nervous system by a mechanism strongly associated with intra-axonal transport. Neurons of the peripheral nervous system are infected first of all, especially cells of the trigeminal ganglia and olfactory cells of nasal mucosa. The virus invades all areas of the central nervous system by neuron-to-neuron spread, using the olfactory and trigeminal pathways (Meyer et al., 2000). In a rabbit model of infection, the olfactory pathway was indeed shown to be the main route of infection of the central nervous system (Lee et al., 1999). Cell-to-cell transmission without extracellular phase and in the absence of specific antibodies has been demonstrated for BoHV-1. It can be postulated that the same mechanism drives the local transfer of BoHV-5 to the central nervous system (Meyer et al., 2000).

In calves, BoHV-5 has been shown to produce fatal meningo-encephalitis after intranasal inoculation or a meningitis-like disease after intravaginal infection because the virus involves the central nervous system (Meyer et al., 2001). Inoculation of conjunctival, nasal and vaginal mucosae provokes conjunctivitis, rhinitis and vulvovaginitis, respectively. Clinical signs of BoHV-5 are characterised by serous nasal discharge and sneezing; apathy and anorexia are also observed.

Neurological signs start with severe depression with anorexia, jaw champing and hypersalivation. In the next phase, calves are affected by muscle trembling, circling to one side, pushing their head against a wall and ataxia, followed by seizure-like episodes and opisthotonos (Meyer et al., 2001). Histological lesions in the brain consist of meningitis, mononuclear perivascular cuffing, neurophagia, satellitosis, gliosis, haemorrhage, necrosis and oedema (Perez et al., 2002).

After primary infection, BoHV-5 latency is established in surviving calves. The classical latency sites are the trigeminal ganglia, but BoHV-5 DNA could also be present throughout the central nervous system during latent infection and probably in nasal and tracheal mucosae, where viral DNA has been detected by PCR (Meyer et al., 2001). Virus reactivation may result in the establishment of latent infection in additional sites of the brain (Vogel et al., 2003). The establishment of latent infection has also been demonstrated in the rabbit model (Caron et al., 2002). Reactivation of BoHV-5 can be provoked by the same glucocorticoid treatment as for BoHV-1: 0.1 mg of dexamethasone per kg body weight for five consecutive days (Meyer et al., 2001).

Caprine herpesvirus 1

The pathogenesis of CpHV-1 infection in goats and BoHV-1 infection in calves is very similar (Engels and Thiry, 2000). Local respiratory or genital infection is followed by a mononuclear cell-associated viremia, which can lead to systemic infection and abortion. The main sources of infection are acutely and latently infected animals. During acute infection, the virus is excreted via ocular, nasal and genital routes. The genital route is thought to be the main entry site of the virus and is thought to be responsible for the maintenance of infection in a herd (Tempesta et al., 2000).

In kids, CpHV-1 causes a systemic disease characterised by high morbidity and mortality rates. Ulcerative and necrotic lesions are distributed throughout the enteric tract (Mettler et al., 1979). In adult goats, the infection leads to vulvovaginitis or balanoposthitis. Abortions associated with CpHV-1 occur during the second half of pregnancy and can be experimentally reproduced after intranasal or intravenous inoculation of pregnant goats (Tempesta et al., 2004; Uzal et al., 2004; Williams et al., 1997). The viral DNA is identified in both the placenta and several inner organs of the aborted foetus (Keuser et al., 2002; Tempesta et al., 2004; Waldvogel et al., 1981).

Natural reactivation from latent CpHV-1 infection can be induced by a physiological stress during the mating season. The hormonal status at oestrus could play a role in the induction of reactivation. Under experimental and natural conditions, the reactivation of a latent virus is difficult (Buonavoglia et al., 1996; Keuser et al., 2004b; Tempesta et al., 2000). Experimental reactivation requires the use of high doses of dexamethasone: six consecutive daily injections at a dose rate of 4.4 mg per kg bodyweight (Buonavoglia et al., 1996). Re-excretion after reactivation is observed in goats with relatively low antibody titres (Tempesta et al., 1998). Viruses are isolated from nasal, genital, rectal and ocular swabs, suggesting several potential latency sites (Buonavoglia et al., 1996; Plebani et al., 1983), such as sacral ganglia where latent CpHV-1 has been detected by PCR (Tempesta et al., 1999a). The site supporting CpHV-1 re-excretion may depend on the route of primary infection. After reactivation of a virus previously inoculated intranasally, the virus is shed from the nose and vagina (Tempesta et al., 1999b). In contrast, after a genital primary infection, reactivated CpHV-1 is only re-excreted from the vagina (Tempesta et al., 2000).

Cervid herpesvirus 1

Primary sites of CvHV-1 infection are the anterior respiratory and ocular mucosae (Keuser et al., 2000). Viruses can be recovered from between two and six days post-infection in nasal and

ocular swabs (Reid et al., 1986). However, a genital transmission cannot be excluded because CvHV-1 has been isolated from red deer sperm (Tisdall and Rowe, 2001). CvHV-1 is responsible for an ocular syndrome in red deer (Inglis et al., 1983). This syndrome is characterised by conjunctivitis and purulent ocular discharge, hypopyon, uniform corneal opacity without ulceration, mucopurulent nasal discharge and photophobia. Moderate swelling of the periorbital tissues and marked oedema of the upper eyelids are also observed (Inglis et al., 1983).

Experimental infection induces hyperthermia, nasal ulceration and conjunctivitis. These clinical signs are mild in comparison with natural infection. CvHV-1 can be also isolated after intramuscular injection with 50 mg of a synthetic glucocorticoid for twelve consecutive days. This result suggests that this virus is able to establish a persistent infection and be reactivated (Ronsholt et al., 1987).

Cervid herpesvirus 2

Reindeer infection by CvHV-2 is asymptomatic (Nettleton et al., 1988a). The first virus isolation was performed on vaginal swabs from reindeer identified as seropositive for BoHV-1. Reindeer were injected intramuscularly on four consecutive days with dexamethasone at a dose rate of 0.1 mg per kg bodyweight (Ek-Kommonen et al., 1986). Therefore, CvHV-2 most likely establishes a latent infection and can be re-excreted in genital secretions. A serological investigation showing a high prevalence in adult reindeer suggests a genital transmission (Nettleton et al., 1988b).

Bubaline and elk herpesviruses 1

Recently, a field strain of BuHV-1 was isolated after a reactivation treatment. Water buffaloes were injected intramuscularly daily for five consecutive days with dexamethasone at a dosage of 4 mg per kg bodyweight. Only slight diarrhoea and serous nasal discharge were observed (De Carlo et al., 2004). Concerning ElkHV-1 infection, pathogenesis and clinical signs remain to be investigated.

Cross-species infection

Cross-infection studies have been performed in order to gain greater knowledge about risks of acute and latent infections in cattle with other ruminant alphaherpesviruses and about potential BoHV-1 reservoirs among ruminant species other than cattle. The results of experimental infections of calves with BoHV-5, CpHV-1, CvHV-1, CvHV-2 and ElkHV-1, and infection of goats, sheep, red deer and reindeer with BoHV-1 are summarised in Tables 2 and 3 (Thiry et al., 2001).

Bovine herpesvirus 1 infection of goats and sheep

Experimental infection of goats with BoHV-1 has clearly shown that this virus is able to infect the heterologous host. This infection leads to mild clinical signs, high levels of BoHV-1 excretion for several days during primary infection and a serological response. BoHV-1 is able to establish a latent infection in the trigeminal ganglia of goats and sheep (Ackermann et al., 1986; Engels et al., 1992; Lehmkuhl et al., 1985; Pirak et al., 1983; Six et al., 2001; Wafula et al., 1985). Reactivation of latent BoHV-1 is obtained by a daily dexamethasone treatment on five consecutive days of 2.5 mg per kg bodyweight in goats (Six et al., 2001) and 0.1 mg per kg bodyweight in sheep (Banks et al., personal communication). Field cases of BoHV-1 latent

infections have also been recorded in goats (Tolari et al., 1990). Although they are usually subclinical in sheep, acute BoHV-1 infections can sometimes be associated with clinical signs, such as acute, fatal pneumonia, or with aborted foetuses (Whetstone and Evermann, 1988). However, sheep do not play a major role in BoHV-1 transmission to calves (Hage et al., 1997).

Bovine herpesvirus 1 infection of red deer and reindeer

Red deer could become infected following a profound BoHV-1 challenge, but titres of excreted virus are very low and almost certainly would not represent a source of contagious spread. Furthermore, there is no evidence that BoHV-1 establishes a latent infection in red deer. Seroconversions to BoHV-1 in red deer do not occur and are most likely the result of a CvHV-1 rather than BoHV-1 infection (Nettleton et al., 1988a; Reid et al., 1986). Another deer species, mule deer (*Odocoileus hemionus*) exhibits a greater susceptibility to BoHV-1 because it actively excretes BoHV-1 and shows seroconversion after experimental inoculation (Nettleton et al., 1988b).

In reindeer, experimental BoHV-1 infection is asymptomatic and does not usually give rise to neutralising antibodies. The titres of nasally excreted viruses are very low and the virus cannot be reactivated experimentally (Thiry et al., 2001).

Bovine herpesvirus 5 infection of sheep

Sheep are susceptible to acute and latent infection with BoHV-5. Experimental inoculation of BoHV-5 shows that this virus is able to infect the heterologous host by invading the central nervous system. Resulting clinical signs are similar to those observed in cattle (Belak et al., 1999). A dexamethasone treatment of 2 mg daily for five consecutive days results in reactivation of the latent infection and virus shedding in lambs (Silva et al., 1999). No BoHV-5 natural infection has yet been reported in sheep.

Caprine herpesvirus 1 infection of calves and sheep

Bovine calves intranasally infected with CpHV-1 have been shown to excrete the virus. CpHV-1 is able to establish a latent infection in trigeminal ganglia. However, reactivation of latent CpHV-1 from calves has not been successfully achieved (Six et al., 2001). In the same way, lambs are susceptible to CpHV-1 infection, but no virus has been isolated after clinical reactivation (Papanastasopoulou et al., 1991). In natural conditions, CpHV-1 virulence is restricted to the natural host (Engels et al., 1992).

Cervid herpesvirus 1 infection of calves

Bovine calves are almost refractory to infection with CvHV-1 by intranasal challenge: neither clinical signs nor virus excretion are observed. Furthermore, when calves are challenged with BoHV-1 after a CvHV-1 infection, they develop pyrexia and the virus is recovered in nasal and ocular swabs, thereby showing that a prior infection with CvHV-1 does not induce any protective immune response (Nettleton et al., 1988a; Reid et al., 1986). In addition, no infection has been noticed in cattle housed in close contact with infected deer (Ronsholt et al., 1987).

Cervid herpesvirus 2 infection of calves

In experimental conditions, cattle can be successfully infected intranasally with CvHV-2

and mild rhinitis is observed. The virus is isolated for six to nine days from nasal and genital swabs (Nettleton et al., 1988b). Therefore, CvHV-2 replication can occur experimentally in bovines, but cattle housed in close contact with infected reindeer are not infected (Ek-Kommonen et al., 1986). These results suggest that the risk of transmission between reindeer and cattle is low in natural conditions.

Elk herpesvirus 1 infection of cattle

Experimentally, cattle are susceptible to infection with ElkHV-1, but do not show any clinical signs. A cow inoculated intranasally has been shown to develop neutralising antibodies, while a genital inoculation failed to induce any serological response in another cow (Deregt et al., 2000). More recently, reactivation from latency was serologically demonstrated in cattle experimentally infected with ElkHV-1 after a daily dexamethasone treatment for five consecutive days of 0.1 mg per kg bodyweight (Deregt et al., 2005). Natural cross-infection has not yet been recorded.

Table 2 – Consequences of infection of calves with ruminant alphaherpesviruses related to bovine herpesvirus 1.

virus	primary infection	serological response	latency	reactivation and reexcretion
BoHV-5	+	+	+	+
CpHV-1	+	+	+	-
CvHV-1	-	-	-	-
CvHV-2	+	+	-	-

Table 3 – Consequences of infection of ruminant species with bovine herpesvirus 1.

host	primary infection	serological response	latency	reactivation and reexcretion
goat	+	+	+	+
sheep	+	+	+	+
red deer	+	-	-	-
reindeer	+	-	-	-

Epidemiology

Bovine herpesvirus 5

Since 1962, several outbreaks of herpesvirus encephalitis have been described and were later on attributed to BoHV-5. In Australia and South America, this infection occurs in cattle not immunised against BoHV-1 and is inducing a major economic problem, mainly in central and southern Brazil and Argentina. Indeed, the morbidity rate may vary between 15 and 50% and the lethal rate is close to 100% (Carillo et al., 1983; Johnston et al., 1962; Perez et al., 2003; Salvador et al., 1998; Weiblen et al., 1989). In USA, BoHV-5 has been associated with field cases of bovine encephalitis (D'Offay et al., 1993). In Europe, BoHV-5 has been isolated only once in Hungary (Bartha et al., 1969), but BoHV-5 infection is probably also present in Germany and eastern European countries (Meyer et al., 2000).

Caprine herpesvirus 1

CpHV-1 was initially isolated in 1975 in California from the tissues of newborn kids

affected with mild to severe enteritis (Saito et al., 1974). In 1979, it was isolated from kids in a herd in the Bregaglia valley of Switzerland (Mettler et al., 1979). Distribution of CpHV-1 infection has not been systematically studied in goats but the virus has been identified in several countries in Europe including Greece, Italy, Spain and Switzerland, as well as in Australia, Canada, New Zealand and the USA (Chenier et al., 2004; Grewal and Wells, 1986; Horner et al., 1982; Keuser et al., 2004b; Koptopoulos et al., 1988; Mettler et al., 1979; Muluneh and Liebermann, 1990; Plebani et al., 1983; Roperto et al., 2000; Saito et al., 1974). The apparent prevalences are variable. In many countries where goats play an economical role, a high seroprevalence is observed: between 30 and 40% in southern Italy and more than 50% in Greece (Guercio et al., 1998; Koptopoulos et al., 1988; Tempesta et al., 1994). CpHV-1 infection has not been identified in Belgium, France (Keuser and Thiry, unpublished results) or the United Kingdom.

Cervid herpesvirus 1

CvHV-1 was initially isolated in 1983 in farmed red deer. The outbreak occurred in a group of 80 red deer in the north of Scotland and 50 to 60 animals exhibited clinical signs (Inglis et al., 1983). More recently, CvHV-1 was identified in New Zealand where the virus was isolated during routine export examination of semen collected from red deer stags (Tisdall and Rowe, 2001). CvHV-1 infection is widespread in Great Britain. Indeed, 35% of animals older than one year are seropositive for CvHV-1 (Nettleton et al., 1986). Moreover, a Czech investigation showed that 71% of red deer imported from Scotland were seropositive (Pospisil et al., 1996). The apparent prevalence of seropositive free ranging deer has been shown to be 11% in Belgium and 1% in France (Thiry et al., 1988). More recently, a further Belgian investigation has shown a seroprevalence of 30% in free ranging adults and 1.4% in calves (Gregoire and Linden, unpublished results). In 1988, 43% of deer in a Belgian farm were shown to be seropositive to CvHV-1 (Thiry, unpublished results). Although the serological analyses were performed with tests detecting anti-BoHV-1 antibodies, they are most suggestive of CvHV-1 infection in wild populations of red deer.

Cervid herpesvirus 2

Serological evidence of exposure of reindeer to a virus related to BoHV-1 has formerly been reported in Canada (El Azhary et al., 1979) and in the USA (Dietrich, 1981). More recently, evidence of alphaherpesvirus infection has been reported in isolated populations of woodland caribou in western Canada (Jordan et al., 2003). In 1982, a prevalence of 23% reindeer seropositive to BoHV-1 was observed in Finnish Lapland (Ek-Kommonen et al., 1982). In Finland, 64% of adult reindeer and 1% of calves have been shown to be seropositive to CvHV-2 (Nettleton et al., 1988b). A Norwegian investigation showed similar results: 60% of reindeer and 15% of calves were shown to be seropositive (Hyllseth et al., 1993). In Norway, during the period 1993-2000, antibodies against BoHV-1 related alphaherpesviruses were found in 28.5% of reindeer (Lillehaug et al., 2003). These results indicate that alphaherpesvirus infections are endemic in reindeer and that CvHV-2 is most likely the herpesvirus responsible for the observed prevalences.

Bubaline and elk herpesviruses 1

BuHV-1 was isolated in 1971 from prepuces of water buffaloes (St George and Philpott, 1972). More recently, a field strain of BuHV-1 has been isolated from buffaloes in Italy (De Carlo et al., 2004). To date, only one case of ElkHV-1 infection has been reported. The virus was isolated from the semen of a North American elk (Deregt et al., 2000).

Diagnosis

The capacity of these related alphaherpesviruses to spread in ruminant populations is a threat to BoHV-1 eradication programmes. Indeed, infection with related viruses could lead to false positive diagnosis of BoHV-1. Moreover, heterologous ruminant species could serve as BoHV-1 reservoirs. Consequently, diagnostic tools able to distinguish between ruminant alphaherpesviruses related to BoHV-1 and BoHV-1 itself are of great interest.

Virological diagnosis

The use of tests based on virus isolation or viral DNA detection can avoid misinterpretation due to these cross-reactions. Restriction endonuclease analysis (REA) performed on viral isolates allows the identification and distinguishing of the related viruses (Brake and Studdert, 1985; Bulach and Studdert, 1990; Buonavoglia et al., 1996; D'Arce et al., 2002; Engels et al., 1981, 1983, 1986, 1987; Pidone et al., 1999; Pratelli et al., 2000; Rimstad et al., 1992; Ronsholt et al., 1987; Vanderplasschen et al., 1993; Williams et al., 1997) and could be used to detect interspecific recombinants (Meurens et al., 2004b). However, this procedure is not convenient for general use in a diagnostic laboratory. Highly specific methods using viral DNA amplification have therefore been developed. Differential PCR assays use pairs of consensus primers selected for the detection of bovine, caprine and cervid herpesviruses, and discrimination is achieved by subsequent REA of PCR products (Lyaku et al., 1996; Ros and Belak, 1999) (Figure 4).

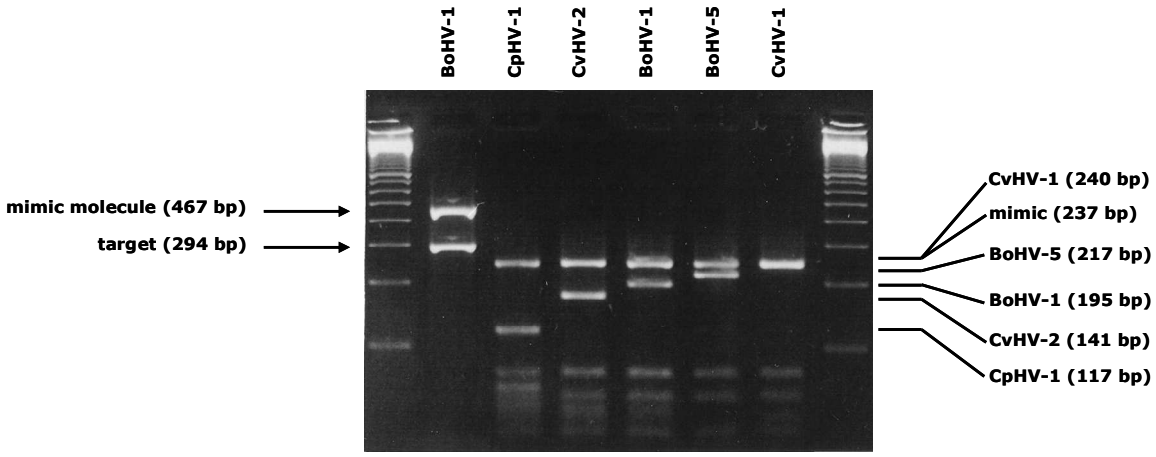

Figure 4 – PCR amplification of ruminant alphaherpesvirus DNA. A mimic molecule is used for control. The final identification of the amplicon is performed by restriction enzyme analysis (reproduced from Ros and Belak (1999), with permission of the American Society for Microbiology).

Another PCR system, using four primer pairs, specifically amplifies a part of the gC gene of each alphaherpesvirus (Ros et al., 1999). A multiplex PCR is available for differentiation between BoHV-1 and BoHV-5 (Alegre et al., 2001). A quantitative real time PCR is available for the detection and quantitation of CpHV-1 DNA in goats (Tempesta et al., 2005).

There also exist monoclonal antibodies that can distinguish between BoHV-1 and BoHV-5 by immunofluorescence, immunoprecipitation or western blot (Friedli and Metzler, 1987; Metzler et al., 1986; Oldoni et al., 2004). More recently, an immunofluorescence assay able to differentiate the five related herpesviruses has been developed. This method is based on the use of four monoclonal antibodies directed against gC, and one against gD, of these related viruses (Keuser et al., 2004a) (Figure 5).

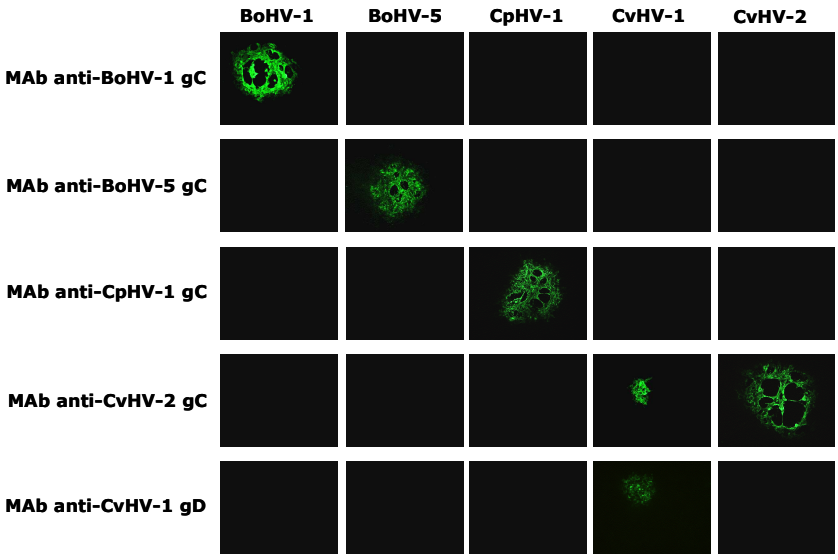

Figure 5 – Indirect immunofluorescence staining of cells infected with ruminant alphaherpesviruses. The primary monoclonal antibodies directed against BoHV-1, BoHV-5, CpHV-1, CvHV-2 and CvHV-1 were detected by FITC-conjugated rabbit immunoglobulin anti-mouse IgG (reproduced from Keuser et al. (2004b), with permission of the American Society for Microbiology).

Serological diagnosis

Serological analysis is very difficult due to the antigenic similarity of ruminant alphaherpesviruses related to BoHV-1. Indeed, these viruses cross-react in ELISA and seroneutralisation tests (Lyaku et al., 1992; Martin et al., 1990; Nixon et al., 1988). The current available serological tests are almost unable to discriminate the related alphaherpesviruses.

A method combining two blocking ELISAs allows a distinction to be made between anti-BoHV-1 and anti-BoHV-5 antibodies in cattle. A serum positive to BoHV-1 in a blocking ELISA based on the recognition of BoHV-1 glycoprotein B is further analysed by a BoHV-1 gE blocking ELISA. Provided there is no vaccination of cattle with a gE negative BoHV-1 vaccine, a negative result in this second test will indicate an infection with BoHV-5. However, the sensitivity and the specificity of this test have been tested only on a very limited number of experimental sera (Wellenberg et al., 2001).

An early study demonstrated a full agreement between seroneutralisation and indirect ELISA for the detection of anti-CpHV-1 antibodies in goats (Plebani et al., 1983). Commercially available BoHV-1 gB blocking ELISA has been tried in other animal species, namely goats and red deer. Compared to CpHV-1 seroneutralisation, this test, applied to the detection of anti-CpHV-1 antibodies in goats, exhibits a relative sensitivity of 95.8% ± 2.4% for experimental sera and 98.7% ± 2.5% for field sera, as well as a relative specificity of 96.9% ± 2.5% for experimental sera and 91.4% ± 9.2% for field sera (Keuser et al., 2003). These good relative intrinsic values are enough to recommend their use at herd level but not on an individual basis. It must be emphasised that this test cannot differentiate between anti-BoHV-1 and anti-CpHV-1 antibodies. The same BoHV-1 gB blocking ELISA has been successfully tested on deer sera to detect antibodies to CvHV-1 by cross-reaction with the same inability to distinguish between anti-BoHV-1 and anti-CvHV-1 antibodies (Reid, personal communication). Any further attempt to develop specific ELISAs has failed to date. The challenge is therefore to develop a differentiating serological test that could be free from high antigenic cross-reactions.

Neutralising antibodies are raised after each alphaherpesvirus infection: for example,

bovine field or experimental sera (Meyer et al., 2000), sera from hyperimmunised rabbits (Bratanich et al., 1991) and monoclonal antibodies raised against gB, gC or gD (Collins et al., 1993; Friedli and Metzler, 1987) exhibit neutralising properties against BoHV-5.

In several combinations, cross-neutralisation assays can differentiate antibodies between the related alphaherpesviruses. A one way cross-neutralisation can be demonstrated for each combination of BoHV-1 and related alphaherpesviruses. Therefore, BoHV-1 specific antiserum neutralises the heterologous virus to a greater extent than anti-sera specific to related alphaherpesviruses have been shown to neutralise BoHV-1 (Deregt et al., 2005; Engels et al., 1983; Meyer et al., 2001; Nettleton et al., 1988a).

Prevention

Although there is no commercially available vaccine against BoHV-5, it is possible to take advantage of a previous immunisation against BoHV-1. Indeed, a primary infection with BoHV-1 affords a cross-protection that protects cattle clinically against a BoHV-5 superinfection (Bratanich et al., 1991; Cascio et al., 1999). This feature can explain the epidemiology of BoHV-5 infection in several continents. Indeed, BoHV-5 can induce outbreaks of meningo-encephalitis in calves not vaccinated against BoHV-1. In North America, IBR vaccination is widely used and sporadic cases of BoHV-5 have only been seen to emerge in calves badly or not immunised against BoHV-1 (D'offay et al., 1993). A recombinant gE negative BoHV-1 vaccine has been shown to be unable to confer full protection against the challenge of BoHV-5 (Spilki et al., 2004). Therefore, the efficacy of the cross-protection afforded by commercial BoHV-1 vaccines needs to be evaluated.

A classical inactivated vaccine confers protection against CpHV-1 infection in goats. This vaccine shows a high immunogenicity and good clinical protection (Tempesta et al., 2001). However, this vaccine has very little chance of being marketed because the veterinary pharmaceutical industry has only a poor interest in the development of vaccines for minor species like goats. As goats are susceptible to BoHV-1, both live and inactivated BoHV-1 vaccines might be tested in goats for their efficacy in the face of a CpHV-1 infection. In this way, a recent preliminary experiment showed that a commercially available live attenuated gE negative BoHV-1 vaccine afforded only a partial protection to intranasally challenged goats (Thiry et al., 2006).

No vaccines are available to protect animals against CvHV-1, CvHV-2, ElkHV-1 or BuHV-1 infections. The main reasons are the low virulence of these viruses and the lack of interest by the industry in these animal species, which are seemingly of minor importance. Currently, no data indicate whether BoHV-1 vaccine is able to protect ruminants infected by their own herpesviruses. However, the close antigenic relationship between these viruses may suggest a cross-protection, but this must, however, be evidenced in challenge experiments.

In the absence of commercialised vaccines, the control of these infections relies on hygienic prophylactic measures: isolation of infected animals and disinfection of farms. Serological diagnosis by a fast and inexpensive method, such as ELISA, is essential to control herds and to give and maintain virus-free status. The removal of seropositive animals considered to be latent carriers, can lead to the forming of seronegative herds, which are likely to be free of virus infection. Such control measures are especially important in deer farming, which is becoming widespread in many countries.

In free-ranging ruminants, control of alphaherpesvirus infection seems extremely difficult

to achieve. The only possibility of doing this relies upon the prevention of extensive contact between animals in order to reduce virus spread.

Conclusion

BoHV-1 is antigenically and genetically related to several ruminant alphaherpesviruses. The existence of antigenic cross-reactions between these viruses and their ability to cross, at least in some instances, the species barrier raise theoretical problems for differential diagnosis. Therefore, it is advisable carefully to evaluate the existence of any other alphaherpesvirus reservoir, both in regions and countries where BoHV-1 infection has been eradicated and in those where the control of IBR is currently or will be undertaken. There must be a requirement to verify that BoHV-1 seropositive ruminants are not infected with BoHV-1 but are infected with their own specific virus. In order to avoid such misdiagnosis, new diagnostic tools are currently being developed. PCR and immunofluorescence tests, using monoclonal antibodies, allow unambiguous discrimination of each ruminant alphaherpesvirus related to BoHV-1. These advances in diagnostic methods must be completed by a better knowledge of the pathogenesis of ruminant alphaherpesvirus infections. The successful control of IBR depends on the parallel surveillance of putative reservoirs of BoHV-1.

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References

Ackermann M., Metzler A.E., McDonaugh H., Bruckner L., Muller H.K., Kihm U., Stellen nichtbovine paarhufer ein IBR-Virus-reservoir dar? I. BHV-1 und CapHV-1 infektions und reaktivierungsversuche an ziegen, virustyp-spezifitat der humoralen antikorper und charakterisierung der viralen antigene, Schweiz. Arch. Tierheilkd. 128 (1986) 557-573.

Alegre M., Nanni M., Fondevila N., Development of a multiplex polymerase chain reaction for the differentiation of bovine herpesvirus-1 and -5, J. Vet. Med. B Infect. Dis. Vet. Public Health 48 (2001) 613-621.

Al-Mubarak A., Chowdhury S., In the absence of glycoprotein I (gI), gE determines bovine herpesvirus type 5 neuroinvasiveness and neurovirulence, J. Neurovirol. 10 (2004) 233-243.

Al-Mubarak A., Zhou Y., Chowdhury S.I., A glycine-rich bovine herpesvirus 5 (BHV-5) gE-specific epitope within the ectodomain is important for BHV-5 neurovirulence, J. Virol. 78 (2004) 4806-4816.

Bagust T.J., Comparison of the biological, biophysical and antigenic properties of four strains of infectious bovine rhinotracheitis herpesvirus, J. Comp. Pathol. 82 (1972) 365-374.

Bagust T.J., Clark L., Pathogenesis of meningo-encephalitis produced in calves by infectious bovine rhinotracheitis herpesvirus, J. Comp. Pathol. 82 (1972) 375-383.

Baranowski E., Dubuisson J., van Drunen Littel-van den Hurk S., Babiuk A.L., Michel A., Pastoret P.-P., Thiry E., Synthesis and processing of bovine herpesvirus-1 glycoprotein H, Virology 206 (1995) 651-654.

Baranowski E., Keil G., Lyaku J., Rijsewijk F.A., van Oirschot J.T., Pastoret P.-P., Thiry E., Structural and functional analysis of bovine herpesvirus 1 minor glycoproteins, Vet. Microbiol. 53 (1996) 91-101.

Bartha A., Hajdu G., Aldasy P., Paczolay G., Occurrence of encephalitis caused by infectious bovine rhinotracheitis virus in calves in hungary, Acta Vet. Acad. Sci. Hung. 19 (1969) 145-151.

Belak K., Kucsera L., Ros C., Kulcsar G., Makranszki L., Soos T., Belak S., Studies on the pathogenicity of bovine herpesvirus type 5 in sheep, Comp. Immunol. Microbiol. Infect. Dis. 22 (1999) 207-220.

Belknap E.B., Collins J.K., Ayers V.K., Schultheiss P.C., Experimental infection of neonatal calves with neurovirulent bovine herpesvirus type 1.3, Vet. Pathol. 31 (1994) 358-365.

Brake F., Studdert M.J., Molecular epidemiology and pathogenesis of ruminant herpesviruses including bovine, buffalo and caprine herpesviruses l and bovine encephalitis herpesvirus, Aust. Vet. J. 62 (1985) 331-334.

Bratanich A.C., Sardi S.I., Smitsaart E.N., Schudel A.A., Comparative studies of BHV-1 variants by in vivo-in vitro tests, Zentralbl. Veterinarmed. B 38 (1991) 41-48.

Bulach D.M., Studdert M.J., Comparative genome mapping of bovine encephalitis herpesvirus, bovine herpesvirus 1, and buffalo herpesvirus, Arch. Virol. 113 (1990) 17-34.

Buonavoglia C., Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Conserva A., Corrente M., Reactivation of caprine herpesvirus 1 in latently infected goats, Comp. Immunol. Microbiol. Infect. Dis. 19 (1996) 275-281.

Caron L., Flores E.F., Weiblen R., Scherer C.F., Irigoyen L.F., Roehe P.M., Odeon A., Sur J.H., Latent infection by bovine herpesvirus type-5 in experimentally infected rabbits: virus reactivation, shedding and recrudescence of neurological disease, Vet. Microbiol. 84 (2002) 285-295.

Carrillo B.J., Ambrogi A., Schudel A.A., Vazquez M., Dahme E., Pospischil A., Meningoencephalitis caused by IBR virus in calves in Argentina, Zentralbl. Veterinarmed. B 30 (1983) 327-332.

Cascio K.E., Belknap E.B., Schultheiss P.C., Ames A.D., Collins J.K., Encephalitis induced by bovine herpesvirus 5 and protection by prior vaccination or infection with bovine herpesvirus 1, J. Vet. Diagn. Invest. 11 (1999) 134-139.

Chenier S., Montpetit C., Helie P., Caprine herpesvirus-1 abortion storm in a goat herd in Quebec, Can. Vet. J. 45 (2004) 241-243.

Chowdhury S.I., Lee B.J., Ozkul A., Weiss M.L., Bovine herpesvirus 5 glycoprotein E is important for neuroinvasiveness and neurovirulence in the olfactory pathway of the rabbit, J. Virol. 74 (2000a) 2094-2106.

Chowdhury S.I., Lee B.J., Onderci M., Weiss M.L., Mosier D., Neurovirulence of glycoprotein C(gC)-deleted bovine herpesvirus type-5 (BHV-5) and BHV-5 expressing BHV-1 gC in a rabbit seizure model, J. Neurovirol. 6 (2000b) 284-295.

Collins J.K., Ayers V.K., Whetstone C.A., van Drunen Littel-van den Hurk S., Antigenic differences between the major glycoproteins of bovine herpesvirus type 1.1 and bovine encephalitis herpesvirus type 1.3, J. Gen. Virol. 74 (1993) 1509-1517.

d'Arce R.C., Almeida R.S., Silva T.C., Franco A.C., Spilki F., Roehe P.M., Arns C.W., Restriction endonuclease and monoclonal antibody analysis of Brazilian isolates of bovine herpesviruses types 1 and 5, Vet. Microbiol. 88 (2002) 315-324.

Davison A.J., Evolution of the herpesviruses, Vet. Microbiol. 86 (2002) 69-88.

De Carlo E., Re G.N., Letteriello R., Del Vecchio V., Giordanelli M.P., Magnino S., Fabbi M., Bazzocchi C., Bandi C., Galiero G., Molecular characterisation of a field strain of bubaline herpesvirus isolated from buffaloes (*Bubalus bubalis*) after pharmacological reactivation, Vet. Rec. 154 (2004) 171-174.

Delhon G., Moraes M.P., Lu Z., Afonso C.L., Flores E.F., Weiblen R., Kutish G.F., Rock D.L., Genome of bovine herpesvirus 5, J. Virol. 77 (2003) 10339-10347.

Deregt D., Jordan L.T., van Drunen Littel-van den Hurk S., Masri S.A., Tessaro S.V., Gilbert S.A., Antigenic and molecular characterization of a herpesvirus isolated from a North American elk, Am. J. Vet. Res. 61 (2000) 1614-1618.

Deregt D., Tessaro S.V., Gilbert S.A., Serological evidence of latency in cattle experimentally infected with elk herpesvirus, Vet. Rec. 156 (2005) 610-611.

Deregt D., Gilbert S.A., Campbell I., Burton K.M., Reid H.W., Hurk S.V., Penniket C., Baxi M.K., Phylogeny and antigenic relationships of three cervid herpesviruses, Virus Res. 114 (2005) 140-148.

Dietrich R.A., Respiratory viruses, in: Dietrich R.A. (Ed.), Alaskan wild life diseases, University of Alaska, Fairbanks, 1981, pp. 28-29.

d'Offay J.M., Mock R.E., Fulton R.W., Isolation and characterization of encephalitic bovine herpesvirus type 1 isolates from cattle in North America, Am. J. Vet. Res. 54 (1993) 534-539.

Egyed L., Brocchi E., Rusvai M., Bartha A., Study of bovine herpesvirus type 1 strains with monoclonal antibodies, Acta Vet. Hung. 40 (1992) 225-230.

Ek-Kommonen C., Veijalainen P., Rantala M., Neuvonen E., Neutralizing antibodies to bovine herpesvirus 1 in reindeer, Acta Vet. Scand. 23 (1982) 565-569.

Ek-Kommonen C., Pelkonen S., Nettleton P.F., Isolation of a herpesvirus serologically related to bovine herpesvirus 1 from a reindeer (Rangifer tarandus), Acta Vet. Scand. 27 (1986) 299-301.

El Azhary M.A., Roy R.S., Frechette J.L., Serological evidence of IBR and BVD infection in caribou (Rangifer tarandus), Vet. Rec. 105 (1979) 336.

Engels M., Ackermann M., Pathogenesis of ruminant herpesvirus infections, Vet. Microbiol. 53 (1996) 3-15.

Engels M., Thiry E., L'infection de la chèvre par l'herpèsvirus caprin de type 1, Point Vét. 31 (2000) 37-42.

Engels M., Steck F., Wyler R., Comparison of the genomes of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis virus strains by restriction endonuclease analysis, Arch. Virol. 6 (1981) 169-174.

Engels M., Gelderblom H., Darai G., Ludwig H., Goat herpesviruses: biological and physicochemical properties, J. Gen. Virol. 64 (1983) 2237-2247.

Engels M., Giuliani C., Wild P., Beck T.M., Loepfe E., Wyler R., The genome of bovine herpesvirus 1 (BHV-1) strains exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization, Virus Res. 6 (1986) 57-73.

Engels M., Loepfe E., Wild P., Schraner E., Wyler R., The genome of caprine herpesvirus 1: genome structure and relatedness to bovine herpesvirus 1, J. Gen. Virol. 68 (1987) 2019-2023.

Engels M., Palatini M., Metzler A.E., Probst U., Kihm U., Ackermann M., Interactions of bovine and caprine herpesviruses with the natural and the foreign hosts, Vet. Microbiol. 33 (1992) 69-78.

Favoreel H.W., Van de Walle G.R., Nauwynck H.J., Pensaert M.B., Virus complement evasion strategies, J. Gen. Virol. 84 (2003) 1-15.

Fehler F., Herrmann J.M., Saalmuller A., Mettenleiter T.C., Keil G.M., Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant, J. Virol. 66 (1992) 831-839.

Friedli K., Metzler A.E., Reactivity of monoclonal antibodies to proteins of a neurotropic bovine herpesvirus 1 (BHV-1) strain and to proteins of representative BHV-1 strains, Arch. Virol. 94 (1987) 109-122.

Gompels U.A., Craxton M.A., Honess R.W., Conservation of glycoprotein H (gH) in herpesviruses: nucleotide sequence of the gH gene from herpesvirus saimiri, J. Gen. Virol. 69 (1988) 2819-2829.

Grewal A.S., Wells R., Vulvovaginitis of goats due to a herpesvirus, Aust. Vet. J. 63 (1986) 79-82.

Griffin A.M., The nucleotide sequence of the glycoprotein gB gene of infectious laryngotracheitis virus: analysis and evolutionary relationship to the homologous gene from other herpesviruses, J. Gen. Virol. 72 (1991) 393-398.

Guercio A., Greco G., Lanizzoto G., Di Marco V., Todaro M., Valutazione della diffusione di anticorpi anti Herpes Virus della capra in allevamenti caprini della Sicilia, Atti. SIPAOC 12 (1998) 138-142.

Hage J.J., Vellema P., Schukken Y.H., Barkema H.W., Rijsewijk F.A., van Oirschot J.T., Wentink G.H., Sheep do not have a major role in bovine herpesvirus 1 transmission, Vet. Microbiol. 57 (1997) 41-54.

Hall W.T., Simmons G.C., French E.L., Snowdon W.A., Asdell M., The pathogenesis of encephalitis caused by the infectious bovine rhinotracheitis virus, Aust. Vet. J. 42 (1966) 229-237.

Horner G.W., Hunter R., Day A.M., An outbreak of vulvovaginitis in goats caused by a caprine herpesvirus, N. Z. Vet. J. 30 (1982) 152.

Hubner S.O., Oliveira A.P., Franco A.C., Esteves P.A., Silva A.D., Spilki F.R., Rijsewijk F.A., Roehe P.M., Experimental infection of calves with gI, gE, US9 negative bovine herpesvirus type 5, Comp. Immunol. Microbiol. Infect. Dis. 28 (2005) 187-196.

Hutchinson L., Goldsmith K., Snoddy D., Ghosh H., Graham F.L., Johnson D.C., Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion, J. Virol. 66 (1992) 5603-5609.

Hyllseth B., Stuen S., Rimstad E., Dahle H.K., Tyler J.C., Serologiske undersokelser over herpesvirus infecksjoner hos reain fra Finnmark og Svalbard, Norsk Veterinaertidsskrift 105 (1993) 733-736.

Inglis D.M., Bowie J.M., Allan M.J., Nettleton P.F., Ocular disease in red deer calves associated with a herpesvirus infection, Vet. Rec. 113 (1983) 182-183.

International Committee on Taxonomy of viruses, in: ICTVdB Index of Viruses, Appendix 00.031 herpesviridae [on line] (2002) http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_herpe.htm [consulted 5 May 2005].

Johnston L., Simmons G., McGavin M., A viral meningo-encephalitis in calves, Aust. Vet. J. 38 (1962) 207-215.

Jones C., Herpes simplex virus type 1 and bovine herpesvirus 1 latency, Clin. Microbiol. Rev. 16 (2003) 79-95.

Jordan L.T., Rettie W.J., Tessaro S.V., Evidence of herpesvirus infection in Woodland Caribou in Saskatchewan, J. Wildl. Dis. 39 (2003) 216-220.

Keuser V., Thiry E., Conséquences de l'infection des cervidés par des alpha-herpèsvirus apparentés au virus de l'IBR, Point Vét. 31 (2000) 219-223.

Keuser V., Gogev S., Schynts F., Thiry E., Demonstration of generalized infection with caprine herpesvirus 1 diagnosed in an aborted caprine fetus by PCR, Vet. Res. Commun. 26 (2002) 221-226.

Keuser V., Infection de la chèvre par l'herpèsvirus caprin 1: diagnostic différentiel et caractérisation de la glycoprotéine D, Doctorate thesis, Liège, Belgium, 2003.

Keuser V., Schynts F., Detry B., Collard A., Robert B., Vanderplasschen A., Pastoret P.-P., Thiry E., Improved antigenic methods for differential diagnosis of bovine, caprine, and cervine alphaherpesviruses related to bovine herpesvirus 1, J. Clin. Microbiol. 42 (2004a) 1228-1235.

Keuser V., Espejo-Serrano J., Schynts F., Georgin J.P., Thiry E., Isolation of caprine herpesvirus type 1 in Spain, Vet. Rec. 154 (2004b) 395-399.

Keuser V., Detry B., Thiry J., de Fays K., Schynts F., Pastoret P.-P., Vanderplasschen A., Thiry E., Characterization of caprine herpesvirus 1 glycoprotein D gene and its translation product, Virus Res. 115 (2006) 112-121.

Konig P., Giesow K., Keil G.M., Glycoprotein M of bovine herpesvirus 1 (BHV-1) is nonessential for replication in cell culture and is involved in inhibition of bovine respiratory syncytial virus F protein induced syncytium formation in recombinant BHV-1 infected cells, Vet. Microbiol. 86 (2002) 37-49.

Koppers-Lalic D., Reits E.A.J., Ressing M.E., Lipinska A.D., Abele R., Koch J., Rezende M.M., Admiraal P., van Leeuwen D., Bienkowska-Szewczyk K., Mettenleiter T.C., Rijsewijk F.A.M., Tampe R., Neefjes J., Wiertz E.J.H.J., Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing, Proc. Natl. Acad. Sci. USA 102 (2005) 5144-5149.

Koptopoulos G., Papanastasopoulou M., Papadopoulos O., Ludwig H., The epizootiology of caprine herpesvirus (BHV-6) infections in goat populations in Greece, Comp. Immunol. Microbiol. Infect. Dis. 11 (1988) 199-205.

Lee B.J., Weiss M.L., Mosier D., Chowdhury S.I., Spread of bovine herpesvirus type 5 (BHV-5) in the rabbit brain after intranasal inoculation, J. Neurovirol. 5 (1999) 474-484.

Lehmkuhl H.D., Cutlip R.C., Protection from parainfluenza-3 virus and persistence of infectious bovine rhinotracheitis virus in sheep vaccinated with modified live IBR-PI-3 vaccine, Can. J. Comp. Med. 49 (1985) 58-62.

Lillehaug A., Vikoren T., Larsen I.L., Akerstedt J., Tharaldsen J., Handeland K., Antibodies to ruminant alpha-herpesviruses and pestiviruses in Norwegian cervids, J. Wildl. Dis. 39 (2003) 779-786.

Liman A., Engels M., Meyer G., Ackermann M., Glycoprotein C of bovine herpesvirus 5 (BHV-5) confers a distinct heparin-binding phenotype to BHV-1, Arch. Virol. 145 (2000) 2047-2059.

Lyaku J.R., Nettleton P.F., Mardsen H., A comparison of serological relationships among five ruminant alphaherpesviruses by ELISA, Arch. Virol. 124 (1992) 333-341.

Lyaku J.R., Vilcek S., Nettleton P.F., Marsden H.S., The distinction of serologically related ruminant alphaherpesviruses by the polymerase chain reaction (PCR) and restriction endonuclease analysis, Vet. Microbiol. 48 (1996) 135-142.

Martin W.B., Castrucci G., Frigeri F., Ferrari M., A serological comparison of some animal herpesviruses, Comp. Immunol. Microbiol. Infect. Dis. 13 (1990) 75-84.

Mettler F., Engels M., Wild P., Bivetti A., Herpesvirus-infektion bei Zieklein in der Schweiz, Schweiz. Arch. Tierheilkd. 121 (1979) 655-662.

Metzler A.E., Schudel A.A., Engels M., Bovine herpesvirus 1: molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease, Arch. Virol. 87 (1986) 205-217.

Meurens F., Influence du délai de surinfection et de la parenté génétique sur la recombinaison de l'herpèsvirus bovin 1, Doctorate thesis, Liège, Belgium, 2004.

Meurens F., Schynts F., Keil G.M., Muylkens B., Vanderplasschen A., Gallego P., Thiry E., Superinfection prevents recombination of the alphaherpesvirus bovine herpesvirus 1, J. Virol. 78 (2004a) 3872-3879.

Meurens F., Keil G.M., Muylkens B., Gogev S., Schynts F., Negro S., Wiggers L., Thiry E., Interspecific recombination between two ruminant alphaherpesviruses, bovine herpesviruses 1 and 5, J. Virol. 78 (2004b) 9828-9836.

Meyer G., Vilcek C., Paces V., O'Hara M.K., Pastoret P.-P., Thiry E., Schwyzer M., Sequence analysis of the bovine herpesvirus type 1 genes homologous to the DNA polymerase (UL30), the major DNA-binding protein (UL29) and the ICP 18.5 assembly protein (UL28) genes of herpes simplex virus, Arch. Virol. 142 (1997) 89-102.

Meyer G., Hanon E., Georlette D., Pastoret P.-P., Thiry E., Bovine herpesvirus type 1 glycoprotein H is essential for penetration and propagation in cell culture, J. Gen. Virol. 79 (1998) 1983-1987.

Meyer G., Bare O., Thiry E., Identification and characterization of bovine herpesvirus type 5 glycoprotein H gene and gene products, J. Gen. Virol. 80 (1999) 2849-2859.

Meyer G., d'Offay J., Thiry E., Les encéphalites à herpèsvirus bovins, Point Vét. 31 (2000) 417-424.

Meyer G., Lemaire M., Ros C., Belak K., Gabriel A., Cassart D., Coignoul F., Belak S., Thiry E., Comparative pathogenesis of acute and latent infections of calves with bovine herpesvirus types 1 and 5, Arch. Virol. 146 (2001) 633-652.

Muluneh A., Liebermann H., Occurrence of caprine herpesvirus (BHV-6) infection in goat population of the GDR, Arch. Exp. Veterinarmed. 44 (1990) 254-257.

Nettleton P.F., Sinclair J.A., Herring J.A., Inglis D.M., Fletcher T.J., Ross H.M., Bonniwell M.A., Prevalence of herpesvirus infection in British red deer and investigations of further disease outbreaks, Vet. Rec. 118 (1986) 267-270.

Nettleton P.F., Thiry E., Reid H.W., Pastoret P.-P., Herpesvirus infections in cervidae, Rev. Sci. Tech. Off. Int. Epiz. 7 (1988a) 977-988.

Nettleton P.F., Ek-Kommonen C., Tanskanen R., Reid H.W., Sinclair J.A., Herring J.A., Studies in the epidemiology and pathogenesis of alphaherpesviruses from red deer (cervus elaphus) and reindeer (rangifer tarandus), The management and Health of Farmed deer (1988b) 143-147.

Nixon P., Edwards S., White H., Serological comparisons of antigenically related herpesviruses in cattle, red deer and goats, Vet. Res. Commun. 12 (1988) 355-362.

Oldoni I., Weiblen R., Inkelmann M.A., Flores E.F., Production and characterization of monoclonal antibodies to a Brazilian bovine herpesvirus type 5, Braz. J. Med. Biol. Res. 37 (2004) 213-221.

Papanastasopoulou M., Koptopoulos G., Lekkas S., Papadopoulos O., Ludwig H., An experimental study on the pathogenicity of the caprine herpesvirus type 1 (CHV-1), Comp. Immunol. Microbiol. Infect. Dis. 14 (1991) 47-53.

Pastoret P.-P., Thiry E., Brochier B., Derboven G., Bovid herpesvirus 1 infection of cattle: pathogenesis, latency, consequences of latency, Ann. Rech. Vét. 13 (1982) 221-235.

Perez S.E., Bretschneider G., Leunda M.R., Osorio E.A., Flores E.F., Odeon A.C., Primary infection, latency, and reactivation of bovine herpesvirus type 5 in the bovine nervous system, Vet. Pathol. 39 (2002) 437-444.

Perez S.E., Vagnozzi A., Sur J.H., Odriozola E., Campero C.M., Odeon A.C., Análisis retrospectivo de casos con diagnóstico de necrosis cerebrocortical y su relación com herpesvirus bovino tipo 5, Rev. Argent. Microbiol. 35 (2003) 69-73.

Pidone C.L., Galosi C.M., Echeverria M.G., Nosetto E.O., Etcheverrigaray M.E., Restriction endonuclease analysis of BHV-1 and BHV-5 strains isolated in Argentina, Zentralbl. Veterinarmed. B 46 (1999) 453-456.

Pirak M., Thiry E., Brochier B., Pastoret P.-P., Infection expérimentale de la chèvre par le virus de la rhinotrachéite infectieuse bovine (Bovine herpesvirus type 1) et tentative de réactivation virale, Rec. Méd. Vét. (1983) 1103-1106.

Plebani G.F., Engels M., Metzler A.E., Wyler R., Caprine herpesvirus in Switzerland: distribution, incidence and latency of infection, Schweiz. Arch. Tierheilkd. 125 (1983) 395-411 (in German).

Pospisil Z., Vyvlecka R., Cihal P., Lany P., Zendulkova D., Detection of herpesvirus serum antibodies in red deer (Cervus elaphus) imported into the Czech Republic, Vet. Med. 41 (1996) 279-282.

Pratelli A., Greco G., Dall'Ara P., Engels M., Tempesta M., Buonavoglia C., Restriction endonuclease analysis of the genome of two Italian caprine herpesvirus 1 strains, Arch. Virol. 145 (2000) 845-851.

Reid H.W., Nettleton P.F., Pow I., Sinclair J.A., Experimental infection of red deer (Cervus elaphus) and cattle with a herpesvirus isolated from red deer, Vet. Rec. 119 (1986) 156-158.

Rimstad E., Krona R., Hyllseth B., Comparison of herpesviruses isolated from reindeer, goats, and cattle by restriction endonuclease analysis, Arch. Virol. 123 (1992) 389-397.

Roizman B., Knipe M.D., Herpes simplex virus and their replication, in: Knipe D.M., Howley P.M. (Eds.), Fields Virology, 4th ed., Lippincott Williams and Wilkins publishers, Philadelphia, 2001, pp. 2399-2461.

Roizman B., Pellett P.E., The family *Herpesviridae*: A brief introduction, in: Knipe D.M., Howley P.M. (Eds.), Fields Virology, 4th ed., Lippincott Williams and Wilkins publishers, Philadelphia, 2001, pp. 2381-2398.

Ronsholt L., Christensen L.S., Bitsch V., Latent herpesvirus infection in red deer: characterization of a specific deer herpesvirus including comparison of genomic restriction fragment patterns, Acta. Vet. Scand. 28 (1987) 23-31.

Roop C., Hutchinson L., Johnson D.C., A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H, J. Virol. 67 (1993) 2285-2297.

Roperto F., Pratelli A., Guarino G., Ambrosio V., Tempesta M., Galati P., Iovane G., Buonavoglia C., Natural caprine herpesvirus 1 (CpHV-1) infection in kids, J. Comp. Pathol. 122 (2000) 298-302.

Ros C., Belak S., Studies of genetic relationships between bovine, caprine, cervine, and rangiferine alphaherpesviruses and improved molecular methods for virus detection and identification, J. Clin. Microbiol. 37 (1999) 1247-1253.

Ros C., Belak S., Characterization of the glycoprotein B gene from ruminant alphaherpesviruses, Virus Genes 24 (2002) 99-105.

Ros C., Riquelme M.E., Forslund K.O., Belak S., Improved detection of five closely related ruminant alphaherpesviruses by specific amplification of viral genomic sequences, J. Virol. Methods 83 (1999) 55-65.

Saito J.K., Gribble D.H., Berrios P.E., Knight H.D., Mc Kercher D.G., A new herpesvirus isolate from goats: Preliminary report, Am. J. Vet. Res. 35 (1974) 847-848.

Salvador S.C., Lemos R.A.A., Riet-Correa F., Osorio A.L.A.R., Meningoencefalite em bovinos causada por bovino-5 no Mato Grosso do Sul and São Paulo, Pesqui. Vet. Bras. 18 (1998) 76-83.

Schudel A.A., Carrillo B.J., Wyler R., Metzler A.E., Infection of calves with antigenic variants of bovine herpesvirus 1 (BHV-1) and neurological disease, J. Vet. Med. B 33 (1986) 303-310.

Schwyzer M., Ackermann M., Molecular virology of ruminant herpesviruses, Vet. Microbiol. 53 (1996) 17-29.

Schynts F., McVoy M.A., Meurens F., Detry B., Epstein A.L., Thiry E., The structures of bovine herpesvirus 1 virion and concatemeric DNA: implications for cleavage and packaging of herpesvirus genomes, Virology 314 (2003) 326-335.

Schynts F., Meurens F., Detry B., Vanderplasschen A., Thiry E., Rise and survival of bovine herpesvirus 1 recombinants after primary infection and reactivation from latency, J. Virol. 77 (2003) 12535-12542.

Silva A.M., Weiblen R., Irigoyen L.F., Roehe P.M., Sur H.J., Osorio F.A., Flores E.F. Experimental infection of sheep with bovine herpesvirus type-5 (BHV-5): acute and latent infection, Vet. Microbiol. 66 (1999) 89-99.

Six A., Banks M., Engels M., Bascunana C.R., Ackermann M., Latency and reactivation of bovine herpesvirus 1 (BHV-1) in goats and of caprine herpesvirus 1 (CapHV-1) in calves, Arch. Virol. 146 (2001) 1325-1335.

Spilki F.R., Silva A.D., Hubner S., Esteves P.A., Franco A.C., Driemeier D., Roehea P.M., Partial protection induced by a BHV-1 recombinant vaccine against challenge with BHV-5., Ann. NY Acad. Sci. 1026 (2004) 247-250.

St George T.D., Philpott M., Isolation of infectious bovine rhinotracheitis virus from the prepuce of water buffalo bulls in Australia, Aust. Vet. J. 48 (1972) 126.

Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Presenza di anticorpi per caprine herpesvirus 1 (CapHV.1) in allevamenti caprini dell'Italia meridionale, Atti. SIPAOC 11 (1994) 121-122.

Tempesta M., Buonavoglia D., Sagazio P., Pratelli A., Buonavoglia C., Natural reactivation of caprine herpesvirus 1 in latently infected goats, Vet. Rec. 143 (1998) 200.

Tempesta M., Pratelli A., Corrente M., Buonavoglia C., A preliminary study on the pathogenicity of a strain of caprine herpesvirus-1, Comp. Immunol. Microbiol. Infect. Dis. 22 (1999a) 137-143.

Tempesta M., Pratelli A., Greco G., Martella V., Buonavoglia C., Detection of caprine herpesvirus 1 in sacral ganglia of latently infected goats by PCR, J. Clin. Microbiol. 37 (1999b) 1598-1599.

Tempesta M., Pratelli A., Normanno G., Camero M., Buonavoglia D. Greco G., Buonavoglia C., Experimental intravaginal infection of goats with caprine herpesvirus 1, J. Vet. Med. B Infect. Dis. Vet. Public Health 47 (2000) 197-201.

Tempesta M., Camero M., Greco G., Pratelli A., Martella V., Buonavoglia C., A classical inactivated vaccine induces protection against caprine herpesvirus 1 infection in goats, Vaccine 19 (2001) 3860-3864.

Tempesta M., Greco G., Pratelli A., Buonavoglia D., Camero M., Martella V., Buonavoglia C., Reactivation of caprine herpesvirus 1 in experimentally infected goats, Vet. Rec. 150 (2002) 116-117.

Tempesta M., Camero M., Sciorsci R.L., Greco G., Minoia R., Martella V., Pratelli A., Buonavoglia C., Experimental infection of goats at different stages of pregnancy with caprine herpesvirus 1, Comp. Immunol. Microbiol. Infect. Dis. 27 (2004a) 25-32.

Tempesta M., Tarsitano E., Camero M., et al.; Short sequence repeats (SSR) in the glycoprotein C gene of caprine herpesvirus type 1 (CpHV.1), Proceedings of the 2nd European congress of virology, Madrid, 2004b, pp. 11-14.

Tempesta M., Decaro N., Elia G., et al., Development of a real time PCR for the detection and quantitation of caprine herpesirus type 1 DNA, in: Nauwynck, H., Favoreel, H. (Ed.), Proc. 2nd ESVV Veterinary Herpesirus Symposium, Ghent, 2005.

Thiry E., Lemaire M., Infection de ruminants par des herpèsvirus hétérologues, Point Vét. 207 (2001) 20-25.

Thiry E., Vercouter M., Dubuisson J., Barrat J., Sepulchre C., Gerardy C., Meersschaert C., Collin B., Blancou J., Pastoret P.-P., Serological survey of herpesvirus infections in wild ruminants of France and Belgium, J. Wildl. Dis. 24 (1988) 268-273.

Thiry E., Lemaire M., Schynts F., Meyer G., Dispas M., Gogev S., Les conséquences de l'infection des bovins par le virus de la rhinotrachéite infectieuse bovine, Point Vét. 199 (1999) 279-285.

Thiry E., Ackermann M., Banks M., et al., Risk evaluation of cross-infection of cattle with ruminant alphaherpesviruses related to bovine herpesvirus type 1, in: R. Körber (Ed.), 3. Internationales Symposium zur BHV-1-/BVD-Bekämpfung, Stendal, 2001, pp. 99-104.

Thiry E., Meurens F., Muylkens B., McVoy M., Gogev S., Thiry J., Vanderplasschen A., Epstein A., Keil G., Schynts F., Recombination in alphaherpesviruses, Rev. Med. Virol. 15 (2005) 89-103.

Thiry E., Muylkens B., Meurens F., Gogev S., Thiry J., Vanderplasschen A., Schynts F., Recombination in the alphaherpesvirus bovine herpesvirus 1, Vet. Microbiol. 113 (2006) 171-177.

Thiry J., Tempesta M., Camero M., Tarsitano E., Bellacicco A.L., Thiry E., Buonavoglia C., A live attenuated glycoprotein E negative bovine herpesvirus 1 vaccine induces a partial cross-protection against caprine herpesvirus 1 infection in goats, Vet. Microbiol. 113 (2006) 303-308.

Tikoo S.K., Campos M., Babiuk L.A., Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control, Adv. Virus. Res. 45 (1995) 191-223.

Tisdall D.J., Rowe S.M., Isolation and characterisation of cervine herpesvirus-1 from red deer semen, New Z. Vet. J. 64 (2001) 111-114.

Tolari F., White H., Nixon P., Isolation and reactivation of bovid herpesvirus 1 in goats, Microbiologica 13 (1990) 67-71.

Uzal F.A., Woods L., Stillian M., Nordhausen R., Read D.H., Van Kampen H., Odani J., Hietala S., Hurley E.J., Vickers M.L., Gard S.M., Abortion and ulcerative posthitis associated with caprine herpesvirus-1 infection in goats in California, J. Vet. Diagn. Invest. 16 (2004) 478-484.

Vanderplasschen A., Bublot M., Pastoret P.-P., Thiry E., Restriction maps of the DNA of cervid herpesvirus 1 and cervid herpesvirus 2, two viruses related to bovine herpesvirus 1, Arch. Virol. 128 (1993) 379-388.

Vogel F.S., Caron L., Flores E.F., Weiblen R., Winkelmann E.R., Mayer S.V., Bastos R.G., Distribution of bovine herpesvirus type 5 DNA in the central nervous systems of latently, experimentally infected calves, J. Clin. Microbiol. 41 (2003) 4512-4520.

Vossen M.T., Westerhout E.M., Soderberg-Naucler C., Wiertz E.J., Viral immune evasion: a masterpiece of evolution, Immunogenetics 54 (2002) 527-542.

Wafula J.S., Mushi E.Z., Wamwayi H., Reaction of goats to infection with infectious bovine rhinotracheitis virus, Res. Vet. Sci. 39 (1985) 84-86.

Waldvogel A., Engels M., Wild P., Stunzi H., Wyler R., Caprine herpesvirus infection in Switzerland: some aspects of its pathogenicity, Zentralbl. Veterinarmed. B 28 (1981) 612-623.

Weiblen R., de Barros C.S., Canabarro T.F., Flores I.E., Bovine meningoencephalitis from IBR virus, Vet. Rec. 24 (1989) 666-667.

Wellenberg G.J., Mars M.H., van Oirschot J.T., Antibodies against bovine herpesvirus (BHV) 5 may be differentiated from antibodies against BHV1 in a BHV1 glycoprotein E blocking ELISA, Vet. Microbiol. 78 (2001) 79-84.

Whetstone C.A., Evermann J.F., Characterization of bovine herpesviruses isolated from six sheep and four goats by restriction endonuclease analysis and radioimmunoprecipitation, Am. J. Vet. Res. 49 (1988) 781-785.

Williams N.M., Vickers M.L., Tramontin R.R., Petrites-Murphy M.B., Allen G.P., Multiple abortions associated with caprine herpesvirus infection in a goat herd, J. Am. Vet. Med. Assoc. 211 (1997) 89-91.

CHAPTER TWO

Objectives

Ruminant alphaherpesviruses related to bovine herpesvirus 1 (BoHV-1) are a cluster of viruses antigenically and genetically closely related (Thiry et al., 2006b). The prototype of this cluster, BoHV-1, is a major pathogen of cattle associated with various clinical manifestations including infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV) (Muylkens et al., 2007). IBR is a disease of major economic concern in many parts of the world and especially in Europe, both in countries where this infection has been eradicated and in those where the control of IBR is currently or will be undertaken (Thiry et al., 1999). The massive use of vaccination allowed a significant reduction of the number of IBR clinical cases. However, the existence of closely related viruses to BoHV-1 is a threat for IBR eradication programmes. Consequently, the main objective of the present work is dedicated to afford a better knowledge of the interaction between alphaherpesviruses and their ruminant hosts in order to contribute to improve the control of IBR.

To meet the objective, two approaches have been developed: the study of the viral diversity aiming to extend both epidemiological and virological data about ruminant alphaherpesviruses related to BoHV-1 and the study of the heterologous protection aiming to protect minor ruminant species by the concept of the "cascade" vaccination.

Illustrating the problematic of the cluster of ruminant alphaherpesviruses related to BoHV-1, an original situation has been described recently in Belgium. During 2001 and 2002 hunting seasons, 28.9% of red deer were detected seropositive to BoHV-1 (Grégoire and Linden, unpublished data). Due to an apparent lack of contact between cattle and red deer, it was suggested that a BoHV-1 related virus was spreading in the Belgian red deer population. Thus, the first isolation of cervid herpesvirus 1 (CvHV-1) in wild fauna is reported, which brings the opportunity to deeper analyse the antigenic, genomic and genetic relationship between BoHV-1 and its related ruminant alphaherpesviruses.

This isolation demonstrates that a ruminant can be strongly identified as BoHV-1 positive while in actual fact it is infected with a related but distinct alphaherpesvirus and this ruminant will be declared as false positive. The problem is even greater when these viruses become latent allowing their possible reactivation and persistence for a very long time in their ecological niches (Thiry et al., 2006b). It is necessary to have tests which can differentiate related alphaherpesviruses that infect different ruminant species. The control of IBR relies on the use of BoHV-1 gB and gE blocking enzyme linked immunosorbent assays (ELISA) in order to differentiate infected and gE-negative vaccinated animals (European Food Safety Authority, 2005). Knowing that CpHV-1 is the most distant virus from BoHV-1, it can be hypothesised that a BoHV-1 gB blocking ELISA detects CpHV-1 but that CpHV-1 infection could be discriminated by a BoHV-1 gE blocking ELISA. CpHV-1 being mainly distributed in the Mediterranean part of Europe as Greece, Spain and Italy, the analysis was performed with field serums collected in France with the aim to update the epidemiological situation of the infection in Europe.

Besides BoHV-1, CpHV-1 is the most relevant infection in Europe but is sadly neglected. The first reason is that economic losses are restricted to a herd level in contrast with IBR that brings an economical impact at a country level. The second reason is that goat is considered as a minor species. In this context, the problem is still not big enough for commercial interest towards vaccine development. The European Union has recently pointed out the problem of minor uses and minor species and allowed off label use of veterinary medicinal products or the use of a product licensed for a major species when an authorised veterinary medicinal product is not available ("cascade" principle). Goat being a minor species and CpHV-1 sharing close antigenic and genetic

properties with BoHV-1, a live attenuated gE-negative BoHV-1 vaccine has been assessed in goats to protect against either a nasal or a genital CpHV-1 infection.

Isolation and characterisation of a ruminant alphaherpesvirus closely related to bovine herpesvirus 1 in a free-ranging red deer

Julien Thiry, Frederik Widén, Fabien Grégoire, Annick Linden, Sándor Belák and Etienne Thiry

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The subfamily of alphaherpesviruses includes a cluster of viruses antigenically and genetically closely related to bovine herpesvirus 1 (BoHV-1): namely bovine herpesvirus 5 (BoHV-5), bubaline herpesvirus 1 (BuHV-1), caprine herpesvirus 1 (CpHV-1), cervid herpesviruses 1 (CvHV-1) and 2 (CvHV-2) and elk herpesvirus 1 (ElkHV-1). Taking into account the serological relationship between these ruminant alphaherpesviruses, several serological surveys demonstrated BoHV-1 related virus infection in ruminant species by using BoHV-1 serological tools. In this way, a recent investigation indicated a high increase of the prevalence in the free-ranging red deer population in Belgium. The current study reports the isolation of a virus closely related to BoHV-1 in a free-ranging red deer. The isolate was antigenically, genomically and genetically characterised by comparison with each BoHV-1 related ruminant alphaherpesvirus. Immunofluorescence assays revealed the isolate was antigenically distinct from bovine and caprine alphaherpesviruses. BamHI and BstEII restriction analyses demonstrated the genomic difference between the isolate and the other BoHV-1 related ruminant alphaherpesviruses. The sequencing of selected parts of UL27 and US8 genes showed a high degree of homologies between each BoHV-1 related ruminant alphaherpesvirus and the isolate. Besides a close relationship between all ruminant alphaherpesviruses, the phylogenetic analysis revealed that the isolate clustered with CvHV-1. Taken together, the obtained data show that a strain of CvHV-1 circulates in wild red deer in continental Europe.

Introduction

The family *Herpesviridae* includes nearly two hundred viruses isolated from various hosts including molluscs, fishes, amphibians, reptiles, birds, mammals and at least one invertebrate. Based on biological and molecular properties, the family has been divided into three subfamilies of viruses, *Alpha-*, *Beta-* and *Gammaherpesvirinae*, which have co-evolved with different host species. Illustrating the concept, several ruminant alphaherpesviruses form a cluster of antigenically and genetically related viruses (Thiry et al., 2006a) Seven alphaherpesviruses belong to this cluster where bovine herpesvirus 1 (BoHV-1), responsible for infectious bovine rhinotracheitis (IBR), a cattle disease of major economic concern in Europe, is the prototype (Muylkens et al., 2007): bovine herpesvirus 5 (BoHV-5) causing meningo-encephalitis in calves (Johnston et al., 1962), bubaline herpesvirus 1 (BuHV-1) responsible for subclinical infections in water buffaloes (St George and Philpott, 1972), caprine herpesvirus 1 (CpHV-1) inducing systemic disease in kids and abortion in adults (Saito et al., 1974), cervid herpesvirus 1 (CvHV-1) responsible for conjunctivitis in red deer (Inglis et al., 1983), cervid herpesvirus 2 (CvHV-2) and elk herpesvirus 1 (ElkHV-1) causing subclinical genital infections in reindeer and elk respectively (Deregt et al., 2000; Ek-Kommonen et al., 1982). Phylogenetic studies of conserved herpesvirus sequences showed that BoHV-5 and BuHV-1 were most closely related to BoHV-1, followed by ElkHV-1, CvHV-1, CvHV-2 and CpHV-1 (Deregt et al., 2000; 2005b; Ros and Belák, 1999). However, BoHV-1 related ruminant alphaherpesvirus are not always restricted to their natural host species. Indeed, buffalo, goat, sheep, red deer and reindeer were successfully infected with BoHV-1 under experimental conditions. Similarly, cattle were shown to be susceptible to BuHV-1, CpHV-1, CvHV-1, CvHV-2 and ElkHV-1 (Thiry et al., 2006a). The cross-serological relationship between these viruses and BoHV-1 was also demonstrated by seroneutralisation and enzyme linked immunosorbent assays (ELISA) (Wellenberg et al., 2001; Lyaku et al., 1992; Martin et al., 1990; Nixon et al., 1988). Consequently, the properties shared by BoHV-1 related alphaherpesviruses can lead to misdiagnosis of BoHV-1 infection which can be considered as a threat to infectious bovine rhinotracheitis eradication programmes (Thiry et al., 2006a).

CvHV-1 was firstly isolated in 1983 from a farmed red deer suffering of ocular lesions. The disease, showing a contagious character, emerged at the end of 1982 in a red deer stag in northern Scotland. Fifty to sixty animals out of 80 exhibited clinical signs at various degrees. A seroneutralisation assay demonstrated the serological relationship of the virus with BoHV-1 (Inglis et al., 1983). Since this isolation, no epidemic of the ocular disease in red deer stags and no severe epidemic in free-ranging animals have been reported. More recently, CvHV-1 was identified in New-Zealand during routine export examination of semen collected from red deer stags (Tisdall and Rowe, 2001). CvHV-1 is responsible for the herpetic conjunctivitis of red deer commonly named ocular syndrome. The disease is characterised by purulent ocular discharge, hypopyon, uniform corneal opacity without ulceration, mucopurulent nasal discharge and photophobia. Moderate swelling of the periorbital tissues and marked oedema of the upper eyelids are also observed (Inglis et al., 1983). The reactivation of CvHV-1 was successfully performed suggesting the persistence of the infection in a latent state (Ronsholt et al., 1987).

Since it was demonstrated that red deer is infected by a herpesvirus, several studies have been initiated to evaluate the percentage of animals seropositive to BoHV-1 and CvHV-1 (Nettleton et al., 1988a; 1988b). The first serological survey revealed that CvHV-1 infection was widespread in Scotland with prevalences of 40% in hill deer and 33% in farmed deer (Nettleton et al., 1986). English farmed deer were also tested for CvHV-1 antibody and presented a prevalence of 14% (Nettleton et al., 1986). In Germany, 12.8% of red deer hunted between 1984 and 1986 were seropositive to BoHV-1 (Kokles et al., 1988) and the serological evidence of a CvHV-1 infection was

reported one year later (Liebermann et al., 1989). More recently, antibodies against BoHV-1 were found in 5.4% red deer from the German region Brandenburg (Muller et al., 1997). In Czech republic, 68% and 71% of red deer were seropositive to BoHV-1 and CvHV-1 respectively (Pospisil et al., 1996) and 0.5% of free-ranging red deer in Norway (Lillehaug et al., 2003). Outside of Europe, CvHV-1 infection was identified in New Zealand (Tisdall and Rowe, 2001; Motha and Jenner, 2001) and 55% of red deer living in United States National Parks were seropositive to BoHV-1 (Aguirre et al., 1995). However, these results only suggest a red deer CvHV-1 infection since the selected tests were not specific to CvHV-1 but to BoHV-1 (Thiry et al., 2006a ; Mollema et al., 2005).

In Belgium, two serological investigations aiming to detect antibodies against BoHV-1 were performed. The first one indicated that 11% of Belgian red deer were seropositive to BoHV-1 or another related herpesvirus (Thiry et al., 1988). In 2001 and 2002, a higher prevalence was noticed with 28.9% of red deer positive to BoHV-1 (Grégoire and Linden, unpublished data). However, since experimental quantification of BoHV-1 transmission in red deer demonstrated that BoHV-1 would probably not survive more than a few decades in red deer populations (Mollema et al., 2005), it is most likely that BoHV-1 antibodies prevalence was due to CvHV-1 and not a BoHV-1 cross-species infection. Regarding these data, it was decided to investigate the presence of an alphaherpesvirus spreading in the Belgian free-ranging red deer population.

Materials and methods

Cells and viruses

The Madin-Darby bovine kidney (MDBK) cell line (ATCC CCL22) was maintained in Earle minimal essential medium (MEM) (Invitrogen, Merelbeke, Belgium) supplemented with 5% of heat-inactivated foetal bovine serum (FBS) (BioWhittaker, Verviers, Belgium) and 2% penicillin (5,000 Units/ml) streptomycin (5,000 μg/ml) (PS) (Invitrogen). The CvHV-1 virus Anlier strain and BoHV-1 Lam 1.1 (Metzler et al., 1985), BoHV-1 K22 1.2a (Kendrick et al., 1958), BoHV-5 N569 (French, 1962), BuHV-1 b6 (St George and Philpott, 1972), CpHV-1 Ba-1 (Buonavoglia et al., 1996), CvHV-1 Banffshire 82 (Inglis et al., 1983), CvHV-2 Salla 82 (Ek-Kommonen et al., 1986), ElkHV-1 (Deregt et al., 2000) virus strains were propagated on MDBK cells in MEM supplemented with 5% FBS. Viral stocks were produced by infection of confluent MDBK cells at a multiplicity of infection (MOI) of 0.1 in MEM supplemented with 2% PS and 5% FBS. When the cytopathic effect reached 90%, culture medium was removed and clarified by centrifugation at 1,500 x *g* for 20 min. The supernatants were aliquoted, frozen at -80°C, and titrated by plaque assay on MDBK cells as previously described (Lemaire et al., 1999).

Viral DNA extraction

Viruses propagated on MDBK cells were clarified by centrifugation at 1,500 x *g* for 20 min. Supernatants were removed and further ultracentrifuged at 100,000 x *g* for 60 min. to pellet virions. Pellets were resuspended in TE buffer (10 mM TrisHCl pH 7.8, 1 mM EDTA) with 0.1% NP40, incubated 30 min at 37°C and ultracentrifuged 2 hours at 100,000 x *g* through a 30% (w/v) sucrose cushion. Pellets were resuspended in TE buffer with 0.5% sodium dodecylsulfate (SDS) and digested with proteinase K (700 µg/ml, Roche Diagnostic) for 2 h. at 56°C. Proteins were precipitated by the addition of half volume ammonium acetate (1.5 M, pH 7.5) and DNA was precipitated by addition of 2 volumes of ethanol. Viral DNA was extracted as previously described (Lemaire et al., 1999).

Restriction enzyme analysis

Viral DNA was submitted to BamHI and BstEII restriction endonucleases (New England Biolabs, England, United Kingdom). The digestion products were electrophoresed in a 0.7% Tris Acetate EDTA gel for 18 h at 40 V/cm and 500 mA. SmartLadder (10 kb; Eurogentec, Liège, Belgium) was used as molecular mass marker.

Immunofluorescence staining

An immunofluorescence assay was performed as described by Keuser and collaborators (2004) with minor modifications. Briefly, MDBK cells grown on glass coverslips were infected with the different viruses and incubated 48 h in MEM containing 5% FBS and 0.6% carboxymethylcellulose. The coverslips with individual plaques were fixed in PBS containing 2% (wt/vol) paraformaldehyde and incubated with undiluted hybridoma supernatant or 1,000-fold-diluted ascitic fluid in PBS containing 5% FBS. As secondary antibodies, FITC-conjugated rabbit anti-mouse IgG (2 µg/ml; Dako) was used. Coverslips were mounted with a Prolong Antifade kit (Molecular Probes Europe BV, Leiden, The Netherlands). Pictures were captured with a charge-coupled device Leica DC 300F camera (with Leica IM 50 V1.20 software) installed on an epifluorescence microscope.

Sequencing and phylogenetic analysis

Two different sets of primers were used in this study. The diagnostic PCR and the sequencing of a 443 bp region of glycoprotein B gene (UL27) was performed by applying the CR30 (5'-TCGAARGCCGAGTACCTGCG-3'; sense; 5' end, position 56,051) and CR31 (5'-CCAGTCCCAGGCRACCGTCAC-3'; antisense; 5' end, position 56,494) primer set (Ros and Belák, 1999). To extend phylogenetic analyses between ruminant alphaherpesviruses and the viral isolate, another set of primers amplifying an estimated 624 bp region of glycoprotein E gene (US8) was designed: ALPHA/US8/914F (5'-CGARACSTGCATCTTYCACC-3'; sense; 5' end, position) and ALPHA/US8/1538R (5'-GGSTCGTTGSTYGGM-3'; antisense; 5' end, position). Fragments resulting from PCR were purified with QIAquick PCR Purification Kit (Quiagen, Venlo, The Netherlands) and cloned using the pGEM®-T Easy vector system (Promega, Leiden, The Nederlands). The sequencing was performed by the GIGA sequencing facility (GIGA, Liège, Belgium). Sequences were assembled and aligned by using the BioEdit Sequence Alignment Editor software (Hall, 1999). Phylogenetic analyses were performed, by distance methods, using the programs dnadist, neighbor and drawgram included in the PHYLIP package (Felsenstein, 1989). The reliability of the trees were assesed by resampling and analysing 1000 random data sets (bootstrapping) using Seqboot and consense software in the PHYLIP package.

Nucleotide accession number

The sequences reported in this paper have been deposited in the GenBank database under the accession numbers EF624466 to EF624479. The following previously GenBank published sequences were used in this study: BoHV-1 K22 UL27 [AF078725.1], BoHV-5 N569 UL27 [AF078726.2], CvHV-1 Banffshire 82 UL27 [AF078729.2] and CvHV-2 Salla 82 UL27 [AF078727.2].

Results

Isolation and identification of a red deer alphaherpesvirus

During the 2004 and 2005 hunting seasons, nasal and genital swabs were collected from free-living red deer in the South of Belgium. A viral isolate (050136) was obtained in MDBK cells inoculated with a nasal swab from a male fawn (*Cervus elaphus*) hunted in Anlier forest. The animal was in good shape and weighed 47 kg. The confirmation of the positive sample was performed by inoculation with the duplicate swab. The virus induced a cytopathic effect morphologically and temporally typical of a herpesvirus (Figure 1a). By PCR (Ros and Belák, 1999), a 443 bp region of glycoprotein B gene (UL27) was amplified (Figure 1b). Taken together, results suggested that the Anlier isolate belonged to the cluster of BoHV-1 related alphaherpesviruses. The analysis of the PCR product sequence showed that the Anlier isolate was close to BoHV-1 related ruminant alphaherpesviruses especially CvHV-1 (data not shown).

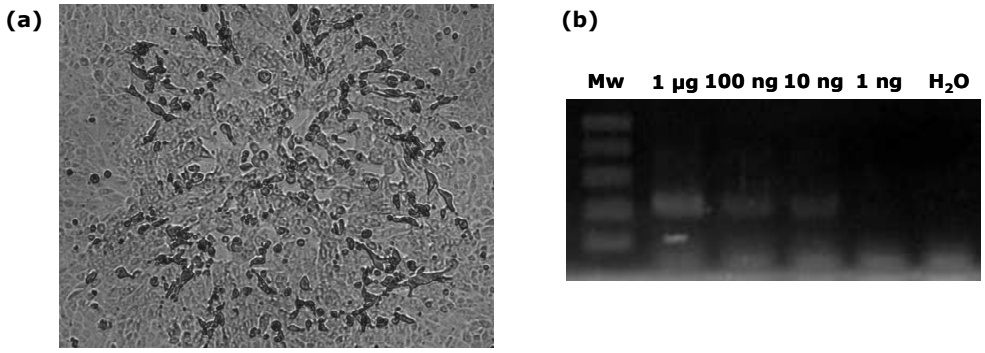

Figure 1 – Isolation and identification of a red deer alphaherpesvirus. (a) Cytopathic effect of the Anlier isolate inoculated onto MDBK cells, overlaid with medium containing 0.6% carboxymethyl cellulose and photographed 72h later. (b) Molecular detection by PCR of the Anlier isolate DNA.

Antigenic characterisation of the Anlier isolate within ruminant alphaherpesviruses

In order to discriminate antigenically the Anlier isolate between bovine, caprine and cervid alphaherpesviruses, five selected monoclonal antibodies were tested by immunofluorescence assay on cells infected separately with the five related alphaherpesviruses: G14G11F5 for BoHV-1, 2915 for BoHV-5, 2E5G5G1 for CpHV-1, 6C2 for CvHV-1 and 5G10 for CvHV-2. Monoclonal antibodies detected their specific viruses as demonstrated by Keuser and collaborators (2004), however, none of them reacted with the Anlier isolate. One can speculate that since CvHV-1 was also isolated from a red deer, CvHV-1 and the Anlier isolate might cross-react serologically. Therefore, other monoclonal antibodies were assessed to detect specifically the Anlier isolate. One monoclonal antibody, 6C3, identified the Anlier isolate. However, a weak reaction was also observed on CvHV-1 or CvHV-2 infected cells (Figure 2).

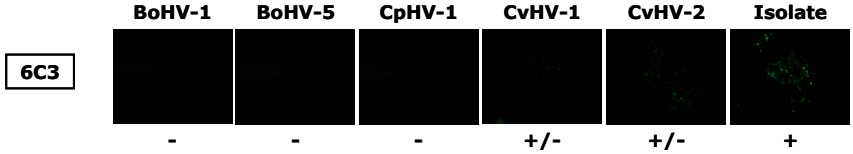

Figure 2 – Indirect immunofluorescence staining of MDBK cells infected with either BoHV-1, BoHV-5, CpHV-1, CvHV-1, CvHV-2, or the Anlier isolate. Cells were incubated until viral plaques were visible and were then treated as described in Materials and Methods. 6C3 is the primary antibody. Stainings were performed three times. Symbols: +, positive signal; -, negative signal; +/-, weak signal.

Taken together, these results demonstrated that the Anlier isolate was antigenically distinct from BoHV-1, BoHV-5 and CpHV-1, but presumably possesses some common epitopes with CvHV-1 and CvHV-2.

Genomic characterisation of a red deer alphaherpesvirus by comparison with ruminant alphaherpesviruses

To avoid any misinterpretation due to antigenic cross reactions, Anlier isolate and ruminant alphaherpesviruses were submitted to restriction enzyme analysis by using two endonucleases, BamHI (Figure 3a) and BstEII (Figure 3b). The patterns were in accordance with the previously published patterns (Engels et al., 1987; Rimstad et al., 1992; Vanderplasschen et al., 1993) and clearly allowed a differentiation between each ruminant alphaherpesvirus as well as between BoHV-1 subtypes 1 and 2 (Figure 3). These data confirmed that the Anlier isolate was genomically distinct from the other related ruminant alphaherpesviruses.

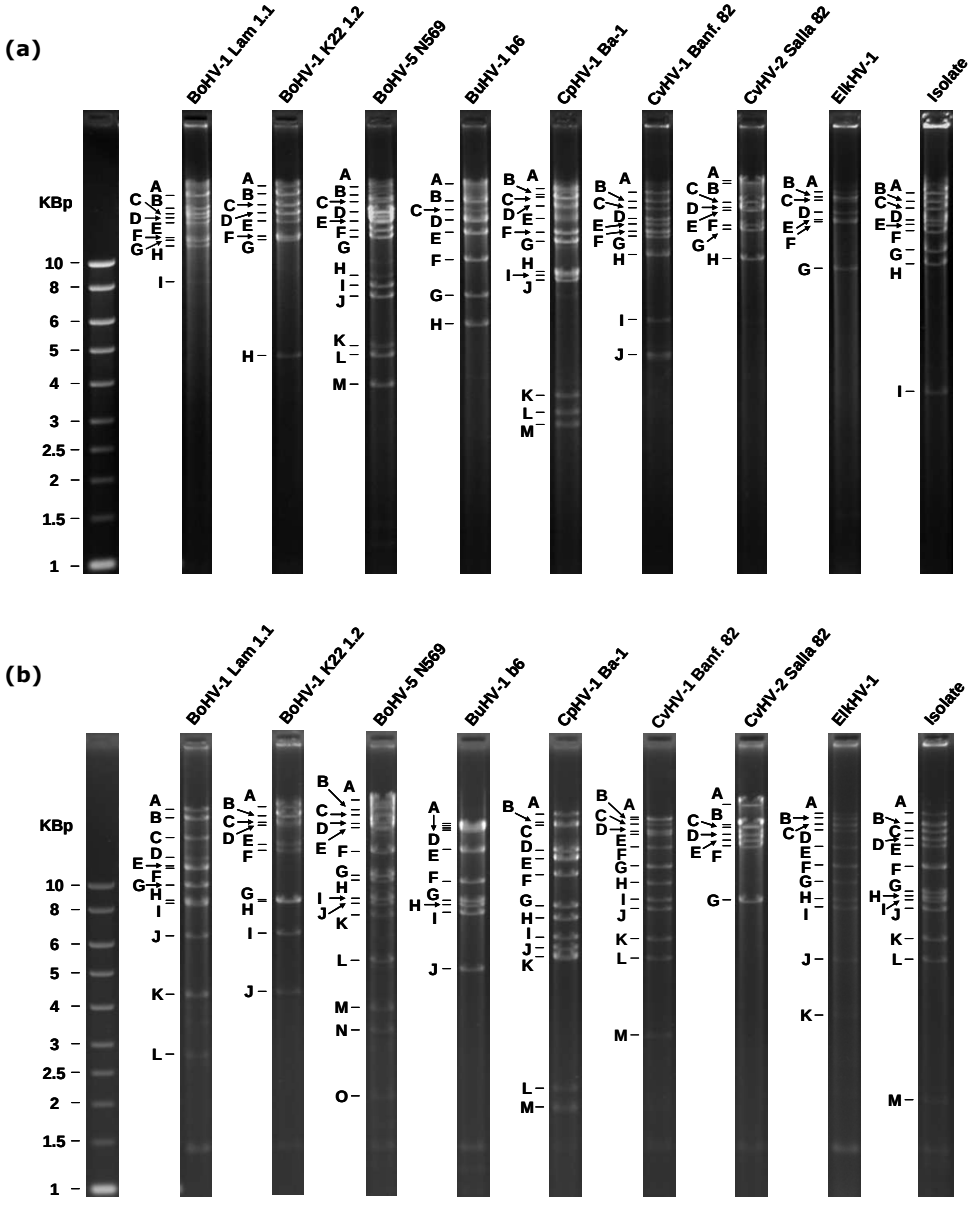

Figure 3 – BamHI (a) and BstEII (b) restriction endonuclease profiles of the Anlier isolate and ruminant alphaherpesviruses.

Phylogenetic relationship between a red deer alphaherpesvirus and ruminant alphaherpesviruses

To further characterise the Anlier isolate, different regions of the genome were sequenced (Figure 4). Partial sequence data from UL27 encoding the glycoprotein B (gB) and US8 encoding the glycoprotein E (gE) were obtained for eight ruminant alphaherpesviruses including the two BoHV-1 subtypes and the Anlier isolate. These sequences represented two different degrees of conservation among ruminant alphaherpesviruses genes.

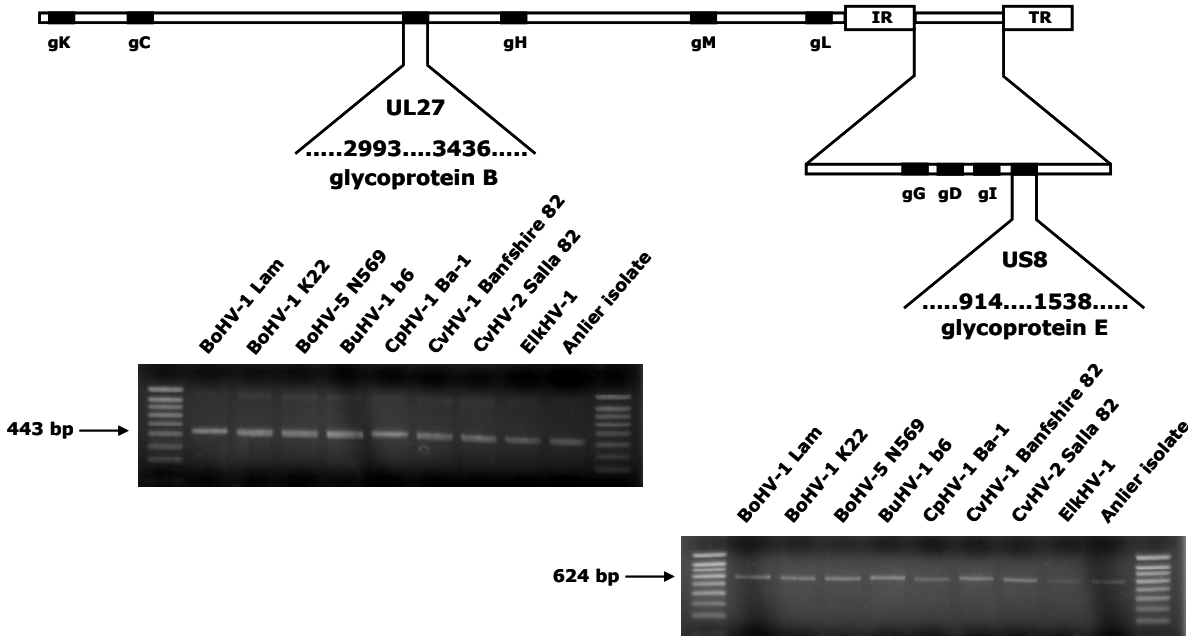

Figure 4 – PCR amplifications of selected parts of UL27 and US8 sequences of the Anlier isolate and ruminant alphaherpesviruses.

The sequence lengths were 444 nucleotides for UL27 and varied between 597 and 609 nucleotides for US8. By analysing multiple nucleotide sequences alignments in the US8 sequence, CvHV-1 and CvHV-2 differed from each other by 96 out of 609 bases, CvHV-1 and ElkHV-1 by 15 out of 609 bases, ElkHV-1 and the Anlier isolate by 22 out of 609 bases, CvHV-2 and the Anlier isolate by 99 out of 609 bases, and CvHV-1 and the Anlier isolate by 16 out of 609 bases (data no shown).

Tables 1 and 2 compare the percentage of nucleotide and amino acid sequence identity between each ruminant alphaherpesvirus.

Phylogenetic analyses were also performed to assess the relationship between the Anlier isolate and other ruminant alphaherpesviruses (Figure 5). BoHV-5 and BuHV-1 clustered together and are the most closely related to BoHV-1 1.1 and BoHV-1 1.2. CpHV-1 is the most diverging ruminant alphaherpesvirus. CvHV-1 is more related to BoHV-1 than ElkHV-1 and CvHV-2 which is the most closely related to CpHV-1. The Anlier isolate clustered with CvHV-1 and the most closely related virus is ElkHV-1. The same topography was obtained for phylogenetic trees based on UL27 (Figure 5a) and US8 nucleotide sequences (Figure 5b).

Taken together, these data revealed high degrees of homology between each ruminant alphaherpesvirus, the highest being observed between CvHV-1 and the Anlier isolate demonstrating the identification of a new CvHV-1 strain.

Table 1 – UL27 nucleotide and amino acid sequence similarities between the Anlier isolate and ruminant alphaherpesviruses.

Nucleotide similarity

Amino acid similarity

UL27	BoHV-1 Lam	BoHV-1 K22	BoHV-5 N569	BuHV-1 b6	CpHV-1 Ba-1	CvHV-1 Banf. 82	CvHV-2 Salla 82	ElkHV-1	CvHV-1 Anlier	SuHV-1 Becker
BoHV-1 Lam		99	95.9	96.6	87.8	94.8	92.3	94.5	94.8	75.4
BoHV-1 K22	100		95.7	96.1	87.8	94.5	92.1	94.5	94.5	75.2
BoHV-5 N569	97.9	97.9		98.6	88.5	96.3	93.6	95.9	96.1	76.3
BuHV-1 b6	97.2	97.2	99.3		88.9	96.8	93.6	96.6	96.8	76.5
CpHV-1 Ba-1	88.5	88.5	88.5	87.8		88	88.7	88.2	87.8	75.6
CvHV-1 Banf. 82	94.5	94.5	95.2	95.9	87.8		95	99.3	99.3	76.5
CvHV-2 Salla 82	91.8	91.8	91.8	91.8	88.5	94.5		95.2	94.3	77.7
ElkHV-1	94.5	94.5	94.5	94.5	88.5	98.6	95.9		98.8	75.9
Isolate	94.5	94.5	95.2	95.2	86.4	98.6	93.2	97.2		75.9
SuHV-1 Becker	70.9	70.9	70.2	70.2	71.6	69.5	70.2	68.9	68.2	

Table 2 – US8 nucleotide and amino acid sequence similarities between the Anlier isolate and ruminant alphaherpesviruses.

Nucleotide similarity

Amino acid similarity

US8	BoHV-1 Lam	BoHV-1 K22	BoHV-5 N569	BuHV-1 b6	CpHV-1 Ba-1	CvHV-1 Banf. 82	CvHV-2 Salla 82	ElkHV-1	CvHV-1 Anlier	SuHV-1 Becker
BoHV-1 Lam		99.4	85.3	86.5	76.2	83.4	76.5	83.7	83.1	50.2
BoHV-1 K22	99		85	86.6	76.3	83.6	76.5	83.9	83.2	50
BoHV-5 N569	76.4	75.9		96.5	74.8	87.3	80.6	88.3	87	53.9
BuHV-1 b6	78.8	79.8	92.7		76.8	89	80.9	90	88.8	54
CpHV-1 Ba-1	59.7	60.1	59.3	61.2		77.8	76.3	78.6	77.8	50.2
CvHV-1 Banf. 82	69.8	70.8	76.4	79.8	60.2		82.6	97.5	97.3	53.2
CvHV-2 Salla 82	64.5	65	67.3	69.8	60.2	70.3		82.7	82.4	52.9
ElkHV-1	69.8	70.8	77.8	81.2	61.2	94.2	70.8		96.2	53.9
Isolate	70.3	71.2	76.4	80.2	57.8	95.1	69.8	92.3		52.4
SuHV-1 Becker	25	25	30.4	30.7	25.8	28.7	25.3	29.1	27.2	

Discussion

The use of a consensus PCR detecting bovine, caprine, red deer and reindeer alphaherpesviruses (Ros and Belák, 1999) has allowed the detection and the identification of the CvHV-1 Anlier strain and its phylogenic position in the BoHV-1 related alphaherpesvirus cluster. No cross-reaction was observed when monoclonal antibodies specific of ruminant alphaherpesviruses were applied to cells infected with the Anlier strain. In contrast, the monoclonal antibody 6C3, described as being specific to CvHV-1 (Keuser et al., 2004), surprisingly detected more specifically the Anlier strain than CvHV-1. The weak reactivity observed with CvHV-2 could result from epitopes common with the Anlier strain. To further characterise the Anlier strain in comparison with ruminant alphaherpesviruses, restriction enzyme analysis were performed. Similarities in the BamHI and BstEII restriction patterns further support that the Anlier isolate is a new strain of CvHV-1.

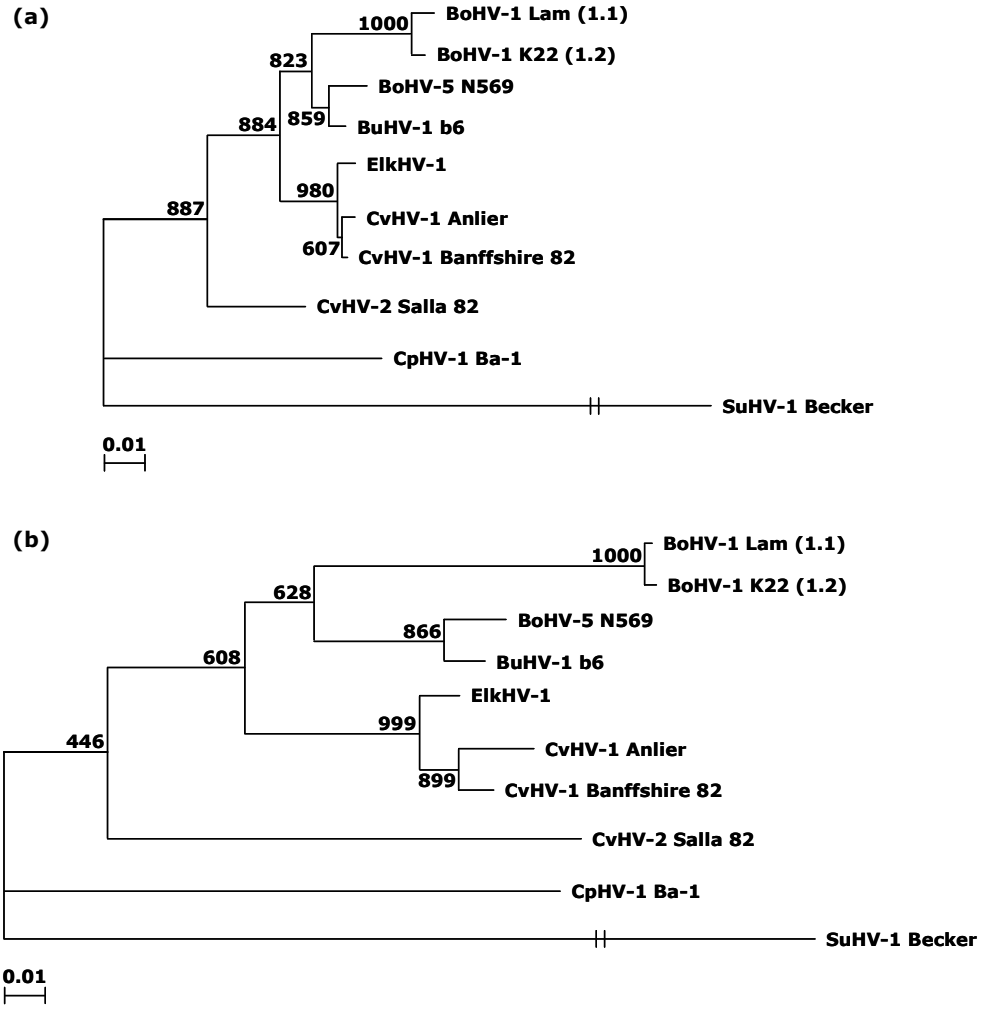

Figure 5 – Phylogenetic relationships between the Anlier isolate and ruminant alphaherpesviruses. Trees were generated by using selected parts of UL27 (a) or US8 (b) nucleotide sequences, and were both rooted by using Suid herpesvirus 1 (SuHV-1) Becker strain gB sequence as an outgroup. The reliability of trees were assesed by resampling and analysing 1000 random data sets (bootstrapping) showed at branches.

When analysing nucleotide and amino acid sequences similarities, the difference in the degree of conservation between UL27 and US8 sequences is demonstrated among BoHV-1 related ruminant alphaherpesviruses, confirming BoHV-5 previously obtained data (Delhon et al., 2003). Such difference in the level of conservation between UL27 and US8 sequences could influence the diagnosis of ruminant alphaherpesvirus infections by both BoHV-1 gB and gE blocking ELISAs. Indeed, experimentally BoHV-5 infected calves were shown to be positive in BoHV-1 gB blocking ELISA and negative in BoHV-1 gE blocking ELISA allowing the discrimination between BoHV-1 and BoHV-5 (Wellenberg et al., 2001). As the highest US8 sequences similarity is observed between BoHV-1 and BoHV-5, it can be hypothesised such methodology serologically discriminate between other heterologous infections, e.g. between BoHV-1 and CpHV-1 infection of goats (Thiry et al., 2006b) or between BoHV-1 and CvHV-1 infection in red deer (Mollema et al., 2005).

Phylogenetic analysis showed that the most closely related viruses to BoHV-1 1.1 and BoHV-1 1.2 are BoHV-5 and BuHV-1 followed by CvHV-1 and ElkHV-1 clustering together and further by CvHV-2 and CpHV-1. These results extend the knowledge on the genetic relatedness of BoHV-1 related ruminant alphaherpesviruses to BuHV-1 and ElkHV-1 (Deregt et al., 2005a; 2005b; Ros and Belák, 1999; De Carlo et al., 2004; Ros and Belák, 2002). Each ruminant alphaherpesvirus

is closely related to each other both for UL27 and US8 sequences similarities confirming the presence of a consistent group of ruminant alphaherpesviruses in the family *Herpesviridae*. Additionally, primers designed to partially amplify US8 bring a new tool in the detection of ruminant alphaherpesvirus infection. Furthermore, they could be used to differentiate wild type BoHV-1 from vaccinal gE-negative BoHV-1 (Schynts et al., 1999).

Genetic analysis of the Anlier strain reveals clear differences with CvHV-1 reference strain (16 out of 609 bases for US8 and 3 out of 444 bases for UL27). However, nothing is known about the potential strain pathogenicity. Moreover, deeper investigations are necessary to assess if this red deer alphaherpesvirus is a new viral subtype. Experimental infection of red deer with CvHV-1 lead to mild clinical signs, such as hyperthermia, nasal ulceration and conjunctivitis, and virus was reisolated from nasal and ocular swabs (Ronsholt et al., 1987; Reid et al., 1986). Natural infection of farmed red deer induced an ocular syndrome associated with various clinical manifestations (Inglis et al., 1983). Here, the Anlier strain was isolated from a nasal swab and the animal did not present any lesions. Moreover, no case of suspicious red deer death was noticed during 2004 and 2005 hunting seasons although a high seroprevalence was observed (Grégoire and Linden, unpublished data). Based on these data, it can be postulated that Anlier strain as several other herpesviruses could induce subclinical respiratory infection. However, it can be also hypothesised that as other herpesviruses too, it may predispose the respiratory tract for secondary bacterial infections or has the capacity by itself to initiate other disease forms (Muylkens et al., 2007). In order to answer these questions, Anlier strain pathogenicity should be investigated by red deer experimental inoculation.

Belgian CvHV-1 emergence might be explained by red deer translocation in Europe. The biogeographic history of European red deer is under human influence (Hartl et al., 2003; Niethammer et al., 1963). During the last one thousand years, there was an extensive trade of the species mainly with the aim to improve the hunted trophy quality (Hartl et al., 2003; Lowe and Gardiner, 1974). The existence of illegal translocation of animals by hunters was recently evidenced in Luxemburg. By using a multilocus genotyping, a red deer group was excluded from the autochthonous population (Frantz et al., 2006) suggesting the presence imported red deer from foreign countries. In the Walloon Region of Belgium, reintroduction of imported animals in wildlife is unclear due to a lack of documentation. An extinction of the species occurred in 1848 and red deer were most likely imported from eastern European countries. In this regard, genetic analysis showed that Scottish CvHV-1 Banffshire 82 strain is different from Belgian Anlier strain. Consequently, viral transmission to a Belgian red deer from a reintroduced Scottish farmed red deer can not be currently supported in absence of molecular epidemiology of the two strains.

In contrast, the hypothesis of an endemic infection occurring in continental Europe has to be considered. A genetic cluster of red deer is distributed from Belgium, across Luxemburg, to Germany (Frantz et al., 2006). Similarly, the presence of genetic units over larger area has also been proven for the Engadin Valley population in eastern Switzerland (Kuehn et al., 2004) and in the Carpathian mountains in Romania (Feulner et al., 2004). It can be hypothesised that animals infected with Anlier strain belong to red deer genetic clusters described in the Czech Republic, Germany, and Hungary and spread the infection. This hypothesis is supported by the high BoHV-1 seroprevalence observed in red deer in these countries (Kokles et al., 1988; Liebermann et al., 1989; Muller et al., 1997; Pospisil et al., 1996). In Scotland, the wild red deer herds have produced the initial stocks for many deer farms (Hamilton, 1994) where the Scottish CvHV-1 Banffshire 82 strain was isolated (Inglis et al., 1983). This strain was probably already established in the wild deer population in Great Britain. As most British populations are non-indigenous (Hartl et al., 2003; Niethammer et al., 1963), the virus could have been introduced a long time ago in the red deer

population in Great Britain from continental Europe and could have diverged from the original virus. The virus could have persisted in small populations because of latency (Ronsholt et al., 1987) and the industrial deer farming facilitated the identification of the virus. However, additional experiments are requested to investigate if the Belgian Anlier strain could be the ancestor of the Scottish Banffshire 82 strain.

Conclusions

According to our knowledge, this is the first report on the isolation of a ruminant alphaherpesvirus from free-ranging red deer. The antigenic, genomic and genetic analysis demonstrated that the detected virus, termed CvHV-1 Anlier strain, is closely related to, but different from the CvHV-1 Banffshire 82 strain. This isolation and our knowledge of red deer population evolution in Europe allow the formulation of an interesting hypothesis on the origin of this virus in red deer knowing CvHV-1 Banffshire 82 strain had been isolated in farmed red deer in Scotland. Furthermore, our investigation brings the first comparative diagnosis of all known ruminant alphaherpesviruses related to BoHV-1 and especially a genetic definition of these viruses.

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References

Aguirre A.A., Hansen D.E., Starkey E.E., McLean R.G., Serologic survey of wild cervids for potential disease agents in selected national parks in the United States, Prev. Vet. Med. 21 (2005) 313–322.

Buonavoglia C., Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Conserva A., Corrente M., Reactivation of caprine herpesvirus 1 in latently infected goats, Comp. Immunol. Microbiol. Infect. Dis. 19 (1996) 275-281.

De Carlo E., Re G.N., Letteriello R., Del Vecchio V., Giordanelli M.P., Magnino S., Fabbi M., Bazzocchi C., Bandi C., Galiero G., Molecular characterisation of a field strain of bubaline herpesvirus isolated from buffaloes (*Bubalus bubalis*) after pharmacological reactivation, Vet. Rec. 154 (2004) 171-174.

Delhon G., Moraes M.P., Lu Z., Afonso C.L., Flores E.F., Weiblen R., Kutish G.F., Rock D.L., Genome of bovine herpesvirus 5, J. Virol. 77 (2003) 10339-10347.

Deregt D., Jordan L.T., van Drunen Littel-van den Hurk S., Masri S.A., Tessaro S.V., Gilbert S.A., Antigenic and molecular characterization of a herpesvirus isolated from a North American elk, Am. J. Vet. Res. 61 (2000) 1614-1618.

Deregt D., Tessaro S.V., Gilbert S.A., Serological evidence of latency in cattle experimentally infected with elk herpesvirus, Vet. Rec. 156 (2005a) 610-611.

Deregt D., Gilbert S.A., Campbell I., Burton K.M., Reid H.W., Hurk S.V., Penniket C., Baxi M.K., Phylogeny and antigenic relationships of three cervid herpesviruses, Virus Res. 114 (2005b) 140-148.

Ek-Kommonen C., Veijalainen P., Rantala M., Neuvonen E., Neutralizing antibodies to bovine herpesvirus 1 in reindeer, Acta Vet. Scand. 23 (1982) 565-569.

Ek-Kommonen C., Pelkonen S., Nettleton P.F., Isolation of a herpesvirus serologically related to bovine herpesvirus 1 from a reindeer (Rangifer tarandus), Acta Vet. Scand. 27 (1986) 299-301.

Engels M., Loepfe E., Wild P., Schraner E., Wyler R., The genome of caprine herpesvirus 1: genome structure and relatedness to bovine herpesvirus 1, J. Gen. Virol. 68 (1987) 2019-2023.

Felsenstein J., PHYLIP--Phylogeny Inference Package, (Version 3.2), Cladistics 5 (1989) 164-166.

Feulner P.G., Bielfeldt W., Zachos F.E., Bradvarovic J., Eckert I., Hartl G.B., Mitochondrial DNA and microsatellite analyses of the genetic status of the presumed subspecies Cervus elaphus montanus (Carpathian red deer), Heredity 93 (2004) 299-306.

Frantz A.C., Pourtois J.T., Heuertz M., Schley L., Flamand M.C., Krier A., Bertouille S., Chaumont F., Burke T., Genetic structure and assignment tests demonstrate illegal translocation of red deer (Cervus elaphus) into a continuous population, Mol. Ecol. 15 (2006) 3191-3203.

French E.L., A specific virus encephalitis in calves: isolation and characterization of the causal agent, Aust. Vet. J. 38 (1962) 216-221.

Hall T.A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucl. Acids Symp. Ser. 41 (1999) 95-98.

Hamilton W.J., The industry, in: Alexander T.L., Buxton D. (Ed.), Management and diseases of deer, The Veterinary Deer Society, London, 1994, pp. 7-13.

Hartl G.B., Zachos F., Nadlinger K., Genetic diversity in European red deer (*Cervus elaphus* L.); anthropogenic influences on natural populations, C. R. Biologies 326 (2003) S37-S42.

Inglis D.M., Bowie J.M., Allan M.J., Nettleton P.F., Ocular disease in red deer calves associated with a herpesvirus infection, Vet. Rec. 113 (1983) 182-183.

Johnston L., Simmons G., McGavin M., A viral meningo-encephalitis in calves, Aust. Vet. J. 38 (1962) 207-215.

Kendrick J.W., Gillespie J.H., Mc Entee K., Infectious pustular vulvovaginitis of cattle, Cornell Vet. 48 (1958) 458-495.

Keuser V., Schynts F., Detry B., Collard A., Robert B., Vanderplasschen A., Pastoret P.-P., Thiry E., Improved antigenic methods for differential diagnosis of bovine, caprine, and cervine alphaherpesviruses related to bovine herpesvirus 1, J. Clin. Microbiol. 42 (2004) 1228-1235.

Kokles R., Dedek J., Loepelmann H., Serologische Untersuchungen auf Infektionen mit dem Virus der infektiösen bovinen Rhinotracheitis/infektiöse Vulvovaginitis und dem Parainfluenza-3-virus bei Rot-, Reh-, Dam- und Muffelwild, Monatsh. Veterinärmed. 43 (1988) 60-63.

Kuehn R., Haller H., Schroeder W., Rottmann O., Genetic roots of the red deer (Cervus elaphus) population in Eastern Switzerland, J. Hered. 95 (2004) 136-143.

Lemaire M., Schynts F., Meyer G., Thiry E., Antibody response to glycoprotein E after bovine herpesvirus type 1 infection in passively immunised, glycoprotein E-negative calves, Vet. Rec. 144 (1999) 172-176.

Liebermann H.D., Tabbaa J., Dedek H., Loepelmann H., Stubbe J., Selbitz H.J., Serologische Untersuchungen auf ausgewählte Virusinfektionen bei Wildwiederkäuern in der DDR, Monatsh. Veterinärmed. 44 (1989) 380-382.

Lillehaug A., Vikoren T., Larsen I.L., Akerstedt J., Tharaldsen J., Handeland K., Antibodies to ruminant alpha-herpesviruses and pestiviruses in Norwegian cervids, J. Wildl. Dis. 39 (2003) 779-786.

Lowe V.P.M., Gardiner A.S., A re-examination of the sub-species of Red deer (Cervus elaphus) with particular reference to the stocks in Britain, J. Zool. Lond. 174 (1974) 185-201.

Lyaku J.R., Nettleton P.F., Mardsen H., A comparison of serological relationships among five ruminant alphaherpesviruses by ELISA, Arch. Virol. 124 (1992) 333-341.

Martin W.B., Castrucci G., Frigeri F., Ferrari M., A serological comparison of some animal herpesviruses, Comp. Immunol. Microbiol. Infect. Dis. 13 (1990) 75-84.

Metzler A.E., Schudel A.A., Engels M., Bovine herpesvirus 1: molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease, Arch. Virol. 87 (1986) 205-217.

Mollema L., Rijsewijk F.A.M., Nodelijk G., de Jong M.C.M., Quantification of the transmission of bovine herpesvirus 1 among red deer (Cervus elaphus) under experimental conditions, Vet. Microbiol. 111 (2005) 25-34.

Motha J., Jenner J., Serological relatedness of cervine herpesvirus-1 and bovine herpesvirus-1 and the prevalence of cervine herpesvirus-1 infection in farmed deer in New Zealand, New Z. Vet. J. 49 (2001) 162-163.

Muller T., Kramer M., Beier D., Untersuchungen zum Vorkommen von Antikorpern gegen ausgewahlte bovine und ovide Viruserkrankungen bei Reh-(Capreolus capreolus), Rot- (Cervus elaphus), Dam-(Dama dama) und Muffelwild (Ovis musimon) im Bundesland Brandenburg, Zeitschrift fur Jagdwissenschaft 43 (1997) 166-175.

Muylkens B., Thiry J., Kirten P., Schynts F., Thiry E., Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis, Vet. Res. 38 (2007) 181-209.

Nettleton P.F., Sinclair J.A., Herring J.A., Inglis D.M., Fletcher T.J., Ross H.M., Bonniwell M.A., Prevalence of herpesvirus infection in British red deer and investigations of further disease outbreaks, Vet. Rec. 118 (1986) 267-270.

Nettleton P.F., Thiry E., Reid H.W., Pastoret P.-P., Herpesvirus infections in cervidae, Rev. Sci. Tech. Off. Int. Epiz. 7 (1988a) 977-988.

Nettleton P.F., Ek-Kommonen C., Tanskanen R., Reid H.W., Sinclair J.A., Herring J.A., Studies in the epidemiology and pathogenesis of alphaherpesviruses from red deer (cervus elaphus) and reindeer (rangifer tarandus), The management and Health of Farmed deer (1988b) 143-147.

Niethammer G., Die Einbürgerung von Säugetieren und Vögeln in Europa. Ergebnisse und Aussichten. Unter Mitarbeit von Niethammer J. und Szijj J., Paul Parey, 1963, Hamburg, Berlin.

Nixon P., Edwards S., White H., Serological comparisons of antigenically related herpesviruses in cattle, red deer and goats, Vet. Res. Commun. 12 (1988) 355-362.

Pospisil Z., Vyvlecka R., Cihal P., Lany P., Zendulkova D., Detection of herpesvirus serum antibodies in red deer (Cervus elaphus) imported into the Czech Republic, Vet. Med. 41 (1996) 279-282.

Reid H.W., Nettleton P.F., Pow I., Sinclair J.A., Experimental infection of red deer (Cervus elaphus) and cattle with a herpesvirus isolated from red deer, Vet. Rec. 119 (1986) 156-158.

Rimstad E., Krona R., Hyllseth B., Comparison of herpesviruses isolated from reindeer, goats, and cattle by restriction endonuclease analysis, Arch. Virol. 123 (1992) 389-397.

Ronsholt L., Christensen L.S., Bitsch V., Latent herpesvirus infection in red deer: characterization of a specific deer herpesvirus including comparison of genomic restriction fragment patterns, Acta. Vet. Scand. 28 (1987) 23-31.

Ros C., Belak S., Studies of genetic relationships between bovine, caprine, cervine, and rangiferine alphaherpesviruses and improved molecular methods for virus detection and identification, J. Clin. Microbiol. 37 (1999) 1247-1253.

Ros C., Belak S., Characterization of the glycoprotein B gene from ruminant alphaherpesviruses, Virus Genes 24 (2002) 99-105.

Saito J.K., Gribble D.H., Berrios P.E., Knight H.D., Mc Kercher D.G., A new herpesvirus isolate from goats: Preliminary report, Am. J. Vet. Res. 35 (1974) 847-848.

Schynts F., Baranowski E., Lemaire M., Thiry E., A specific PCR to differentiate between gE negative vaccine and wildtype bovine herpesvirus type 1 strains, Vet. Microbiol. 66 (1999) 187-195.

St George T.D., Philpott M., Isolation of infectious bovine rhinotracheitis virus from the prepuce of water buffalo bulls in Australia, Aust. Vet. J. 48 (1972) 126.

Thiry E., Vercouter M., Dubuisson J., Barrat J., Sepulchre C., Gerardy C., Meersschaert C., Collin B., Blancou J., Pastoret P.-P., Serological survey of herpesvirus infections in wild ruminants of France and Belgium, J. Wildl. Dis. 24 (1988) 268-273.

Thiry J., Keuser V., Muylkens B., Meurens F., Gogev S., Vanderplasschen A., Thiry E., Ruminant alphaherpesviruses related to bovine herpesvirus 1, Vet. Res. 37 (2006a) 169-190.

Thiry J., Tempesta M., Camero M., Tarsitano E., Bellacicco A.L., Thiry E., Buonavoglia C., A live attenuated glycoprotein E negative bovine herpesvirus 1 vaccine induces a partial cross-protection against caprine herpesvirus 1 infection in goats, Vet. Microbiol. 113 (2006b) 303-308.

Tisdall D.J., Rowe S.M., Isolation and characterisation of cervine herpesvirus-1 from red deer semen, New Z. Vet. J. 64 (2001) 111-114.

Vanderplasschen A., Bublot M., Pastoret P.-P., Thiry E., Restriction maps of the DNA of cervid herpesvirus 1 and cervid herpesvirus 2, two viruses related to bovine herpesvirus 1, Arch. Virol. 128 (1993) 379-388.

Wellenberg G.J., Mars M.H., van Oirschot J.T., Antibodies against bovine herpesvirus (BHV) 5 may be differentiated from antibodies against BHV1 in a BHV1 glycoprotein E blocking ELISA, Vet. Microbiol. 78 (2001) 79-84.

Serological evidence of caprine herpesvirus 1 infection in Mediterranean France

Julien Thiry, Claude Saegerman, Christophe Chartier, Pascale Mercier, Véronique Keuser and Etienne Thiry

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Caprine herpesvirus 1 (CpHV-1) is responsible of a systemic disease in kids and genital diseases inducing abortions in adult goats. In Europe, CpHV-1 is widespread in Mediterranean countries such as Greece, Italy and Spain. As France is geographically close to these countries, a survey was conducted to investigate the presence of CpHV-1 in goats in a Mediterranean department (Corse-du-Sud) and in continental departments (Dordogne and Vendée) of this country. Taking into account the close antigenic and genetic relationships between bovine herpesvirus 1 (BoHV-1) and CpHV-1, the serological detection was performed by using BoHV-1 glycoproteins B (gB) and E (gE) blocking ELISAs. The analysis of 2,548 serum samples in a BoHV-1 gB blocking ELISA revealed that a ruminant alphaherpesvirus infection related to BoHV-1 was widespread in Corse-du-Sud whereas no positive animals was detected in Dordogne and Vendée. Furthermore, the specificity and the sensitivity of the BoHV-1 gB blocking ELISA to detect a BoHV-1 related infection in goats were evaluated. A subsequent analysis by a BoHV-1 gE blocking ELISA demonstrated that 22.6% of gB-positive serum samples were also gE-positive. Cross-seroneutralisation assays afforded the unambiguous identification of antibodies against CpHV-1 in gB-positive goats. The likely presence of CpHV-1 in Corse-du-Sud supported by a high seroprevalence (61.9%) in all investigated flocks extends the number of countries infected with CpHV-1. Moreover, the difference observed between Corse-du-Sud and Dordogne and Vendée suggests that CpHV-1 is more prevalent in Mediterranean countries or regions than in central and northern Europe.

Introduction

Caprine herpesvirus 1 (CpHV-1) causes mainly a systemic infection in neonatal kids. Although most infections in adults are subclinical, CpHV-1 can provoke vulvovaginitis (Grewal and Wells, 1986), balanoposthitis (Tarigan et al., 1987), and abortion (Keuser et al., 2002). Although the economical impact of the infection has not been determined yet at the level of a country, it may be important in infected flocks because of abortions, returns to oestrus, and neonatal mortality (Thiry et al., 2007a). CpHV-1 was first isolated in 1975 in California from newborn kids showing enteritis (Saito et al., 1974). Five years later, it was recovered in the Bregaglia valley of Switzerland from kids affected with a similar disease (Mettler et al., 1979). The distribution of CpHV-1 infection has not been systematically studied in goats but has been worldwide identified (Thiry et al., 2007a) as in Europe (Buonavoglia et al., 1996; Keuser et al., 2004; Koptopoulos et al., 1988; Mettler et al., 1979; Muluneh and Liebermann, 1990), Australia (Grewal and Wells, 1986), Canada (Chenier et al., 2004), New Zealand (Horner et al., 1982) and USA (Saito et al., 1974). In many countries where goats play an economical role, a high prevalence is observed: more than 50% in Greece (Koptopoulos et al., 1988), between 30 and 60% in southern Italy (Guercio et al., 1998; Tempesta et al., 1994; Thiry et al., 2006c) and 21% in Spain (Keuser et al., 2004; Thiry et al., 2006c). The CpHV-1 infection was identified neither in Belgium nor in France (Thiry et al., 2006c). Therefore, in Europe, although the first isolation was performed in Switzerland, CpHV-1 infection seems to be more prevalent in Mediterranean than in Northern European countries.

On the basis of its antigenic and genetic properties, CpHV-1 belongs to a cluster of ruminant alphaherpesviruses closely related to bovine herpesvirus 1 (BoHV-1) (Thiry et al., 2006a). BoHV-1 is responsible of diseases of major economic concern in many parts of the world and especially in Europe. The infection causes a variety of clinical diseases in cattle including infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV) (Muylkens et al., 2007). In order to detect and control IBR, specific molecular and serological diagnostic tests were developed. Currently, the serological diagnosis is based on the use of commercial BoHV-1 glycoprotein B (gB) and glycoprotein E (gE) blocking enzyme-linked immunosorbent assays (ELISA) which are fast, inexpensive and simple methods for screening large numbers of test samples (Kramps et al., 2004). The existence of the consistent group of ruminant alphaherpesviruses closely related to BoHV-1 leads to a theoretical problem to distinguish serologically between these viruses (Thiry et al., 2006a). When they are analysed by ELISA or seroneutralisation, BoHV-1 and related ruminant alphaherpesviruses cross-react due to antigenic cross-relationships (Lyaku et al., 1992; Martin et al., 1990; Nixon et al., 1988). Thus, the use of a BoHV-1 gB blocking ELISA allows the identification of such a ruminant alphaherpesvirus infection but is not able to make a differentiation.

The current study aims to investigate the epidemiological situation in France, a country which includes both Mediterranean and northern areas, in order to study if CpHV-1 prevalence is different in Mediterranean than in Northern Europe. Furthermore, the results of this survey were improved by the validation of the serological assay in order to detect a BoHV-1 related infection and especially a CpHV-1 infection in goats. The combination with a BoHV-1 gE blocking ELISA could lead to distinguish between CpHV-1 and BoHV-1 as demonstrated for bovine herpesvirus 5 (BoHV-5) (Wellenberg et al., 2001). Cattle experimentally infected with BoHV-5 are detected seropositive by a BoHV-1 gB blocking ELISA and seronegative by a BoHV-1 gE blocking ELISA (Wellenberg et al., 2001). BoHV-5 being the alphaherpesvirus the most closely related to BoHV-1 (Ros et Belák, 1999 ; 2002) and showing antigenic differences for gE (Kaashoek et al., 1995 ; Wellenberg et al., 2001), it could be hypothesised that CpHV-1 gE has more pronounced antigenic differences and

may allow the differentiation from BoHV-1 by using BoHV-1 gB and gE blocking ELISA. As BoHV-1 gB blocking ELISA is used as a screening tool to detect antibodies against a related BoHV-1 infection, the validation of this test in goats was evaluated under field and experimental conditions.

Materials and methods

Flocks, animals and serums samples

Field serum samples were collected in several French flocks in Corse-du-Sud (department 2A), in Dordogne (department 24) and in Vendée (department 85). Goat flock size ranged from 7 to 304 adult animals over than 6 months of age. All flocks were composed of dairy breeds and produced milk for dairies or for on-farm cheese making. The sampling size corresponded to the whole adult flock size. The experimental serum samples came from four Belgian goats intranasally inoculated with the CpHV-1 E/CH strain at a dose of 10^6 plaque forming units (PFU) per nostril (Keuser et al., 2002) and two Danish calves intranasally inoculated with the BoHV-1 Iowa strain at a dose of $5x10^7$ PFU per nostril (Muylkens et al., 2006). Prior to inoculation, absence of antibodies against CpHV-1 in goats was confirmed by serum neutralisation assay (Keuser et al., 2002) and absence of antibodies against BoHV-1 in calves was confirmed by BoHV-1 gB blocking ELISA (Muylkens et al., 2006).

Enzyme linked immunosorbent assay (ELISA)

To detect antibodies in serum samples, a BoHV-1 gB blocking ELISA (SERELISA^{TM} IBR/IPV gB Ab Mono Blocking, Synbiotics Europe, Lyon, France) and a BoHV-1 gE blocking ELISA (Herdchek Anti-IBR gE, Idexx, Germany) were used following the instructions of the manufacturers. Serum samples were analysed in duplicate and the average was used to calculate the competition percentage. The cut off was fixed to 45% of competition. Serum samples identified as doubtful were analysed in a second round, when identified twice doubtful, serum samples were considered as positive.

Serum neutralisation assay

Serum samples were heated at 56°C for 30 min. Serial twofold dilutions of each serum sample were incubated at 37°C for 2 h. in the presence of 75 PFU of CpHV-1 Ba-1 strain (Buonavoglia et al., 1996) or 12 h. in the presence of 75 PFU of BoHV-1 Iowa strain (Miller and van der Maaten, 1984). Each virus-serum dilution was then dispensed into 96 wells microplates containing Madin Darby Bovine Kidney (MDBK, ATCC CCL-22) cells grown as previously described (Lemaire et al., 1999). Readings were made after incubation for 3 days at 37°C. The titres were expressed as the reciproqual of the highest dilution of serum sample inhibiting cytopathic effect.

Statistical analysis

Statistical analyses were carried out in STATA/SE 8 (StataCorp, 2003).

Results

Serums from flocks sampled in Corse-du-Sud (n=1,430), Dordogne (n=997) and Vendée (n=121) departments in France were tested in a BoHV-1 gB blocking ELISA. The apparent

prevalences are given in Table 1. In Corse-du-Sud, all flocks tested were seropositive. The within-herd prevalence ranged from 38.7% to 73.7% with an individual prevalence of 57.9% (95% confident interval (CI): 55.3%-60.5%). In Dordogne and Vendée, serum samples were consistently negative.

In regards to these data, the specificity and the sensitivity of the BoHV-1 gB blocking ELISA to detect a BoHV-1 related infection in goats were evaluated with a cut-off of 45% of competition. On the basis of 1,118 serum samples from Dordogne and Vendée, regions presumably free of BoHV-1 related virus infection in goats, the measured specificity was 100% (95% CI: 99.67%-100%). The sensitivity of the ELISA was assessed by testing serum samples obtained from serial bleedings of four goats experimentally infected with CpHV-1 (n=31). All serum samples but two taken at day 7 post infection were detected positive. The measured sensitivity was 93.5% (95% CI: 78.6%-99.2%).

The true prevalences were calculated for each region (Table 1) according the following equation: True Prevalence = (Apparent Prevalence + Specificity - 1) / (Sensitivity + Specificity - 1) (Toma et al., 2001). In Corse-du-Sud, the within-herd prevalence ranged from 41.36% to 78.86% with an individual prevalence of 61.93% (95% CI: 59.13%-64.68%). Dordogne and Vendée obviously kept their free status.

Table 1 - Serological prevalence of CpHV-1 infection in Corse-du-Sud, Dordogne and Vendée.

Department	Flocks	Animals [a]	Apparent prevalence [b]	True prevalence [b, c]
Corse-du-Sud	A	126	72.22 (63.54-79.83)	77.22 (67.93-85.37)
	B	143	49.65 (41.19-58.13)	53.06 (44-62.13)
	C	114	54.39 (44.79-63.74)	58.13 (47.85-68.14)
	D	118	73.73 (64.83-81.40)	78.84 (69.31-87.05)
	E	75	54.67 (42.75-66.21)	58.43 (45.67-70.78)
	F	144	55.56 (47.05-63.83)	59.38 (50.27-68.24)
	G	91	41.76 (31.50-52.57)	44.61 (33.63-56.18)
	H	195	66.67 (59.58-73.24)	71.28 (63.69-78.31)
	I	181	38.67 (31.54-46.18)	41.30 (33.67-49.34)
	J	102	63.72 (53.61-73.02)	68.12 (57.30-78.08)
	K	141	65.96 (57.51-73.72)	70.52 (61.47-78.82)
	Total	1430	57.90 (55.29-60.48)	61.89 (59.09-64.65)
Dordogne	L	22	0 (0-12.7)	0 (0-13.50)
	M	304	0 (0-0.99)	0 (0-0.96)
	N	115	0 (0-2.57)	0 (0-2.65)
	O	288	0 (0-0.99)	0 (0-0.96)
	P	15	0 (0-18.1)	0 (0-19.28)
	Q	56	0 (0-5.2)	0 (0-5.47)
	R	7	0 (0-35)	0 (0-37.37)
	S	82	0 (0-3.6)	0 (0-3.76)
	T	51	0 (0-5.7)	0 (0-6.01)
	U	30	0 (0-9.5)	0 (0-10.07)
	V	27	0 (0-10.5)	0 (0-11.14)
	Total	997	0 (0-0.30)	0 (0-0.22)
Vendée	W	121	0 (0-2.44)	0 (0-2.52)

[a] Number of goats analysed in BoHV-1 gB blocking ELISA.
[b] Seroprevalence expressed in percent with 95% confidence interval in brackets (exact binomial).
[c] The true prevalence was measured according to a specificity of 100% and a sensitivity of 93.5%.

The distribution of the data obtained from the investigated goat population clearly illustrates that a BoHV-1 gB blocking ELISA significantly detects a BoHV-1 related infection in goats (Figure 1). The goat population in Corse-du-Sud presents a bimodal distribution (Figure 1a) contrarily to the non-infected population in Dordogne and Vendée (Figure 1b) and the experimental infected population (Figure 1c).

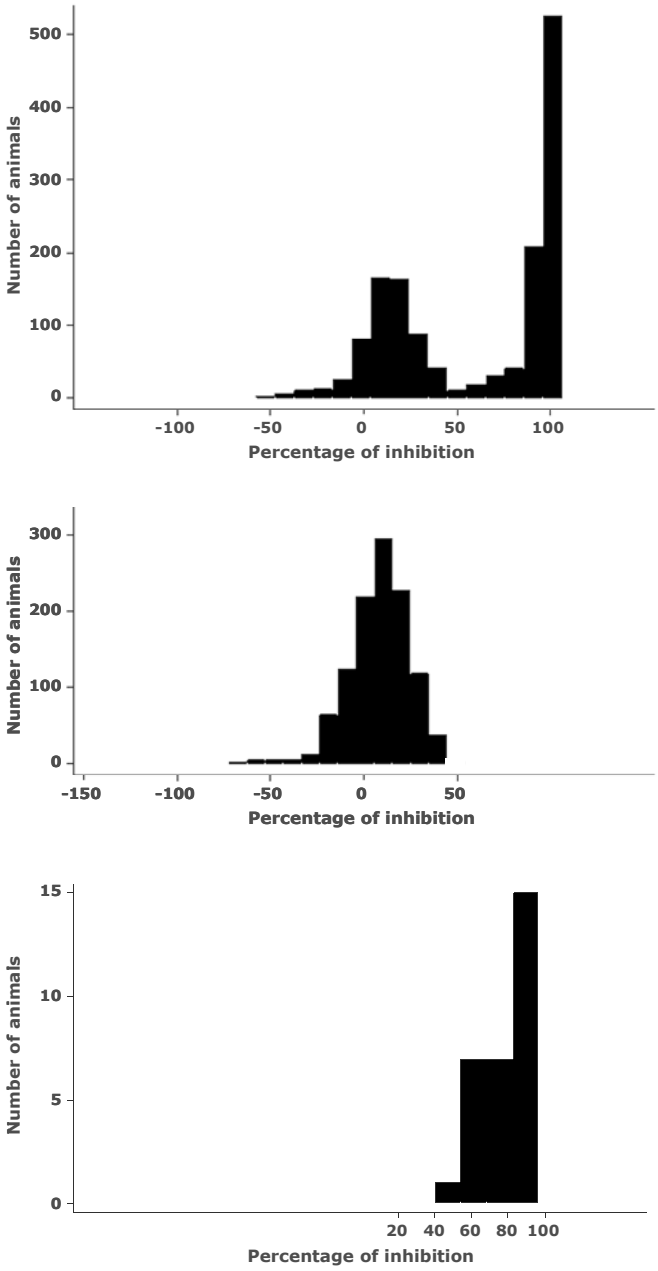

Figure 1 – Distribution of goat population in Corse-du-Sud (a), Dordogne and Vendée (b), and in experimental conditions (c), in regards to the percentage of inhibition from a BoHV-1 gB blocking ELISA. The experimental conditions are explained in the section materials and methods.

A BoHV-1 gE blocking ELISA was applied on serum samples detected positive in the BoHV-1 gB blocking ELISA (gB-positive) to fully distinguish BoHV-1 from a related infection. The analysis revealed that 22.6% of gB-positive serum samples in Corse-du-Sud were positive in the BoHV-1 gE blocking ELISA (gE-positive) (Table 2).

Table 2 - Serological analysis in BoHV-1 gE blocking ELISA of serum samples detected positive in BoHV-1 gB blocking ELISA.

Flocks	Animals [a]	gE-Positive	gE-Doubtful
A	91	15	4
B	71	11	9
C	62	14	7
D	87	18	6
E	41	7	4
F	80	8	8
G	38	7	0
H	130	10	10
I	70	11	9
J	65	4	2
K	93	14	9
Total	828	119	68

[a] Number of goats detected positive in BoHV-1 gB blocking ELISA.

In this context, different categories of serum samples were tested by a serum neutralisation assay (Table 3). Cross-seroneutralisation assays showed that serums from CpHV-1 experimentally infected goats neutralise CpHV-1 to a much greater extent than BoHV-1. Such one way cross-seroneutralisation was observed with gB-positive serum samples and affords evidences that the infection serologically detected in goats by the BoHV-1 gB blocking ELISA is a CpHV-1 infection (Table 3).

Discussion

The serological diagnosis of ruminant alphaherpesviruses related to BoHV-1 is made difficult by their antigenic cross-relationships. The existence of cross-reactions in serological assays as seroneutralisation and the ability of these viruses to cross, at least in some instances, the species barrier raise theoretical issues for differential diagnosis (Thiry et al., 2006a). The currently available serological tests are almost unable to discriminate related ruminant alphaherpesviruses. The current study reports the serological evidence of CpHV-1 infection occurring in Corse-du-Sud, a Mediterranean French department situated in Corsica, by applying a BoHV-1 gB blocking ELISA which has been evaluated in experimental conditions for sensitivity and under field conditions for specifity to detect CpHV-1 in goat species. Cross-seroneutralisation assays confirmed that antibodies detected were directed against CpHV-1 and not against BoHV-1.

The analysis of goat serum samples in a BoHV-1 gB blocking ELISA revealed that BoHV-1 related infection did not occur in Dordogne and Vendée, or only occurred at a low prevalence, not detected by our survey. In contrast, a high seroprevalence was observed in Corse-du-Sud. The gB-positive serum samples most likely come from animals infected with CpHV-1. Indeed, several studies showed that gB is relatively well conserved within related ruminant alphaherpesviruses (Ros and Belák, 2002; Thiry et al., 2007b). Thus, BoHV-1 gB may include epitopes which most of them are shared with CpHV-1; antibodies directed against CpHV-1 may cross react in an ELISA using BoHV-1 gB as antigen as demonstrated for BoHV-5 (Wellenberg et al., 2001). The analysis of serum samples from CpHV-1 experimentally infected goats in a BoHV-1 gB blocking ELISA demonstrated a good sensitivity. Furthermore, the serum neutralisation assay demonstrated that CpHV-1 was indeed neutralised with both field and experimental goat serum samples.

Table 3 - Cross serum neutralisation assay of different categories of serum samples against BoHV-1 and CpHV-1.

Categories of serum samples	Neutralising antibody titre against	
	CpHV-1	BoHV-1
Goats experimentally infected with CpHV-1	1:32	1:8
	1:64	1:8
Calves experimentally infected with BoHV-1	<1:4	1:32
	<1:4	1:32
gB-negative/gE-negative [a]	<1:4	<1:4
	<1:4	<1:4
gB-positive/gE-negative [a]	1:8	<1:4
	1:16	<1:4
	1:16	<1:4
	1:32	1:4
	1:32	1:8
	1:64	1:4
	1:64	1:8
	1:64	1:16
gB-positive/gE-doubtful [a]	1:4	<1:4
	1:8	<1:4
	1:8	<1:4
	1:16	<1:4
	1:16	1:4
	1:16	1:4
	1:32	<1:4
	1:64	1:16
	1:64	1:32
	1:128	1:16
gB-positive/gE-positive [a]	1:8	<1:4
	1:8	<1:4
	1:16	<1:4
	1:16	1:4
	1:32	<1:4
	1:64	1:4
	1:64	1:8
	1:64	1:8
	1:64	1:16
	1:64	1:16

[a] Serum samples detected negative, doubtful or positive in BoHV-1 gB and gE blocking ELISA.

The use of a BoHV-1 gE blocking ELISA could lead to the discrimination between BoHV-1 and other ruminant alphaherpesvirus infections as demonstrated for BoHV-5 (Wellenberg et al., 2001). BoHV-5 is one of the ruminant alphaherpesviruses the most related to BoHV-1 whereas CpHV-1 seems to be the most distantly related to BoHV-1 (Ros and Belák, 1999; 2002). Recent partial sequencing of US8 gene encoding gE confirmed the genetic divergence within the cluster of BoHV-1 related ruminant alphaherpesviruses (Thiry et al., 2007b). At the same level, BoHV-5 shows differences in the gE antigenicity (Kaashoek et al., 1995; Wellenberg et al., 2001). Therefore, serum samples from goats seropositive to BoHV-1 gB were expected to be negative in a BoHV-1 gE blocking ELISA. Surprisingly, 22.6% of gB-positive serum samples were also positive in BoHV-1 gE blocking ELISA. A first explanation would be that gE-positive goats could have been infected with BoHV-1. Nevertheless, natural infections of goats with BoHV-1 have been only seldomly reported (Tolari et al., 1990 ; Whetstone et Evermann, 1988), whereas goats have been

successfully infected experimentally with BoHV-1 inducing a serologic response and establishment of a latent infection (Six et al., 2001). In Corsica, the direct contact between cattle and goats is incidental because flocks and herds are not mixed although sharing the same rangeland pastures in mountains and plains. Therefore, the transmission of BoHV-1 from cattle to goats would be only exceptional, such cross-infection being very rare, and a BoHV-1 infection in goats is not likely to occur. The very high seroprevalence observed in the current study strongly supports a large circulation of CpHV-1. Furthermore, the differentiation between BoHV-1 and CpHV-1 can be made taking profit of a one way cross-neutralisation (Engels et al., 1983). In the current study, serum samples neutralised CpHV-1 to a much greater extent than BoHV-1 and afforded the ultimate identification of a CpHV-1 infection. On the other hand, goat population in Corsica is almost exclusively composed by the local breed underlining the quasi-absence of introduction of animals from the continental France or from Italy for ages (Hugot and Bouche, 1999). The only and limited importations are from southern continental departments as Var or Bouches-du-Rhône in particular with Rove breed animals (Gauthier, personal communication).

As a BoHV-1 infection is not a likely explanation, the detection of gE-positive and gE-negative results could be explained by large differences in the antigenicity of gE epitopes as described for Pseudorabies virus (Jacobs and Kimman, 1994). Furthermore, BoHV-1 strains that do not express a particular gE-epitope in cell culture can induce antibodies that are detected in a BoHV-1 gE blocking ELISA (van Oirschot et al., 1999). In contrast, the gE-positivity observed could reflect the level of immunisation induced after a CpHV-1 infection. As demonstrated for a BoHV-1 infection, a primary infection leads to a high production of antibody (Muylkens et al., 2007). Therefore, when a serum is found highly positive by both BoHV-1 gB and gE blocking ELISA, goats could be highly immunised after a recent CpHV-1 infection or reactivation. Contrarily, a lower antibody response could be the result of a previous infection leading to latent persistence. Indeed, latency is closely associated with long-term maintenance of BoHV-1 neutralising antibodies during two to three years after infection (Kaashoek et al., 1996). CpHV-1 could follow the same pattern of evolution and latently infected goats could have lower antibody levels associated with gB-positive and gE-negative results.

BoHV-1 marker vaccines made of a mutant with a deletion in one of the non-essential genes have been developed to be used in eradication campaigns of IBR initiated in several European countries. The commonest used vaccines are either attenuated or killed viruses carrying a deletion in the gene encoding the glycoprotein E (Bosch et al., 1996; Kaashoek et al., 1994). The control of the infection relies on the use of BoHV-1 gB and gE blocking ELISA in order to differentiate infected and gE-negative vaccinated animals (European Food Safety Authority, 2005). In the context of the IBR certification in France, some cattle have been identified seropositive to BoHV-1 from herds qualified as free of IBR. An attempted explanation would be the cross-infection of cattle with a related ruminant alphaherpesvirus as for example CpHV-1 (Thiry et al., 2006b). The obtained results do not support this hypothesis. Indeed, CpHV-1 infection is absent in continental France or the seroprevalence is at least very low. Nevertheless, the current sampling is restricted to Dordogne and Vendée, and therefore cannot rule out the presence of CpHV-1 infection in other French regions especially in the Mediterranean part of the country as CpHV-1 infection has been identified in Corsica in the current study.

As demonstrated for BoHV-1 (Kaashoek et al., 1996), CpHV-1-specific humoral response is an indicator of a CpHV-1 infection that has taken place. In addition, specific antibody titres could persist in goats for many years, detection of latently infected animals and flocks by serological tests is therefore reliable and of great diagnostic relevance. Moreover, a gB-positive/gE-negative result could not be attributed to a BoHV-5 cross-infection because BoHV-5 infection has never been

described in Europe so far. In regards to the specificity and the sensitivity measured, a BoHV-1 gB blocking ELISA is a useful tool to detect CpHV-1 infection in a flock. A negative result in BoHV-1 gE blocking ELISA could differentiate BoHV-1 from CpHV-1 infection in goats. In the case of a positive result, a cross-seroneutralisation assay will identify the infection. A continuous monitoring of the epidemiology of ruminant infection with CpHV-1 is essential in European countries involved in IBR control and eradication in order to identify potential BoHV-1 related virus reservoirs which could be implicated in IBR misdiagnosis.

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References

Bosch J.C., Kaashoek M.J., Kroese A.H., van Oirschot J.T., An attenuated bovine herpesvirus 1 marker vaccine induces a better protection than two inactivated marker vaccines, Vet. Microbiol. 52 (1996) 223-234.

Buonavoglia C., Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Conserva A., Corrente M., Reactivation of caprine herpesvirus 1 in latently infected goats, Comp. Immunol. Microbiol. Infect. Dis. 19 (1996) 275-281.

Chenier S., Montpetit C., Helie P., Caprine herpesvirus-1 abortion storm in a goat herd in Quebec, Can. Vet. J. 45 (2004) 241-243.

Engels M., Gelderblom H., Darai G., Ludwig H., Goat herpesviruses: biological and physicochemical properties, J. Gen. Virol. 64 (1983) 2237-2247.

European Food Safety Authority, Definition of a BoHV-1-free animal and a BoHV-1-free holding, and the procedures to verify and maintain this status, EFSA Journal 311 (2005) 1-65.

Grewal A.S., Wells R., Vulvovaginitis of goats due to a herpesvirus, Aust. Vet. J. 63 (1986) 79-82.

Guercio A., Greco G., Lanizzoto G., Di Marco V., Todaro M., Valutazione della diffusione di anticorpi anti Herpes Virus della capra in allevamenti caprini della Sicilia, Atti. SIPAOC 12 (1998) 138-142.

Horner G.W., Hunter R., Day A.M., An outbreak of vulvovaginitis in goats caused by a caprine herpesvirus, N. Z. Vet. J. 30 (1982) 152.

Hugot S., Bouche R., Regard sur les pratiques de l'élevage extensif de la chèvre corse : préalable à la mise en place d'un schéma de sélection, Options Méditerranéennes 38 (1999) 137-143.

Jacobs L., Kimman T.G., Epitope-specific antibody response against glycoprotein E of pseudorabies virus, Clin. Diagn. Lab. Immunol. 1 (1994) 500-505.

Kaashoek M.J., Moerman A., Madic J., Rijsewijk F.A., Quak J., Gielkens A.L., van Oirschot J.T., A conventionally attenuated glycoprotein E-negative strain of bovine herpesvirus type 1 is an efficacious and safe vaccine, Vaccine 12 (1994) 439-444.

Kaashoek M.J., Moerman A., Madic J., Weerdmeester K., Maris-Veldhuis M., Rijsewijk F.A., van Oirschot J.T., An inactivated vaccine based on a glycoprotein E-negative strain of bovine herpesvirus 1 induces protective immunity and allows serological differentiation, Vaccine 13 (1995) 342-346.

Kaashoek M.J., Rijsewijk F.A., Oirschot J.T., Persistence of antibodies against bovine herpesvirus 1 and virus reactivation two to three years after infection, Vet. Microbiol. 53 (1996) 103-110.

Keuser V., Gogev S., Schynts F., Thiry E., Demonstration of generalized infection with caprine herpesvirus 1 diagnosed in an aborted caprine fetus by PCR, Vet. Res. Commun. 26 (2002) 221-226.

Keuser V., Espejo-Serrano J., Schynts F., Georgin J.P., Thiry E., Isolation of caprine herpesvirus type 1 in Spain, Vet. Rec. 154 (2004) 395-399.

Koptopoulos G., Papanastasopoulou M., Papadopoulos O., Ludwig H., The epizootiology of caprine herpesvirus (BHV-6) infections in goat populations in Greece, Comp. Immunol. Microbiol. Infect. Dis. 11 (1988) 199-205.

Kramps J.A., Banks M., Beer M., Kerkhofs P., Perrin M., Wellenberg G.J., Oirschot J.T., Evaluation of tests for antibodies against bovine herpesvirus 1 performed in national reference laboratories in Europe, Vet. Microbiol. 102 (2004) 169-181.

Lemaire M., Schynts F., Meyer G., Thiry E., Antibody response to glycoprotein E after bovine herpesvirus type 1 infection in passively immunised, glycoprotein E-negative calves, Vet. Rec. 144 (1999) 172-176.

Lyaku J.R., Nettleton P.F., Mardsen H., A comparison of serological relationships among five ruminant alphaherpesviruses by ELISA, Arch. Virol. 124 (1992) 333-341.

Martin W.B., Castrucci G., Frigeri F., Ferrari M., A serological comparison of some animal herpesviruses, Comp. Immunol. Microbiol. Infect. Dis. 13 (1990) 75-84.

Mettler F., Engels M., Wild P., Bivetti A., Herpesvirus-infektion bei Zieklein in der Schweiz, Schweiz. Arch. Tierheilkd. 121 (1979) 655-662.

Miller J.M., Van der Maaten M.J., Reproductive tract lesions in heifers after intrauterine inoculation with infectious bovine rhinotracheitis virus, Am. J. Vet. Res. 45 (1984) 790-794.

Muluneh A., Liebermann H., Occurrence of caprine herpesvirus (BHV-6) infection in goat population of the GDR, Arch. Exp. Veterinarmed. 44 (1990) 254-257.

Muylkens B., Meurens F., Schynts F., Farnir F., Pourchet A., Bardiau M., Gogev S., Thiry J., Cuisenaire A., Vanderplasschen A., Thiry E. Intraspecific bovine herpesvirus 1 recombinants carrying glycoprotein E deletion as a vaccine marker are virulent in cattle, J. Gen. Virol. 87 (2006) 2149-2154.

Muylkens B., Thiry J., Kirten P., Schynts F., Thiry E. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis, Vet. Res. 38 (2007) 181-209.

Nixon P., Edwards S., White H., Serological comparisons of antigenically related herpesviruses in cattle, red deer and goats, Vet. Res. Commun. 12 (1988) 355-362.

Ros C., Belák S., Studies of genetic relationships between bovine, caprine, cervine, and rangiferine alphaherpesviruses and improved molecular methods for virus detection and identification, J. Clin. Microbiol. 37 (1999) 1247-1253.

Ros C., Belák S., Characterization of the glycoprotein B gene from ruminant alphaherpesviruses, Virus Genes 24 (2002) 99-105.

Saito J.K., Gribble D.H., Berrios P.E., Knight H.D., Mc Kercher D.G., A new herpesvirus isolate from goats: Preliminary report, Am. J. Vet. Res. 35 (1974) 847-848.

Six A., Banks M., Engels M., Bascunana C.R., Ackermann M., Latency and reactivation of bovine herpesvirus 1 (BHV-1) in goats and of caprine herpesvirus 1 (CapHV-1) in calves, Arch. Virol. 146 (2001) 1325-1335.

StataCorp, Stata Statistical Software: Release 8, Stata Corporation, College Station, Texas, 2003.

Tarigan S., Webb R.F., Kirkl D., Caprine herpesvirus from balanoposthitis, Aust. Vet. J. 64 (1987) 321.

Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Presenza di anticorpi per caprine herpesvirus 1 (CapHV.1) in allevamenti caprini dell'Italia meridionale, Atti. SIPAOC 11 (1994) 121-122.

Thiry J., Keuser V., Muylkens B., Meurens F., Gogev S., Vanderplasschen A., Thiry E., Ruminant alphaherpesviruses related to bovine herpesvirus 1, Vet. Res. 37 (2006a) 169-190.

Thiry J., Tempesta M., Camero M., Tarsitano E., Bellacicco A.L., Thiry E., Buonavoglia C., A live attenuated glycoprotein E negative bovine herpesvirus 1 vaccine induces a partial cross-protection against caprine herpesvirus 1 infection in goats, Vet. Microbiol. 113 (2006b) 303-308.

Thiry J., Keuser V., Schynts F., Chartier C., Tempesta M., Espejo-Serrano J., Saegerman C., Thiry E. Evaluation de la prévalence sérologique de l'infection à herpèsvirus caprin 1 dans le sud-ouest de l'Europe, Epidémiol. Et Santé Anim. 49 (2006c) 55-58.

Thiry J., Engels M., Tempesta M., Thiry E. Caprine herpesvirus 1 infection, in: Lefèvre P.C., Blancou J., Chermette R. (Eds), Infectious and Parasitic Diseases of Livestock, Lavoisier Editions, Provigny, France, 2007a, in press.

Thiry J., Widén F., Grégoire F., Linden A., Belák S., Thiry E., Isolation and characterisation of a ruminant alphaherpesvirus closely related to bovine herpesvirus 1 in a free-ranging red deer, BMC Vet. Res. (2007b) 3:26.

Tolari F., White H., Nixon P., Isolation and reactivation of bovid herpesvirus 1 in goats, Microbiologica 13 (1990) 67-71.

Toma B., Dufour B., Sanaa M., Bénet J.J., Shaw A., Moutou F., Louza A., Epidémiologie appliquée à la lutte collective contre les maladies transmissibles majeures, Association pour l'étude de l'épidémiologie des maladies animales, Maisons-Alfort, 2001, pp. 49-78.

van Oirschot J.T., Kaashoek M.J., Maris-Veldhuis M.A., Rijsewijk F.A., Strains of bovine herpesvirus 1 that do not express an epitope on glycoprotein E in cell culture still induce antibodies that can be detected in a gE-blocking ELISA, Vet. Microbiol. 65 (1999) 103-113.

Wellenberg G.J., Mars M.H., van Oirschot J.T., Antibodies against bovine herpesvirus (BHV) 5 may be differentiated from antibodies against BHV1 in a BHV1 glycoprotein E blocking ELISA, Vet. Microbiol. 78 (2001) 79-84.

Whetstone C.A., Evermann J.F., Characterization of bovine herpesviruses isolated from six sheep and four goats by restriction endonuclease analysis and radioimmunoprecipitation, Am. J. Vet. Res. 49 (1988) 781-785.

A live attenuated glycoprotein E negative bovine herpesvirus 1 vaccine induces a partial cross-protection against caprine herpesvirus 1 infection in goats

Julien Thiry, Maria Tempesta, Michele Camero, Elvira Tarsitano, Anna Lucia Bellacicco, Etienne Thiry and Canio Buonavoglia

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Taking into account the close antigenic relationship between bovine herpesvirus 1 (BoHV-1) and caprine herpesvirus 1 (CpHV-1), a live attenuated glycoprotein E (gE) negative BoHV-1 vaccine was assessed in goats with the aim to protect against CpHV-1 infection. Vaccine safety was evaluated by intranasal inoculation of two groups of goats with either a gE-negative BoHV-1 vaccine or a virulent BoHV-1. The length of viral excretion and the peak viral titre were reduced with the gE-negative vaccine. To assess the efficacy, two goats were inoculated intranasally twice two weeks apart with a gE-negative BoHV-1 vaccine. Four weeks later, immunised and control goats were challenged with CpHV-1. A two $\log_{10}$ reduction in the peak viral titre was observed and the challenge virus excretion lasted two days more in immunised than in control goats. These data indicate the safety and the partial efficacy of a live attenuated gE-negative BoHV-1 vaccine intranasally administrated in goats.

Introduction

Among the subfamily *Alphaherpesvirinae*, bovine herpesvirus 1 (BoHV-1) is a major pathogen of cattle responsible of infectious bovine rhinotracheitis (IBR). This disease is of major economic concern in Europe and this situation led to the development of BoHV-1 marker vaccines based on the deletion in the glycoprotein E (gE) gene. Such gE-negative vaccines either attenuated or inactivated have proven their safety and efficacy in the target bovine species (van Oirschot et al., 1996). They can reduce severity of disease, virus shedding, and virus circulation in a population (van Oirschot et al., 1996).

Several ruminant alphaherpesviruses, and among other caprine herpesvirus 1 (CpHV-1) are antigenically and genetically closely related to BoHV-1. The existence of antigenic cross-reactions between these viruses and their ability to cross the species barrier raise theoretical problems for the differential diagnosis and the detection of any other virus reservoir, both in regions and countries where BoHV-1 infection has been eradicated and in those where the control of IBR is currently or will be undertaken (Thiry et al., 2001).

CpHV-1 is responsible of severe economic losses in Mediterranean countries. In kids, CpHV-1 is responsible of a systemic disease characterised by high morbidity and mortality, and ulcerative and necrotic lesions throughout the enteric tract (Mettler et al., 1979). In adult goats, infections of the reproductive tract as vulvovaginitis, balanoposthitis or spontaneous abortion are observed (Williams et al., 1997; Uzal et al., 2004). A classical inactivated vaccine has been developed. It shows a high immunogenicity and confers a good clinical protection (Tempesta et al., 2001). However, this vaccine has very little chance to be marketed because the veterinary pharmaceutical industry has only poor interest in the development of vaccines for minor species like goats.

Taking into account the issue of ruminant alphaherpesvirus diagnosis, the economical constraints of the veterinary pharmaceutical industry and the well-being of animals, we hypothesised that the intranasal use of an already licensed live attenuated gE-negative BoHV-1 vaccine could be an alternative tool to protect goats against CpHV-1 infection. Therefore, we decided to assess the safety and the efficacy of a live attenuated gE-negative BoHV-1 vaccine in goats, firstly by comparison with a virulent BoHV-1 and, secondly by immunisation followed by a CpHV-1 challenge.

Materials and methods

Animals

Nine goats, approximately 4-5 years of age, were used. All goats were originated from two CpHV-1 seronegative Italian flocks. Prior to inoculation, absence of antibodies against BoHV-1 and CpHV-1 was confirmed by serum neutralisation assay.

Cells and viruses

The Madin-Darby bovine kidney (MDBK) cell line (ATCC CCL22) was maintained as previously described (Tempesta et al., 2001). The BoHV-1 virus Ciney strain, characterised as highly virulent (Lemaire et al., 1999), the challenge CpHV-1 virus Ba-1 strain (Buonavoglia et al., 1996) and the BoHV-1 virus Los Angeles strain (Madin et al., 1956) used for serum neutralisation

assays were produced and titrated as described by Tempesta et al. (2001). The gE-negative BoHV-1 vaccine virus used for immunisation is the commercial vaccine Bayovac® IBR-marker vivum (Bayer, Italy).

Experimental design

Safety assessment

Six goats were randomly allocated into two groups of three goats. These groups were inoculated intranasally in the form of aerosol and received 2 ml per nostril of virulent BoHV-1 Ciney at a dose of $10^{4.8}$ $TCID_{50}$ or 2 ml per nostril of vaccine gE-negative BoHV-1 at a dose of $10^{4.8}$ $TCID_{50}$. Goats were clinically examined and rectal temperatures were measured daily for 21 days. Nasal and vaginal swabs were collected daily for 21 days. Blood samples were collected daily for 21 days for virological findings and weekly during the whole experiment at days 0, 7, 14, 21, 28, 35, 42 and 49 for serology.

Efficacy assessment

Two goats were inoculated intranasally in the form of aerosol twice two weeks apart and received 2 ml per nostril of vaccine gE-negative BoHV-1 at a dose of $10^{4.8}$ $TCID_{50}$. One goat was kept uninoculated as negative control. On day 28, all goats were challenged by the intranasal route with 10^{7} $TCID_{50}$ of CpHV-1 Ba-1 strain. Goats were clinically examined daily from day -1 (before first immunisation) up to day 7, from day 13 (before second immunisation) up to day 21, and from day 27 (before the challenge) up to day 42. Rectal temperatures and nasal and vaginal swabs were collected with the same time schedule. Blood samples were collected with the same time schedule for virological findings and weekly during the whole experiment at days 0, 7, 14, 21, 28, 35, 42 and 49 for serology.

Virus isolation, polymerase chain reaction and serum neutralisation assays

The virus isolation was performed as described by Tempesta et al. (2001). Buffy coats were separated from blood by centrifugation in presence of lympholyte (Cedarlane, Canada). Sample preparation and polymerase chain reaction (PCR) were performed as previously described with minor modifications (Vilcek et al., 1993; Tempesta et al., 1999). Serum neutralisation assays were performed as described by Tempesta et al. (2001). The titre of each serum was expressed as the highest serum dilution which neutralised the virus.

Results

Safety assessment

Clinical examination

The infection with the virulent BoHV-1 Ciney or the gE-negative BoHV-1 vaccine did not induce any undesirable local or systemic reaction. Goats inoculated with the gE-negative BoHV-1 did not show any clinical sign of disease. The mean rectal temperature was normal. On the opposite, hyperthermia was noticed in the group inoculated with the BoHV-1 Ciney (Figure 1). Clinical signs as diarrhoea or vaginal hyperaemia were also observed in this group.

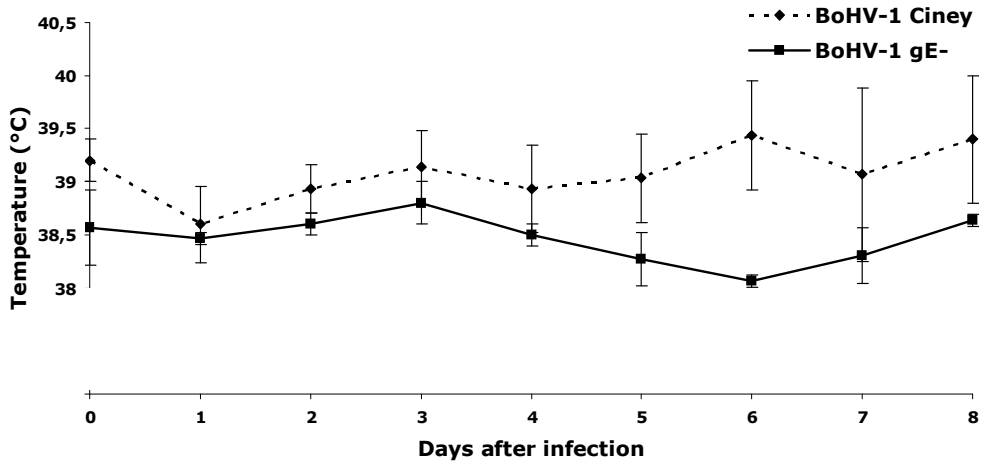

Figure 1 – Mean rectal temperatures after intranasal infection. Goats were inoculated at day 0 with the virulent Ciney or the gE-negative vaccine BoHV-1 strain.

Virus isolation and detection of viral DNA by PCR

Goats infected with the gE-negative BoHV-1 vaccine excreted approximately 2 to 4 log_{10} less viruses than those infected with the virulent BoHV-1 Ciney. The length of the virus excretion was 12 days shorter for animals infected with the vaccine virus (Figure 2).

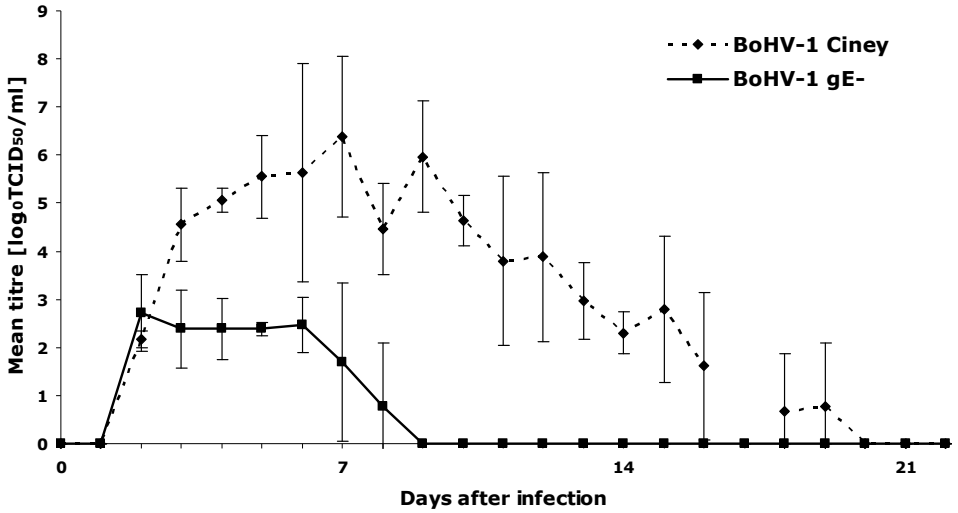

Figure 2 – Mean titres of BoHV-1 in nasal swabs after intranasal infection. Goats were inoculated at day 0 with the virulent Ciney or the gE-negative vaccine BoHV-1 strain. Titres are expressed as log_{10} tissue culture infectious dose 50 ($TCID_{50}$) per 1 ml of nasal secretions.

The virus was also detected by PCR in nasal swabs from each goat. Buffy coat samples were consistently negative for animals inoculated with the gE-negative BoHV-1. On the opposite, virus was recovered from days 5 up to 9 in buffy coats from goats inoculated with the BoHV-1 Ciney. In vaginal swabs, virus was not isolated by cell culture and not detected by PCR in both groups.

Immune responses

The BoHV-1 neutralising antibody titres of goats inoculated with the virulent BoHV-1 Ciney were below 1:2 at days 0 and 7. At day 14, titres increased to 1:2 and, at days 21, 28, 35,

42 and 49, to 1:4 and 1:8. BoHV-1 neutralising antibodies were not detected in goats inoculated with the gE-negative BoHV-1 vaccine.

Efficacy assessment

Clinical examination

After challenge of both immunised and control goats with CpHV-1 Ba-1, goats expressed only very few clinical signs, especially hyperthermia was noticed in each goat and lasted an average of ten days (data not shown).

Virus isolation and detection of viral DNA by PCR

Following the first immunisation (day 0) with the gE-negative BoHV-1 vaccine, virus was isolated by cell culture and detected by PCR in nasal swabs. The peak viral titre was $10^{4.8}$ $TCID_{50}$ per 1 ml of nasal secretions. Virus was not recovered from vaginal swabs and buffy coats. After the second immunisation (day 14), all samples were consistently negative (Figure 3).

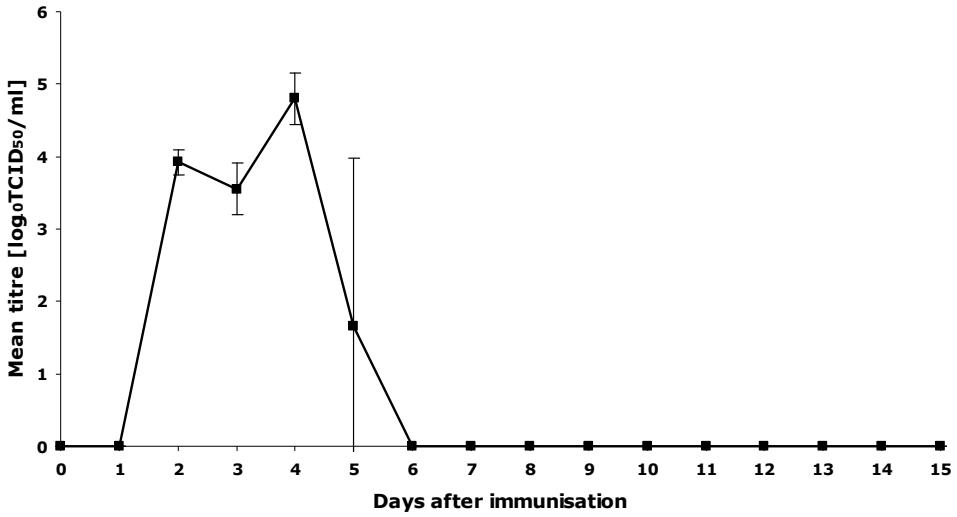

Figure 3 – Mean titres of BoHV-1 in nasal swabs after intranasal immunisation. Goats were immunised intranasally twice two weeks apart (days 0 and 14) with the gE-negative BoHV-1 vaccine strain. Titres are expressed as log_{10} tissue culture infectious dose 50 ($TCID_{50}$) per 1 ml of nasal secretions.

Two weeks later (day 28), control and immunised goats were challenged with the CpHV-1 Ba-1. A two log_{10} reduction in the peak viral titre was observed in nasal swabs from immunised goats. Virus excretion lasted 2 days more in immunised animals than in the control goat (Figure 4). The virus was isolated in cell culture and detected by PCR in buffy coats (days 3 to 5 post challenge), nasal and vaginal swabs from control and immunised goats.

Immune responses

After the first (day 0) and the second immunisations (day 14) with the gE-negative BoHV-1 vaccine, the BoHV-1 neutralising antibody titres of goats were below 1:2. During days post-challenge (day 28), every goat seroconverted. A sharp increase in titres was observed in all immunised goats: 1:4 at day 35, 1:32 and 1:64 at days 42 and 49. The non immunised control goat showed a similar increase in BoHV-1 neutralising antibodies: 1:4 at day 35, 1:64 at days 42 and 49.

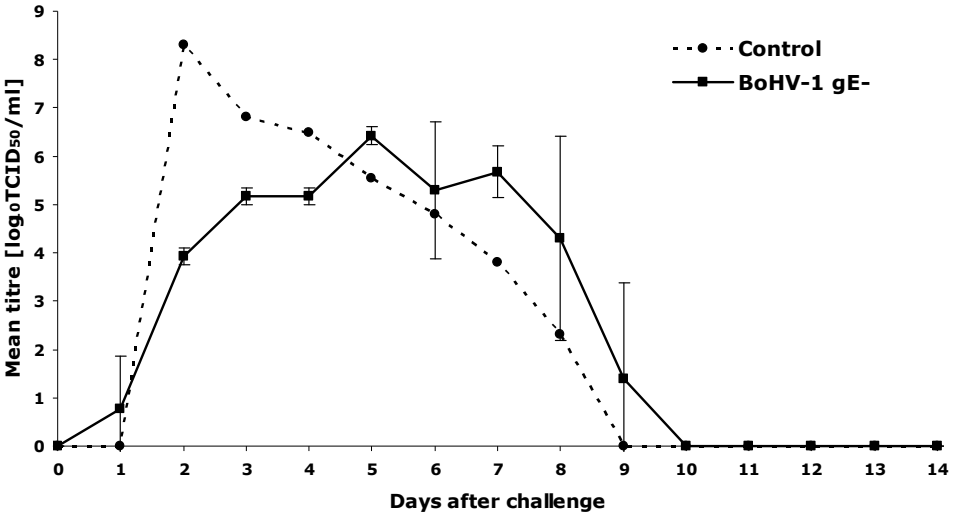

Figure 4 – Mean titres of CpHV-1 in nasal swabs after intranasal challenge (day 0) with the Ba-1 CpHV-1 strain. Goats were previously immunised intranasally twice two weeks apart with the gE-negative BoHV-1 vaccine strain. A non immunised control goat was inoculated at the same time. Titres are expressed as $\log_{10}$ tissue culture infectious dose 50 ($TCID_{50}$) per 1 ml of nasal secretions.

Discussion

The safety of a gE-negative BoHV-1 vaccine was assessed in goats by comparison with a virulent BoHV-1. Absence of adverse effects and local or systemic clinical signs suggested that the use of gE-negative BoHV-1 vaccine was safe in goats. On the opposite, animals inoculated with a virulent BoHV-1 showed an increase of the length of virus excretion and developed mild clinical signs. The susceptibility of goats to BoHV-1 is in accordance with previously described results (Six et al., 2001) and goat would be suggested as a less expensive animal model to study the *in vivo* properties of BoHV-1 infection.

In addition, the efficacy of a gE-negative BoHV-1 vaccine was assessed in goats by an immunisation experiment followed by a CpHV-1 challenge. After challenge, the reduction of the peak viral titre suggests that a gE-negative BoHV-1 vaccine could induce a cross-protection against CpHV-1 infection. However, the challenge virus excretion lasted two days more in immunised than in control goats indicating that the gE-negative BoHV-1 vaccine did not afford a sufficient protection. These results must be confirmed in a next experiment involving a higher number of animals. Both in safety and efficacy assessments, BoHV-1 neutralising antibody titres suggest a weak humoral immune response. It could be explained by a weak replication of BoHV-1 in goats. The development of a goat specific interferon-gamma assay could afford more information on the acquired cell-mediated immunity to gE-negative BoHV-1 immunisation and CpHV-1 infection.

Besides prevention of CpHV-1 induced disease, control of CpHV-1 infection by vaccination would be helpful in countries involved in BoHV-1 eradication in cattle while possessing a significant population of goats. Indeed, such vaccination could reduce the circulation of CpHV-1 in goats. In consequence, goats would be less infected by an alphaherpesvirus close to BoHV-1 and therefore less involved in BoHV-1 misdiagnosis due to CpHV-1 infection. Another issue linked to the use of gE-negative virus vaccine would be its hypothetical latent infection in goats. BoHV-1 can indeed establish latency in this species, but with a poor reactivation success rate (Engels et al., 1992; Six et al., 2001). Moreover, gE-negative BoHV-1 are also less effective in reactivation and reexcretion (Mars et al., 2000; Lemaire et al., 2001). The risk of reactivation and reexcretion of a gE-negative BoHV-1 in goats is therefore very low.

In conclusion, the obtained data, although limited, clearly demonstrated the safety of the intranasal use of a gE-negative marker vaccine in goats. However, the efficacy assessment showed that a live attenuated gE-negative BoHV-1 vaccine can afford only a partial cross-protection against CpHV-1 infection in goats.

Acknowledgements

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References

Buonavoglia C., Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Conserva A., Corrente M., Reactivation of caprine herpesvirus 1 in latently infected goats, Comp. Immunol. Microbiol. Infect. Dis. 19 (1996) 275-281.

Engels M., Palatini M., Metzler A.E., Probst U., Kihm U., Ackermann M., Interactions of bovine and caprine herpesviruses with the natural and the foreign hosts, Vet. Microbiol. 33 (1992) 69-78.

Lemaire M., Schynts F., Meyer G., Thiry E., Antibody response to glycoprotein E after bovine herpesvirus type 1 infection in passively immunised, glycoprotein E negative calves, Vet. Record 144 (1999) 172-176.

Lemaire M., Schynts F., Meyer G., Georgin J.-P., Baranowski E., Gabriel A., Ros C., Belak S., Thiry E., Latency and reactivation of a glycoprotein E negative bovine herpesvirus type 1 vaccine: influence of virus load and effect of specific maternal antibodies, Vaccine 19 (2001) 4795-4804.

Madin S.H., McKercher D.G., York C.J., Isolation of the infectious bovine rhinotracheitis virus, Science 124 (1956) 721-722.

Mars M.H., De Jong M.C., van Oirschot J.T., A gE-negative bovine herpesvirus 1 vaccine strain is not re-excreted nor transmitted in an experimental cattle population after corticosteroid treatments, Vaccine 18 (2000) 1975-1981.

Mettler F., Engels M., Wild P., Bivetti A., Herpesvirus-infektion bei Zieklein in der Schweiz, Schweiz. Arch. Tierheilkd. 121 (1979) 655-662.

Six A., Banks M., Engels M., Bascunana C.R., Ackermann M., Latency and reactivation of bovine herpesvirus 1 (BHV-1) in goats and of caprine herpesvirus 1 (CapHV-1) in calves, Arch. Virol. 146 (2001) 1325-1335.

Tempesta M., Pratelli A., Corrente M., Buonavoglia C., A preliminary study on the pathogenicity of a strain of caprine herpesvirus-1, Comp. Immunol. Microbiol. Infect. Dis. 22 (1999) 137-143.

Tempesta M., Camero M., Greco G., Pratelli A., Martella V., Buonavoglia C., A classical inactivated vaccine induces protection against caprine herpesvirus 1 infection in goats, Vaccine 19 (2001) 3860-3864.

Thiry E., Ackermann M., Banks M., Belak K., Belak S., Campbell I., Ek-Kommonen C., Engels M., Meyer G., Reid H., Ros C., Six A., Risk evaluation of cross-infection of cattle with ruminant alphaherpesviruses related to bovine herpesvirus type 1, in: R. Körber (Ed.), 3. Internationales Symposium zur BHV-1-/BVD-Bekämpfung, Stendal, 2001, pp. 99-104.

Uzal F.A., Woods L., Stillian M., Nordhausen R., Read D.H., Van Kampen H., Odani J., Hietala S., Hurley E.J., Vickers M.L., Gard S.M., Abortion and ulcerative posthitis associated with caprine herpesvirus-1 infection in goats in California, J. Vet. Diagn. Invest. 16 (2004) 478-484.

van Oirschot J.T., Kaashoek M., Rijsewijk F.A.M., Advances in the development and evaluation of bovine herpesvirus 1 vaccines, Vet. Microbiol. 53 (1996) 43-54.

Vilcek S., Detection of the bovine herpesvirus-1 (BHV-1) genome by PCR, J. Virol. Methods 41 (1993) 245-247.

Williams N.M., Vickers M.L., Tramontin R.R., Petrites-Murphy M.B., Allen G.P., Multiple abortions associated with caprine herpesvirus infection in a goat herd, J. Am. Vet. Med. Assoc. 211 (1997) 89-91.

Clinical protection against caprine herpesvirus 1 genital infection by intranasal administration of a live attenuated glycoprotein E negative bovine herpesvirus 1 vaccine

Julien Thiry, Maria Tempesta, Michele Camero, Elvira Tarsitano, Benoît Muylkens, François Meurens, Etienne Thiry and Canio Buonavoglia

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Caprine herpesvirus 1 (CpHV-1), responsible of various clinical manifestations in goats, is antigenically and genetically closely related to bovine herpesvirus 1 (BoHV-1). On the basis of these common properties, a live attenuated glycoprotein E (gE) negative BoHV-1 vaccine was assessed in goats by an intranasal immunisation twice three weeks apart followed by a subsequent CpHV-1 intravaginal challenge which is the natural route of infection in goats. To analyse the safety and the efficacy of this marker vaccine, two groups of goats served as controls: one was immunised with a virulent CpHV-1 and one was kept uninoculated until the challenge. The vaccine did not induce any undesirable local or systemic reaction and goats did not excrete gE-negative BoHV-1. After challenge, a significant reduction in disease severity was observed in immunised goats. Moreover, goats immunised with either gE-negative BoHV-1 or CpHV-1 exhibited a significant reduction in the length and the peak of viral excretion. Antibodies neutralising both BoHV-1 and CpHV-1 were raised in immunised goats. These data show that the intranasal application of a live attenuated gE-negative BoHV-1 vaccine is able to afford a clinical protection and a reduction of virus excretion in goats challenged by a CpHV-1 genital infection.

Introduction

The subfamily *Alphaherpesvirinae* includes a cluster of closely related ruminant viruses with bovine herpesvirus 1 (BoHV-1) as prototype (Thiry et al., 2006a). BoHV-1, a major cattle pathogen, is typically responsible of infectious bovine rhinotracheitis (IBR) causing severe economic losses in livestock (Muylkens et al., 2007). Since its isolation, several conventional vaccines have been developed. These vaccines usually prevented clinical signs and reduced the amount of excreted viruses. However, there was still a need for improvements in order to use them in control and/or eradication programmes (van Oirschot et al., 1996). Therefore, BoHV-1 marker vaccines comprising attenuated or killed mutants with a deletion in one of the non-essential genes (gE) were developed and eradication campaigns were initiated in many European countries. They have proven their safety and efficacy in the target bovine species since they are efficacious at reducing disease severity, virus shedding, and circulation in a population (Mars et al., 2000; 2001).

Caprine herpesvirus 1 (CpHV-1) is associated with two different syndromes in goats, a lethal systemic disease in kids (Saito et al., 1974; Van der Lugt and Randles, 1993) and a genital disease leading to balanoposthitis (Tarigan et al., 1987), vulvovaginitis (Grewal and Wells, 1986) and abortion (Keuser et al., 2002) in adults. These clinical signs and the virus presence in nasal, ocular, rectal and vaginal samples suggest both the venereal transmission as the principal virus entry route and infection persistence within herds (Tempesta et al., 1998; 1999a). The genital tropism of CpHV-1 was confirmed by the detection of viral DNA in sacral ganglia of latently infected goats (Tempesta et al., 1999b). According to serological investigations, the infection occurs worldwide with highest prevalences observed in Mediterranean countries (Kao et al., 1985; Keuser et al., 2004; Koptopoulos et al., 1988; Muluneh et al., 1990; Plebani et al., 1983; Thiry et al., 2006c). However, the economical losses due to CpHV-1 infection are probably underestimated. To date, a classical inactivated vaccine has been developed (Tempesta et al., 2001; Camero et al., 2007), however, it can not be licensed since the market of veterinary medicinal products for minor species, like goats, is not economically profitable. Consequently, the control of this infection still relies on hygienic prophylactic measures (Thiry et al., 2006a).

BoHV-1 and CpHV-1 are antigenically and genetically closely related (Thiry et al., 2006a). This relationship was originally demonstrated by serological assays (Kao et al., 1985; Berrios et al., 1975; Mettler et al., 1979; Ackermann et al., 1986) and lately by phylogenetic analysis (Ros and Belak, 1999; 2002; Ros et al., 1999). These viruses are able to some extent to cross the species barrier and establish infection in heterologous animal species (Pirak et al., 1983; Engels et al., 1992). Experimental reactivation of latent infection of BoHV-1 in goats was successfully performed (Six et al., 2001). Moreover, it has been recently demonstrated that a live attenuated gE-negative BoHV-1 vaccine was partially able to protect goats against nasal CpHV-1 infection (Thiry et al., 2006b).

In the following study, it is hypothesised that an intranasal administration (of a bovine vaccine) could afford a protection against the clinical genital infection. Indeed, for many years, the upper respiratory mucosa has been proven to be suitable for vaccine delivery. The recent advances in the study of the mucosal immune system strengthen this mode of administration as being a very effective route for vaccination for both peripheral and mucosal immunity (Davis, 2001). In human, nasal mucosa can serve as an efficient site for the induction of specific IgA and IgG responses in vaginal secretions (Bergquist et al., 1997; Johansson et al., 2001). The goat genital tract might employ similar homing mechanisms as those of the upper respiratory tract and therefore could receive primed immune cells from the nasopharynx-associated lymphoid tissue (NALT) (Brandtzaeg

et al., 1999). Therefore, it was decided to investigate gE-negative BoHV-1 intranasal route of vaccination in goats with the aim to protect this species against CpHV-1 genital infection.

Materials and methods

Cells and viruses

The Madin-Darby bovine kidney (MDBK) cell line (ATCC CCL22) was maintained in Dulbecco-Minimal Essential Medium (D-MEM) supplemented with 10% of foetal bovine serum (FBS). The challenge CpHV-1 Ba-1 strain (Buonavoglia et al., 1996) was produced by infection of MDBK cells in D-MEM supplemented with 10% of FBS. At 72 h after infection, culture medium was removed and clarified by centrifugation at 1,500 x g for 20 min. Supernatants were divided into aliquots, frozen at -80°C and titrated by tissue culture infectious dose 50 ($TCID_{50}$) method on MDBK cells. The gE-negative BoHV-1 vaccine virus strain used for immunisation is the commercial vaccine Rispoval® IBR-marker vivum (Pfizer Animal Health). The BoHV-1 Iowa (Miller and van der Maaten, 1984) and the CpHV-1 Ba-1 strains were used for serum neutralisation assays.

Experimental design

Nine dairy Alpine, Ionica, Maltese and Saanen crossbred goats, approximately 4-5 years of age, were used. All goats were originated from a CpHV-1 seronegative flock in Italy. Prior to inoculation, absence of antibodies against BoHV-1 and CpHV-1 was confirmed by serum neutralisation assay. The goats were randomly divided in three groups of three goats. Each group was separated in different airspaces. Two groups were inoculated intranasally by aerosolization twice three weeks apart as follows: group 1 served as positive control and received 2 ml per nostril of virulent CpHV-1 Ba-1 at a dose of $10^{5.25}$ $TCID_{50}$/50µl, and group 2 received 2 ml per nostril of gE-negative BoHV-1 vaccine at a dose of $10^{4.25}$ $TCID_{50}$/50µl. Group 3 served as negative control and was kept uninoculated before challenge. On day 42, all goats were challenged by the intravaginal route with 4 ml of virulent CpHV-1 Ba-1 ($10^{6.25}$ $TCID_{50}$/50µl). All precautions were taken to avoid viral spread. Clothes and boots were changed before entering any stable. For handling, new gloves were used between groups. Clinical monitoring and all sampling procedures were carried out in a blind manner.

Goats were clinically examined daily from day -1 (before infection) up to day 21 following the challenge and rectal temperatures were also measured up to day 16 post-challenge. Clinical observations were carried out at approximately the same time everyday and by the same scientist throughout the study. Clinical monitoring included the following clinical signs: apathy, anorexia, vaginal haemorrhage, vaginal discharge, pain, hyperemia of vulva and vagina, oedema of vulva and vagina, number of lesions in vulva and vagina. A clinical score from 0 to 2 was given for each clinical parameter except temperature. Scores quantifying the oedema of vulva and vagina were multiplied by 2. Scores quantifying the number of lesions in vulva and vagina were multiplied by 3.

Blood samples for serology were collected from the jugular vein of animals weekly during the whole experiment at days 0, 7, 14, 21, 28, 35, 42, 49, 56 and 63 after the primary immunisation. Serums obtained after centrifugation were stored at -20°C until analysis. Heparinised blood samples for buffy coat extraction, nasal and vaginal swabs were collected daily up to day 14 after first immunisation, up to day 14 after second immunisation, and during 14 days post-challenge, using one swab per animal, swabbing deeply into each nostril or vagina.

Viral characterisation

Samples were immersed in 1 ml of D-MEM and centrifuged at 5,000 x g for 5 min. The supernatant was then treated with a 10% antibiotics mixture (5,000 UI/ml penicillin, 2,500 µg/ml streptomycin, 10 µg/ml amphotericin) for 30 min at room temperature and titrated by the $TCID_{50}$ method on MDBK cells cultured in 96-well microtitre plates. The excess of samples was stored frozen at -80°C. Cells were examined daily for cytopathic effect (CPE). The virus titre was expressed as $TCID_{50}$ per 50 µl of secretion. Buffy coats were separated from blood by centrifugation in presence of lympholyte (Cedarlane, Canada). Sample preparation and polymerase chain reaction (PCR) were performed as previously described with minor modifications (Tempesta et al., 1999a; Vilcek, 1993). Viral DNA were prepared from supernatants of MDBK cell cultures infected with viruses isolated on days 1 and 4 after challenge (Keuser et al., 2004). Two µg of DNA were submitted to *Bst*EII restriction analysis (New England Biolabs) and DNA fragments were separated in a 0.7% Tris Acetate EDTA gel for 22 h at 30 V/cm and 500 mA.

Serological analysis

Serial twofold dilutions of each serum were mixed with either 100 $TICD_{50}$ of BoHV-1 Iowa strain or 100 $TICD_{50}$ of CpHV-1 Ba-1 strain in 96-well microtitre plates. The plates were held for 90 min at room temperature and 20,000 MDBK cells were then added to each well. Analysis was done after three days of incubation at 37°C in presence of 5% CO_2. The titre of each serum was expressed as the highest serum dilution which neutralised the virus in 50% of the wells (Lemaire et al., 2001). The BoHV-1 gE blocking ELISA (Herdchek Anti-IBR gE, Idexx, Germany) was used following the manufacturer instructions. Serums were analysed in duplicate.

Statistical analysis

Statistical comparisons in the clinical, virological and serological data were performed in the form of mixed models for repeated measurements by SAS procedure (procedure MIXED) (Littell et al., 1998).

Results

Clinical and viral responses after intranasal immunisation

Goats intranasally inoculated with virulent CpHV-1 or gE-negative BoHV-1 vaccine kept a good general state of health. No signs of severe disease as anorexia, apathy, oedema or lesions were observed. The gE-negative BoHV-1 immunisation did not induce any undesirable local or systemic reaction and goats did not show any clinical sign of disease. On the opposite, goats inoculated with CpHV-1 expressed mild clinical signs as hyperemia and nasal discharge. Based on the mean rectal temperature, the statistical analysis revealed significant differences between groups ($p<0.005$). From day 3 after immunisation, the mean temperatures of CpHV-1 inoculated goats were higher than the temperature of gE-negative BoHV-1 immunised goats ($p<0.001$) (data not shown).

Following the first immunisation (day 0), only CpHV-1 was excreted by goats (Figure 1). CpHV-1 was isolated in cell culture and was detected by PCR in nasal swabs but was not recovered from vaginal swabs and buffy coats. The peak viral titre was $10^{6.6}$ $TCID_{50}$ per 50 µl of nasal secretions. After the second immunisation (day 21), both samples from gE-negative BoHV-1 and

from CpHV-1 infected goats were consistently negative by isolation in cell culture and detection by PCR.

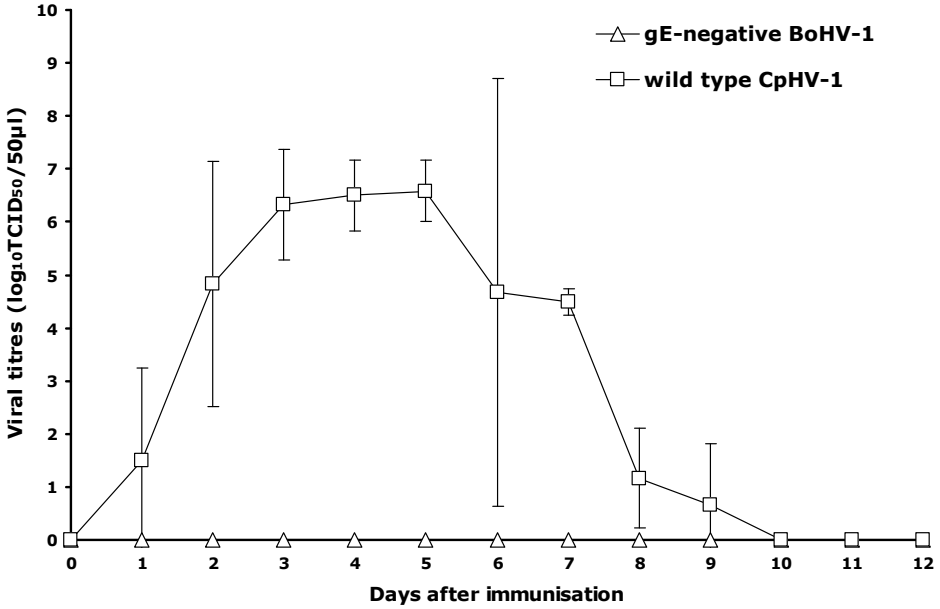

Figure 1 – Mean titres of BoHV-1 or CpHV-1 in nasal swabs recorded after intranasal immunisation. Goats were immunised intranasally with either live attenuated gE-negative BoHV-1 vaccine or virulent CpHV-1 Ba-1. Titres are expressed as $\log_{10}$ $TCID_{50}$ per 50 µl of nasal secretions.

Viral excretion after CpHV-1 intravaginal challenge

Three weeks after the second immunisation, control and immunised goats were intravaginally challenged with the virulent CpHV-1 Ba-1 strain (Figure 2).

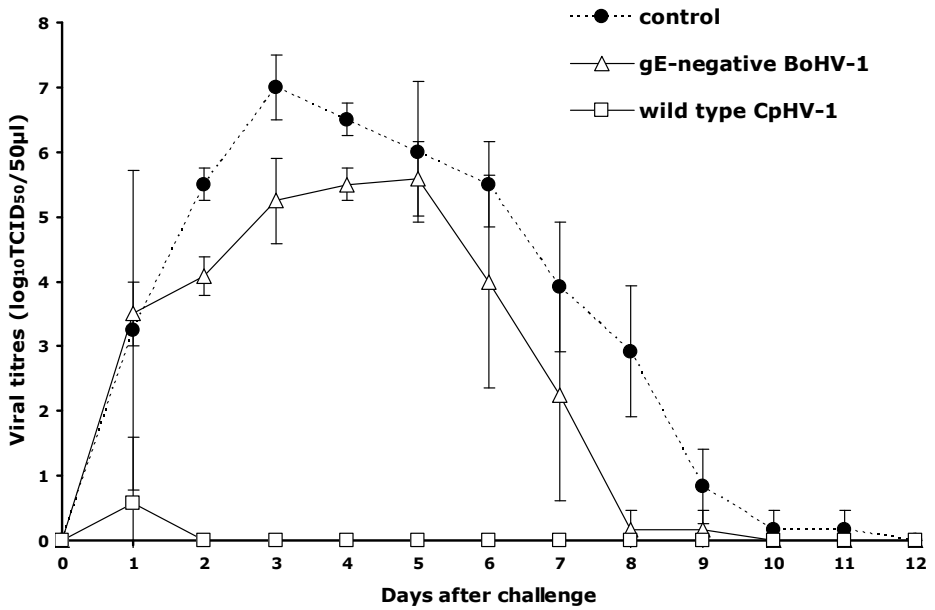

Figure 2 – Mean titres of CpHV-1 in vaginal swabs recorded after CpHV-1 Ba-1 intravaginal challenge. Goats were previously immunised intranasally with either live attenuated gE-negative BoHV-1 vaccine or virulent CpHV-1 Ba-1. The control group was not inoculated with any preparation. Titres are expressed as $\log_{10}$ $TCID_{50}$ per 50 µl of vaginal secretions.

The mean CpHV-1 titres in vaginal swabs were significantly different ($p<0.0001$) between groups immunised with either gE-negative BoHV-1 vaccine or virulent CpHV-1 and the non-

immunised group. The lowest excretion titres were obtained in the CpHV-1 immunised group compared to gE-negative BoHV-1 immunised or non-immunised groups ($p<0.0001$). The immunisation with gE-negative BoHV-1 vaccine decreased the mean challenge virus excretion titres: 1.42 log on day 2, 1.75 log on day 3, 1.5 log on day 6, 1.66 log on day 7, 2.75 on day 8, except on days 4, 5 and 9 after challenge where the mean excretion titres, although not significantly different, were lower than the mean excretion titres obtained on the same day in non-immunised goats. Furthermore, the gE-negative BoHV-1 vaccine shortened the challenge strain shedding. While no viral shedding was detected on day 12 in non-immunised group, goats from the gE-negative BoHV-1 immunised group excreted the challenge CpHV-1 during a significantly shorter time period. The shortest virus shedding, up to day 1 after challenge, was detected in the CpHV-1 immunised group (Figure 2).

On days 1 and 4 after challenge, viruses from one goat in each group were further propagated individually and characterised using restriction enzyme analysis. The *Bst*EII profiles confirmed that viruses excreted after challenge by goats immunised with gE-negative BoHV-1 vaccine were CpHV-1 challenge Ba-1 strain (data not shown).

Clinical protection against CpHV-1 intravaginal challenge

Groups intranasally immunised with either virulent CpHV-1 or gE-negative BoHV-1 vaccines were protected against the clinical form of the genital CpHV-1 infection (Figure 3). Consequently, the clinical score of each group was significantly lower ($p<0.0001$) than in the non-immunised group. The statistical analysis revealed no significant difference between the gE-negative BoHV-1 immunised and the CpHV-1 immunised groups ($p=0.06$) except at days 5 and 6 after challenge. At these days, goats immunised with gE-negative BoHV-1 vaccine showed mild oedema or vulva hyperemia.

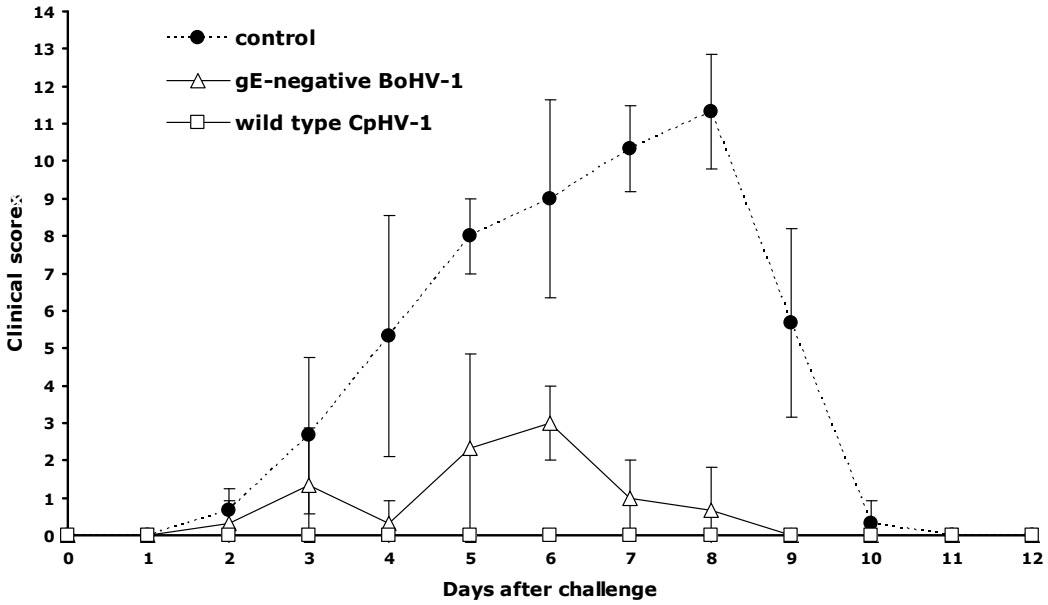

Figure 3 –Mean clinical scores recorded after CpHV-1 Ba-1 intravaginal challenge. Goats were previously immunised intranasally with either live attenuated gE-negative BoHV-1 vaccine or virulent CpHV-1 Ba-1. The control group was not inoculated with any preparation.

The mean rectal temperature of immunised groups was significantly lower ($p<0.0001$) than those of the non-immunised group except at the challenge peak (days 3 and 5 after challenge). Additionally, the group inoculated with virulent CpHV-1 exhibited a significant lower

temperature ($p<0.0001$) than the gE-negative BoHV-1 vaccine immunised group. Overall, goats did not show any sign of hyperthermia (data not shown).

Immune responses after immunisation and challenge

In goats immunised with either virulent CpHV-1 or gE-negative BoHV-1 vaccine, CpHV-1 neutralising antibodies were observed from day 14 after the first immunisation (Figure 4). No significant difference between groups was noticed. After the second immunisation, a sharp increase in CpHV-1 neutralising antibody titres was observed in the CpHV-1 immunised group. In contrast, goats immunised with gE-negative BoHV-1 did not show any boost of the primary immune response (Figure 4). Following the CpHV-1 intravaginal challenge, the mean CpHV-1 neutralising antibody titres were significantly different ($p<0.0001$) between groups immunised with gE-negative BoHV-1 or CpHV-1 and the non-immunised group. Interestingly, goats immunised with gE-negative BoHV-1 vaccine showed an increase in CpHV-1 neutralising antibodies, but their neutralising titres were much lower than that of CpHV-1 immunised goats (Figure 4). Neutralising antibody titres were lower against BoHV-1 than CpHV-1 in all groups (data not shown). Moreover, all animals remained negative with the BoHV-1 gE blocking ELISA.

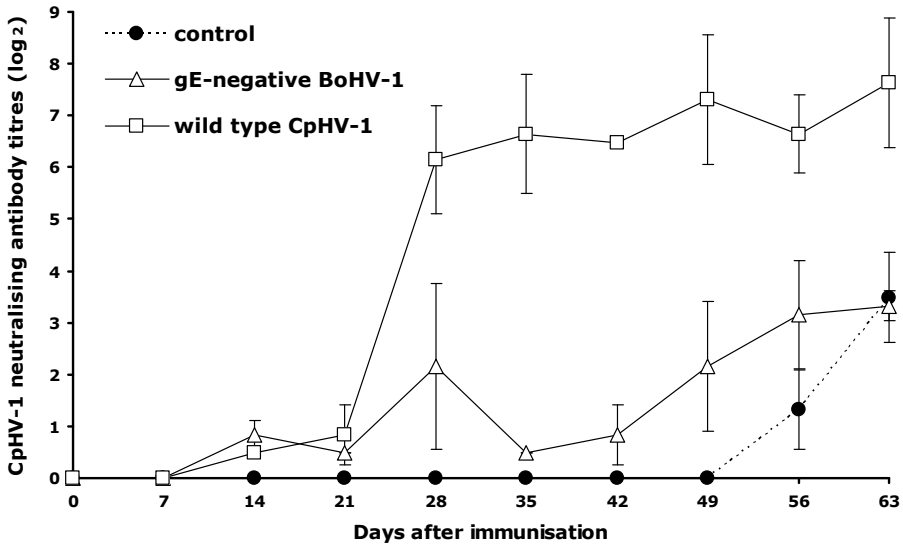

Figure 4 – Evolution of CpHV-1 neutralising antibody titres in goats intranasally immunised on day 0 with either live attenuated gE-negative BoHV-1 vaccine or virulent CpHV-1 Ba-1. The control group was not inoculated with any preparation. Goats were intravaginally challenged with virulent CpHV-1 Ba-1 on day 42. Neutralising titres are expressed as the initial dilution of serum that neutralised 50% of wells, calculated using the Spearman-Kärber method.

Discussion

Taking advantage of the susceptibility of goats to BoHV-1 (Thiry et al., 2006b), the efficacy of a live attenuated gE-negative BoHV-1 vaccine was assessed in goats after two intranasal administrations followed by a subsequent CpHV-1 intravaginal challenge. The intranasal use of a gE-negative BoHV-1 vaccine enabled a cross-protection against CpHV-1 genital infection which is the natural route of infection in goats. As observed in human, nasal mucosa can serve as an efficient site for the induction of a specific protective response in the genital tract. It is likely a consequence of the induction of specific IgA and IgG responses in vaginal secretions. The presence of specific antibody secreting cells (ASCs) in the genital tract have been demonstrated after nasal vaccination in mice (Eriksson et al., 1998; Johansson et al., 1998). In another study, it has been

shown that T lymphocyte homing to the genital mucosa requires the interaction of integrins $\alpha_L\beta_2$ and $\alpha_4\beta_1$ with endothelial intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (VCAM-1), respectively (Perry et al., 1998; Johansson et al., 1999). Since both nasal and genital mucosa express VCAM-1, this adressin could be involved in the homing of specific ASCs to the genital tract. Moreover, chemokine like CC chemokine ligand 28, which is expressed in both tissues, could interact with the chemokine receptor 10 expressed on nasal ASCs and be involved too in the homing of specific ASCs to the genital area (Johansen et al., 2005; Meurens et al., 2007). Although the underlying mechanism was not investigated in this study, it can be speculated that such pathways could be involved in the current protection.

Following the first immunisation, the safety of the gE-negative BoHV-1 vaccine in goats was evidenced by the absence of side effects and local or systemic reactions. Interestingly, goats did not excrete gE-negative BoHV-1 although a low level of BoHV-1 excretion was observed previously (Thiry et al., 2006b). A weaker replication of gE-negative BoHV-1 could account for this result. Nevertheless, the presence of neutralising antibodies against BoHV-1 after the first and second immunisations suggests the replication of gE-negative BoHV-1 in goats. It can be hypothesised that the first immunisation induced a strong mucosal immunity leading to the neutralisation of newly replicated viruses after the second immunisation. The absence of detection of anti-gE antibodies is consistent with the absence of gE in the bovine vaccine and shows that the antigenicity of gE is different in BoHV-1 and CpHV-1.

Another issue to consider is the possible establishment of gE-negative BoHV-1 in a latent state in vaccinated goats. Indeed, BoHV-1 is able to establish latency in goats but with a poor reactivation success rate (Engels et al., 1992; Six et al., 2001). However, gE-negative BoHV-1 is less effective in reactivation and reexcretion than wild type viruses in calves (Mars et al., 2000; Lemaire et al., 2001). Therefore, the risk of reactivation and reexcretion of a gE-negative BoHV-1 in goats is low. Otherwise, the vaccination could also lead to the emergence of new recombinant viruses. Indeed, despite the fact that in the subfamily *Alphaherpesvirinae*, viruses of different species show very few sequence similarities to allow homologous recombination, several interspecific recombinants were isolated *in vitro* (Thiry et al., 2005). Natural recombinants between equid herpesviruses 1 and 4 were, for example, recently identified (Pagamjav et al., 2005). Therefore, the question of recombinants rising from cross-infection of CpHV-1 infected goats with BoHV-1 needs to be considered. Among the cluster of ruminant alphaherpesviruses related to BoHV-1, only two recombinant viruses between BoHV-1 and BoHV-5 were isolated, and any recombinant between BoHV-1 and less closely related CpHV-1 and CvHV-2 was detected *in vitro* (Meurens et al., 2004). Consequently, in regards of these data and especially the low level of excretion, the vaccination described here is likely to be completely safe.

The reduction of the clinical score was considered as the most relevant parameter showing the efficacy of nasal immunisation against CpHV-1. Goats immunised intranasally with gE-negative BoHV-1 vaccine were clinically protected. Moreover, the difference between goats immunised with either gE-negative BoHV-1 or CpHV-1 was not statistically significant. The gE-negative BoHV-1 vaccine was not only effective in preventing development of genital disease upon challenge, but also in significantly reducing the magnitude and the duration of challenge CpHV-1 excretion. A high protection against clinical signs was also observed after immunisation by intranasal infection with CpHV-1. However, in natural conditions, the same kind of protection is not likely to be reached because the main route of transmission is venereal instead of respiratory (Tempesta et al., 1998; 1999a). The current CpHV-1 vaginal challenge used in this assay was even more severe than in previous experiments (Tempesta et al., 2000; 2002) and this result brings a good validation of the current study. The significant differences observed between immunised and

non-immunised groups have been obtained with a relatively low number of animals, therefore despite a lower power of the statistical test. These data allowed the identification of a strong effect of vaccination with the live attenuated gE-negative BoHV-1 vaccine (Howell, 1997). Moreover, nasal vaccination is an interesting alternative for inducing specific antibody responses in female genital tract, both for convenience and because the outcome of vaginal vaccination might be dependent on the time point in the oestral cycle for vaccine administration (Johansson et al., 2001).

Concerning infection control, such vaccination could bring several advantages. Indeed, the existence of antigenic cross-reactions between ruminant alphaherpesviruses related to BoHV-1 and their ability to cross the species barrier raise theoretical problems for the differential diagnosis and the detection of any other virus reservoir, both in regions and countries where BoHV-1 infection has been eradicated and in those where the control of IBR is currently or will be undertaken (Muylkens et al., 2007). The use of such vaccination could reduce the circulation of CpHV-1 in goats which would be therefore less involved in BoHV-1 misdiagnosis due to infection with a closely related alphaherpesvirus. Moreover, the development of new vaccines in order to protect minor species against infection causing economical and management problems meets a poor interest from the pharmaceutical industry. In this context, a classical inactivated vaccine inducing a good protection against CpHV-1 infection in goats was developed but was not licensed (Tempesta et al., 2001; Camero et al., 2007). Consequently, it was required to investigate the capacity of an already licensed bovine vaccine to induce a cross-protection against a related virus infection in goats according to the principle of the cascade. The European Union has recently pointed out the requirements of medicinal veterinary products for minor uses and minor species, as goats for example (Committee for Veterinary Medicinal Products, 2004). The results obtained in this study clearly show that a bovine vaccine can be safely and efficiently used in goats.

Conclusion

Regarding the issue of ruminant alphaherpesvirus diagnosis, the economical constraints of the veterinary pharmaceutical industry and the well-being of animals, this study brings an expected tool for the CpHV-1 induced disease prevention. Indeed, the intranasal administration of a live attenuated gE-negative BoHV-1 vaccine protects goats clinically and virologically against CpHV-1 genital infection which is the natural route of infection in goats. In addition, the current study emphasises the interest of studying intranasal vaccination approaches against genitally transmitted infections through the mucosal immune system.

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References

Ackermann M., Metzler A.E., McDonaugh H., Bruckner L., Muller H.K., Kihm U., Stellen nichtbovine paarhufer ein IBR-Virus-reservoir dar? I. BHV-1 und CapHV-1 infektions und reaktivierungsversuche an ziegen, virustyp-spezifitat der humoralen antikorper und charakterisierung der viralen antigene, Schweiz. Arch. Tierheilkd. 128 (1986) 557-573.

Bergquist C., Johansson E.L., Lagergard T., Holmgren J., Rudin A., Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina, Infect. Immun. 65 (1997) 2676-2684.

Berrios P.E., McKercher D.G., Knight H.D., Pathogenicity of a caprine herpesvirus, Am. J. Vet. Res. 36 (1975) 1763-1769.

Brandtzaeg P., Baekkevold E.S., Farstad I.N., Jahnsen F.L., Johansen F.E., Nilsen E.M., Yamanaka T., Regional specialization in the mucosal immune system: what happens in the microcompartments?, Immunol. Today 20 (1999) 141-151.

Buonavoglia C., Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Conserva A., Corrente M., Reactivation of caprine herpesvirus 1 in latently infected goats, Comp. Immunol. Microbiol. Infect. Dis. 19 (1996) 275-281.

Camero M., Bellacicco A.L., Tarsitano E., Decaro N., Martella V., Tempesta M. Buonavoglia C., Intravaginal administration of an inactivated vaccine prevents lesions induced by caprine herpesvirus-1 in goats, Vaccine 25 (2007) 1658-1661.

Committee for Veterinary Medicinal Products, Position paper regarding availability of products for minor uses and minor species (MUMS), in: European Medicine Agency (Ed.), EMEA/CVMP/477/03/final, Luxembourg, 2004, pp. 1-13.

Davis S.S., Nasal vaccines, Adv. Drug Deliv. Rev. 51 (2001) 21-42.

Engels M., Palatini M., Metzler A.E., Probst U., Kihm U., Ackermann M., Interactions of bovine and caprine herpesviruses with the natural and the foreign hosts, Vet. Microbiol. 33 (1992) 69-78.

Eriksson K., Quiding-Jarbrink M., Osek J., Moller A., Bjork S., Holmgren J., Czerkinsky C., Specific-antibody-secreting cells in the rectums and genital tracts of nonhuman primates following vaccination, Infect. Immun. 66 (1998) 5889-5896.

Grewal A.S., Wells R., Vulvovaginitis of goats due to a herpesvirus, Aust. Vet. J. 63 (1986) 79-82.

Guercio A., Greco G., Lanizzoto G., Di Marco V., Todaro M., Valutazione della diffusione di anticorpi anti Herpes Virus della capra in allevamenti caprini della Sicilia, Atti. SIPAOC 12 (1998) 138-142.

Howell D.C., Power considerations in terms of sample size, Statistical Methods for Psychology, 4th ed., Duxbury, 1997, pp. 226.

Johansen F.E., Baekkevold E.S., Carlsen H.S., Farstad I.N., Soler D., Brandtzaeg P., Regional induction of adhesion molecules and chemokine receptors explains disparate homing of human B cells to systemic and mucosal effector sites: dispersion from tonsils, Blood 106 (2005) 593-600.

Johansson E.L., Rask C., Fredriksson M., Eriksson K., Czerkinsky C., Holmgren J., Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates, Infect. Immun. 66 (1998) 514-520.

Johansson E.L., Rudin A., Wassen L., Holmgren J., Distribution of lymphocytes and adhesion molecules in human cervix and vagina, Immunology 96 (1999) 272-277.

Johansson E.L., Wassen L., Holmgren J., Jertborn M., Rudin A., Nasal and vaginal vaccinations have differential effects on antibody responses in vaginal and cervical secretions in humans, Infect. Immun. 69 (2001) 7481-7486.

Kao M., Leiskau T., Koptopoulos G., Papadopoulos O., Horner G.W., Hyllseth B. et al., Goat herpesvirus infections: a survey on specific antibodies in different countries. In: Pastoret P-P, Thiry E, Saliki JT, editors. Immunity to herpesvirus infections of domestic animals. Luxembourg, Report EUR 9737 EN, 1985:93-97.

Keuser V., Gogev S., Schynts F., Thiry E., Demonstration of generalized infection with caprine herpesvirus 1 diagnosed in an aborted caprine fetus by PCR, Vet. Res. Commun. 26 (2002) 221-226.

Keuser V., Espejo-Serrano J., Schynts F., Georgin J.P., Thiry E., Isolation of caprine herpesvirus type 1 in Spain, Vet. Rec. 154 (2004) 395-399.

Koptopoulos G., Papanastasopoulou M., Papadopoulos O., Ludwig H., The epizootiology of caprine herpesvirus (BHV-6) infections in goat populations in Greece, Comp. Immunol. Microbiol. Infect. Dis. 11 (1988) 199-205.

Lemaire M., Schynts F., Meyer G., Georgin J.P., Baranowski E., Gabriel A., Ros C., Belak S., Thiry E., Latency and reactivation of a glycoprotein E negative bovine herpesvirus type 1 vaccine: influence of virus load and effect of specific maternal antibodies, Vaccine 19 (2001) 4795-4804.

Littell R.C., Henry P.R., Ammerman C.B., Statistical analysis of repeated measures data using SAS procedures, J. Anim. Sci. 76 (1998) 1216-1231.

Mars M.H., De Jong M.C., van Oirschot J.T., A gE-negative bovine herpesvirus 1 vaccine strain is not re-excreted nor transmitted in an experimental cattle population after corticosteroid treatments, Vaccine 18 (2000) 1975-1981.

Mars M.H., De Jong M.C., Franken P., van Oirschot J.T., Efficacy of a live glycoprotein E-negative bovine herpesvirus 1 vaccine in cattle in the field, Vaccine 19 (2001) 1924-1930.

Mettler F., Engels M., Wild P., Bivetti A., Herpesvirus-Infektion bei Zicklein in der, Schweiz Arch. Tierheilkd. 121 (1979) 655-662.

Meurens F., Keil G.M., Muylkens B., Gogev S., Schynts F., Negro S., Wiggers L., Thiry E., Interspecific recombination between two ruminant alphaherpesviruses, bovine herpesviruses 1 and 5, J. Virol. 78 (2004) 9828-9836.

Meurens F., Whale J., Brownlie R., Dybvig T., Thompson D.R., Gerdts V., Expression of mucosal chemokines TECK/CCL25 and MEC/CCL28 during fetal development of the ovine mucosal immune system, *Immunology* 120 (2007) 544-555.

Miller J.M., van der Maaten M.J., Reproductive tract lesions in heifers after intrauterine inoculation with infectious bovine rhinotracheitis virus, Am. J. Vet. Res. 45 (1984) 790-794.

Muluneh A., Liebermann H., Occurrence of caprine herpesvirus (BHV-6) infection in goat population of the GDR, Arch. Exp. Veterinarmed. 44 (1990) 254-257.

Muylkens B., Thiry J., Kirten P., Schynts F., Thiry E., Bovine herpesvirus 1 and infectious bovine rhinotracheitis, Vet. Res. 38 (2007) 181-209.

Pagamjav O., Sakata T., Matsumura T., Yamaguchi T., Fukushi H., Natural recombinant between equine herpesviruses 1 and 4 in the ICP4 gene, Microbiol. Immunol. 49 (2005) 167-179.

Perry L.L., Feilzer K., Portis J.L., Caldwell H.D., Distinct homing pathways direct T lymphocytes to the genital and intestinal mucosae in Chlamydia-infected mice, J. Immunol. 160 (1998) 2905-2914.

Pirak M., Thiry E., Brochier B., Pastoret P.-P., Infection expérimentale de la chèvre par le virus de la rhinotrachéite infectieuse bovine (Bovine herpesvirus type 1) et tentative de réactivation virale, Rec. Méd. Vét. (1983) 1103-1106.

Plebani G.F., Engels M., Metzler A.E., Wyler R., Caprine herpesvirus in Switzerland: distribution, incidence and latency of infection, Schweiz. Arch. Tierheilkd. 125 (1983) 395-411.

Ros C., Belak S., Studies of genetic relationships between bovine, caprine, cervine, and rangiferine alphaherpesviruses and improved molecular methods for virus detection and identification, J. Clin. Microbiol. 37 (1999) 1247-1253.

Ros C., Riquelme M.E., Forslund K.O., Belak S., Improved detection of five closely related ruminant alphaherpesviruses by specific amplification of viral genomic sequences, J. Virol. Methods 83 (1999) 55-65.

Ros C., Belak S., Characterization of the glycoprotein B gene from ruminant alphaherpesviruses, Virus Genes 24 (2002) 99-105.

Saito J.K., Gribble D.H., Berrios P.E., Knight H.D., Mc Kercher D.G., A new herpesvirus isolate from goats: Preliminary report, Am. J. Vet. Res. 35 (1974) 847-848.

Six A., Banks M., Engels M., Bascunana C.R., Ackermann M., Latency and reactivation of bovine herpesvirus 1 (BHV-1) in goats and of caprine herpesvirus 1 (CapHV-1) in calves, Arch. Virol. 146 (2001) 1325-1335.

Tarigan S., Webb R.F., Kirkland D., Caprine herpesvirus from balanoposthitis, Aust. Vet. J. 64 (1987) 321.

Tempesta M., Buonavoglia D., Sagazio P., Pratelli A., Buonavoglia C., Natural reactivation of caprine herpesvirus 1 in latently infected goats, Vet. Rec. 143 (1998) 200.

Tempesta M., Pratelli A., Corrente M., Buonavoglia C., A preliminary study on the pathogenicity of a strain of caprine herpesvirus-1, Comp. Immunol. Microbiol. Infect. Dis. 22 (1999a) 137-143.

Tempesta M., Pratelli A., Greco G., Martella V., Buonavoglia C., Detection of caprine herpesvirus 1 in sacral ganglia of latently infected goats by PCR, J. Clin. Microbiol. 37 (1999b) 1598-1599.

Tempesta M., Pratelli A., Normanno G., Camero M., Buonavoglia D. Greco G., Buonavoglia C., Experimental intravaginal infection of goats with caprine herpesvirus 1, J. Vet. Med. B Infect. Dis. Vet. Public Health 47 (2000) 197-201.

Tempesta M., Camero M., Greco G., Pratelli A., Martella V., Buonavoglia C., A classical inactivated vaccine induces protection against caprine herpesvirus 1 infection in goats, Vaccine 19 (2001) 3860-3864.

Tempesta M., Greco G., Pratelli A., Buonavoglia D., Camero M., Martella V., Buonavoglia C., Reactivation of caprine herpesvirus 1 in experimentally infected goats, Vet. Rec. 150 (2002) 116-117.

Thiry E., Meurens F., Muylkens B., McVoy M., Gogev S., Thiry J., Vanderplasschen A., Epstein A., Keil G., Schynts F., Recombination in alphaherpesviruses, Rev. Med. Virol. 15 (2005) 89-103.

Thiry J, Keuser V, Muylkens B, Meurens F, Gogev S, Vanderplasschen A, Thiry E., Ruminant alphaherpesviruses related to bovine herpesvirus 1, Vet. Res. 37 (2006a) 169-90.

Thiry J., Tempesta M., Camero M., Tarsitano E., Bellacicco A.L., Thiry E., Buonavoglia C., A live attenuated glycoprotein E negative bovine herpesvirus 1 vaccine induces a partial cross-protection against caprine herpesvirus 1 infection in goats, Vet. Microbiol. 113 (2006b) 303-308.

Thiry J., Keuser V., Schynts F., Chartier C., Tempesta M., Espejo-Serrano J. et al. Evaluation de la prévalence sérologique de l'infection par l'herpèsvirus caprin 1 dans le sud-ouest de l'Europe, Epidémiol. Santé Anim., 49 (2006c) 55-58.

Van der Lugt J.J., Randles J.L., Systemic herpesvirus infection in neonatal goats, J. S. Afr. Vet. Assoc. 64 (1993) 169-171.

van Oirschot J.T., Kaashoek M.J., Rijsewijk F.A., Advances in the development and evaluation of bovine herpesvirus 1 vaccines, Vet. Microbiol. 53 (1996) 43-54.

Vilcek S., Detection of the bovine herpesvirus-1 (BHV-1) genome by PCR, J. Virol. Methods 41 (1993) 245-247.

CHAPTER FIVE

General Discussion

Ruminant herpesviruses have been widely described because they are causative agents of diseases of economic importance (Engels and Ackermann, 1996; Schwyzer and Ackermann, 1996; Thiry et al., 2006b). Illustrating the spectacular evolutionary success of the family *Herpesviridae* (Davison, 2002), several ruminant herpesviruses are able to infect heterologous species leading to various clinical signs or subclinical infections. For example, sheep is susceptible to BoHV-1 but the infection is asymptomatic (Whetstone and Evermann, 1988). In contrast, the development of a severe illness can occur as for example Aujeszky's disease, a fatal meningoencephalitis in cattle caused by suid herpesvirus 1, a pig alphaherpesvirus (Wittmann, 1989). In the same way, equine herpesvirus 1 causes nervous and ocular syndromes in alpacas and llamas in South America (Rebhun et al., 1988). The determination of the spectrum of herpesvirus susceptible species is made more difficult by the existence of viruses closely related as, for example, the cluster of ruminant alphaherpesviruses related to BoHV-1.

In order to characterise the relationship existing between BoHV-1 and its related viruses, phylogenetic analyses were performed by sequencing a selected part of UL27 encoding glycoprotein B and US8 encoding glycoprotein E. The choice of these genes was determined by their degree of divergence observed when comparing BoHV-1 and BoHV-5 genomes (Delhon et al., 2003). The obtained data confirm the genetic relatedness of the members of this cluster. BoHV-5 and BuHV-1 are the most closely related viruses to BoHV-1, followed by CvHV-1 and ElkHV-1 clustering together and with CvHV-2 and CpHV-1. The exact knowledge of the evolutionary relationships among these viruses request complete genome sequencing but this analysis is complicated by the large size of herpesvirus genome. Another possibility would be to compare different regions distributed along the genome of each virus as demonstrated by the study of the BoHV-4 evolution history (Dewals et al., 2006). Such analysis could bring interesting data about events that occurred in the past between herpesviruses and their ruminant hosts. Although humans certainly play an important role with the extensive trade of ruminants, this question has to be raised when knowing that BoHV-1 is worldwide distributed (Muylkens et al., 2007), BoHV-5 is mainly distributed in South America (Meyer et al., 2007), but has been isolated in Australia and USA; BuHV-1 was isolated in Australia (Bulach and Studdert, 1990) and its presence is demonstrated in Italy (De Carlo et al., 2004) and highly suspected in Argentina (Romera, personal communication); CpHV-1, CvHV-1 and CvHV-2 are present in Europe (Thiry et al., 2006c; Inglis et al., 1983. Ek-kommonen et al., 1986) and ElkHV-1 was recovered from a North American elk in Canada (Deregt et al., 2000).

The present work gives a contribution to the knowledge of the natural history of the cluster of ruminant alphaherpesviruses related to BoHV-1. The first isolation of CvHV-1 in a free-ranging red deer linked to the knowledge of the evolution of red deer populations in Europe allowed an interesting hypothesis about the origin of this virus. The genetic analysis showed that the virus isolated in Anlier was different from the virus isolated in Scotland. Moreover, in absence of clinical signs observed in the hunted fawn sampled, it is difficult to confirm the presence of a new viral subtype because nothing is known about the pathogenicity. The data suggest the existence of a new strain of CvHV-1 which is named Anlier. In regards of the observed prevalence and the presence of red deer over large area, this virus could be responsible of an endemic infection spreading in Europe. Indeed, the prevalence of a BoHV-1 related alphaherpesvirus infection in red deer ranged between 5.4 to 71% in Czech Republic (Pospisil et al., 1996), Germany (Muller et al., 1997) and Hungary (Kalman and Egyed, 2005) as well as 28.9% in Belgium (Grégoire and Linden, unpublished data). Moreover, the same genetic cluster of red deer is present through Central and Western Europe (Feulner et al., 2004; Frantz et al., 2004; Kuehn et al., 2004). This explanation of the emergence of CvHV-1 Anlier in Belgium is more likely than the result of an illegal translocation of a Scottish farmed red deer in wildlife. However, molecular epidemiologic study of several isolates

of the two strains is required to firmly assess if the Belgian CvHV-1 Anlier strain could be the ancestor of the Scottish CvHV-1 Banffshire 82 strain. This goal will be difficult to achieve due to the need to search for viral infections in wildlife.

Until now, no severe epidemic resulting from herpesvirus infections was identified in wild fauna. Nevertheless, several free ranging ruminants are susceptible to these viruses which are presumably responsible for sporadic diseases. Such infections have low clinical consequences but their epidemiologic role is important. The infection of free ranging animals contributes to maintain viruses in a specific region and within a population that could become a virus reservoir for domestic ruminants. The current development of breeding of ruminants taken from wild fauna has to take into account these infections. Indeed, the increase of farmed animal density allows the emergence of epidemic diseases whereas they should be sporadic in natural conditions and go unnoticed. Moreover, the proximity with domestic animals enhances the risk of transmission of these viruses to recently introduced animals. Thus, it should not be true to reduce the impact of herpesvirus infections in free ranging ruminants to a simple circulation of viruses in a wild population resulting from a transmission by domestic animals. In the same way, the situation observed in domestic ruminants can not be transferred to free ranging ruminants. When viruses are antigenically and genetically closely related, serological misdiagnosis can occur because the context leads to conclude that the wild species is infected by a virus specific of a domestic species.

The best example of this misinterpretation is the situation observed in Finland in 1982. An epidemiological survey in the Finnish reindeer population showed that 23% of animals were seropositive to BoHV-1, while all cattle were seronegative (Ek-Kommonen et al., 1982). These data strongly suggested a BoHV-1 infection of reindeer with an absence of transmission to cattle due to an apparent lack of contact between the two ruminant species. This hypothesis was rapidly rejected. From a BoHV-1 seropositive reindeer, a new virus was isolated and further characterised as CvHV-2. This infection provided a likely explanation of the presence of anti-CvHV-2 antibodies cross-reacting with BoHV-1 in reindeer (Ek-Kommonen et al., 1986). In spite of this epidemiologic situation in reindeer, the Finnish cattle population maintained an IBR free status.

Another example revealing the importance of clarifying the cause of potential serological cross-relationships with BoHV-1 is the difficulty encountered by the French veterinary authorities to differentiate true and false BoHV-1 positive cattle in the framework of an IBR herd certification. In France, a herd gets label "A" after repeated serological investigations leading to a so-called "IBR-free status". However, cattle purchased from these herds are sporadically identified as seropositive to BoHV-1 (Petit, 2002; Thiry et al., 2007a). Among other hypotheses, one explanation of this "false" seropositivity would be the infection with a BoHV-1 related virus that has crossed the species barrier.

As demonstrated in the first study, BoHV-5 is the most closely related virus to BoHV-1. However, BoHV-5 was never identified in Europe, except once in Hungary (Bartha et al., 1969). ElkHV-1 has been only identified in Canada (Deregt et al., 2000). In Europe, these two viruses cannot therefore contribute to IBR misdiagnosis, contrarily to the other four ruminant alphaherpesviruses. In regards to the low number of buffalo livestock in France, the hypothesis of BuHV-1 infection of cattle could be rejected. The transmission of CvHV-1 in cattle would be only exceptional due to the weak susceptibility of cattle to CvHV-1 (Ronsholt et al., 1987) and the seldom epidemiologic situation where bovine and red deer could meet each other. In the same way, it was demonstrated that BoHV1 will probably not survive longer than a few decades (several times the mean deer lifetime) in red deer populations (Mollema et al., 2005). As reindeer are not present in France, CvHV-2 infection can be also excluded. Interestingly, the Finnish situation

demonstrated that the presence of infected reindeer did not present a risk factor of BoHV-1 seropositive cattle. Consequently, the only virus that could be responsible of IBR misdiagnosis in France is CpHV-1 because goats and cattle can be in close contact in some field situations.

The results obtained in the second study did not support this hypothesis. Indeed, CpHV-1 infection is either absent or at a very low prevalence in continental France. The current sampling is restricted to Dordogne and Vendée, but a previous seroepidemiologic study also failed to identify seropositive goats in mainland France (Thiry et al., 2006d). Contrarily, a very high seroprevalence of CpHV-1 was measured in the Corse-du-Sud department. In Corsica, the contact between cattle and goats is very limited because flocks and herds are not mixed although they share the same rangeland pastures in mountains and plains. Therefore, the transmission of CpHV-1 from goats to cattle would be only exceptional, such cross-infection being very rare, and a BoHV-1 infection in goats is not likely to occur.

The use of a BoHV-1 gB blocking ELISA to investigate the epidemiologic situation of CpHV-1 in France was motivated by the presence of anti-gB antibodies at high titres, their early onset and persistence after infection in cattle (Kramps et al., 1994) but primarily because gene encoding gB is one of the most conserved within the cluster of ruminant alphaherpesviruses related to BoHV-1 as demonstrated in the first study. The statistical analysis measured a specificity of 100% and a sensitivity of 93.5%. These results are similar than those obtained by the inter-laboratory comparison tests revealing a specificity of 99% and a sensitivity of 96% for the detection of antibodies against BoHV-1 (Kramps et al., 2004). The only minor point that can be discussed is the sensitivity assessment resulting from serial bleeding of CpHV-1 infected goats. It would be useful to measure it from naturally CpHV-1 infected goats or at least one bleeding of several experimentally CpHV-1 infected goats. However, the cross serum neutralisation showed that all gB-positive serum samples neutralised to a greater extent CpHV-1 than BoHV-1. Therefore, the BoHV-1 gB blocking ELISA can be validated for the detection of CpHV-1 infected goats in flocks.

The BoHV-1 gB blocking ELISA was shown to be the most sensitive test to detect antibodies against BoHV-1 followed by indirect ELISA and serum neutralisation assay in contrast with BoHV-1 gE blocking ELISA showing a lower analytical sensitivity (de Wit et al., 1998; Beer et al., 2003; Kramps et al., 2004). The differentiation expected between BoHV-1 and CpHV-1 immunisation was not observed when using the BoHV-1 gE blocking ELISA. 22.6% of gB and CpHV-1 positive goat serum samples did react in the BoHV-1 gE blocking ELISA. Several hypotheses are suggested to explain the gE-positive results. First, the level of immunisation induced after CpHV-1 infection or the long-term maintenance of lower antibody titres after latent establishment could be an explanation (Kaashoek et al., 1996). Another phenomenon could be a steric hindrance due to antibody binding to neighbouring epitopes on gE preventing the interaction with the specific antibody, as demonstrated for the attenuated strain not expressing a particular gE-epitope (van Oirschot et al., 1999). Differences in the antigenicity of gE or either in vitro and in vivo gE expression has also to be taken into account (Wellenberg et al., 2001). Contrarily, the incidental occurrence of weak false-positive reactions when testing serum samples that have not been frozen or heat-inactivated before the assay (Beer et al., 2003; Isa et al., 2003) has to be rejected. In the current study, all serum samples were frozen, thawed and incubated 30 min. at 56°C, therefore, the diagnostic sensitivity was not affected and the results obtained were relevant. In regards to those, it is interesting to discuss the method described by Wellenberg and collaborators (2001) able to differentiate anti-BoHV-5 from anti-BoHV-1 antibodies. They obtained weak positive reactions in the BoHV-1 gE blocking ELISA with experimental BoHV-5 calves serum samples. Here, the analysis of serum samples from experimentally CpHV-1 infected goats always leaded to gE-negative results (Keuser and Thiry, unpublished results) in contrast to field serum

samples. It would be therefore of interest to analyse BoHV-5 positive field serum samples in order to see if a clear differentiation can be made.

The present work also afforded data about differential and comparative diagnosis of each ruminant alphaherpesvirus related to BoHV-1. Thus, the detection and the identification of the CvHV-1 Anlier strain demonstrated the speed and the efficiency of the consensus PCR developed by Ros and Belák (1999) and confirmed it as a useful diagnostic tool to detect bovine, caprine, red deer and reindeer alphaherpesviruses from field samples. The primer set designed to partly amplify US8 can also be used to detect the related viruses but would be more appropriate for monitoring the spread of live marker vaccine and the gE genotype of viral field isolate (Schynts et al., 1999) as well as the differentiation between these latter and wildtype BoHV-1. Another diagnostic method applied was the immunofluorescence assay developed by Keuser and collaborators (2004a). Attempted reactions between viruses and their specific monoclonal antibody were observed but no reaction was detected with CvHV-1 Anlier. Believing that epitopes of the two CvHV-1 strains could be different, the monoclonal antibody 6C3, which is different from the CvHV-1 monoclonal antibody 6C2, was assessed. By ELISA and flow cytometry, 6C2 detected specifically CvHV-1 but not CvHV-2. Identical results were obtained by ELISA for 6C3 but not by flow cytometry whereas 6C3 detected only weakly CvHV-1 (Keuser et al., 2004a). Following this reasoning, the antigenic differentiation of the two CvHV-1 strains was enabled. The first comparison of restriction patterns of all ruminant alphaherpesviruses was also performed and demonstrated that these related viruses can be clearly differentiated from each other at a genomic level.

Diagnosis and vaccination are complementary tools of control of infectious diseases. Thus, in the context of IBR control and eradication, some European countries have applied a strategy named "Differentiation of Infected from Vaccinated Animals" (Vannier et al., 2007). BoHV-1 marker vaccines carrying a deletion of the gE gene were developed to prevent BoHV-1 infection. The use of these vaccines together with a serological detection of gE-specific antibodies in a BoHV-1 gE blocking ELISA allows the discrimination between infected and vaccinated animals (Muylkens et al., 2007). Such scheme was developed because IBR is a widespread disease with severe economic impact in the European cattle industry. Contrarily to BoHV-1, CpHV-1 infection is not responsible for important issues of commercial interest which could justify a specific vaccine development. However, the serological evidence of CpHV-1 in France extended its geographic distribution in Mediterranean Europe and therefore CpHV-1 appears to be the most relevant ruminant alphaherpesvirus infection besides BoHV-1. Taking into account the close antigenic and genetic relationships between BoHV-1 and CpHV-1, a live attenuated gE-negative BoHV-1 vaccine was assessed in order to protect goats against CpHV-1 infection. The choice of this live vaccine was based on previous studies showing its efficacy and safety in cattle. Indeed, the live attenuated gE-negative vaccine induced immunity within a few days after vaccination (Kaashoek and van Oirschot, 1996). Moreover, the immunity conferred by the live vaccine is large and includes humoral, cell-mediated and mucosal immune responses.

The virulence of gE-negative BoHV-1 had not been determined in goats although natural BoHV-1 infection in goats was already reported (Tolari et al., 1990; Whetstone and Evermann, 1988) and was successfully reproduced in experimental conditions (Six et al., 2001). The current safety assessment demonstrated the absence of adverse effects and local or systemic reactions. The difference of excretion between goats inoculated with either virulent BoHV-1 or gE-negative BoHV-1 vaccine was similar to that was described in cattle (Lemaire et al., 2000). Interestingly, viral excretion by nasal routes in goats suggested a similar pathogenesis of BoHV-1 infection as in cattle. Consequently, goats could be proposed as an alternative model to study virus host interactions. Unfortunately, the immune response seems to be different in the heterologous species

in regards to the weak level of serological reaction observed and therefore could not be analysed with this model. Moreover, the susceptibility of goats to a gE-negative BoHV-1 showed that gE is not an important component involved in the outcome of BoHV-1 infection in the heterologous host contrarily that was demonstrated for gC (Engels et al., 1992). This reasoning could be extended to gI because associated with gE in a functional complex allowing the cell-to-cell spread (Tyborowska et al., 2000; Baranowski et al., 1996). Together, these data demonstrated the utility to vaccinate goats with a live attenuated BoHV-1 gE-negative firstly to protect against CpHV-1 infection and secondly to avoid serological misdiagnosis in the rare cases where goats can be cross-infected with BoHV-1.

The experimental challenge design of the efficacy assessment was similar to those having demonstrated a protection against intranasal CpHV-1 infection by a classical inactivated vaccine (Tempesta et al., 2001). In the preliminary experiment, when goats were immunised twice two weeks a part with the live attenuated gE-negative BoHV-1 vaccine, only a partial cross-protection was afforded against CpHV-1 intranasal challenge. Such nasal infection is rarely reported in natural conditions. The genital mucosa is the principal entry route as demonstrated by the isolation of CpHV-1 DNA in sacral ganglia of latently infected goats (Tempesta et al., 1999b). Therefore, the best method to assess the efficacy of the gE-negative BoHV-1 vaccine was to induce a CpHV-1 intravaginal challenge which was even more severe in the present work than in previous studies (Tempesta et al., 2000; 2001; 2002). The most relevant parameter observed was the reduction of the clinical score. Associated with the absence of gE-negative BoHV-1 shedding after immunisation and a significant reduction of challenge virus excretion, the intranasal inoculation of a live attenuated gE-negative BoHV-1 vaccine appeared to be completely safe and induces both clinical and partial virological protection against CpHV-1 genital infection.

Several studies have investigated the mucosal delivery of vaccines to protect the genital tract against sexually transmitted infectious diseases. The rationale for such studies is based mainly on the concept of a common mucosal immune system (Mestecky et al., 1994). Intranasal immunisation has been commonly used as a mean of mucosal vaccine delivery to provide immune protection to local and distal mucosal surfaces. It is an interesting alternative both for convenience and because the outcome of vaginal vaccination might be dependent on the time point in the oestral cycle for vaccine administration (Johansson et al., 2001). In human, the best known genital herpesvirus is human herpesvirus 2 (HHV-2) which shares the same peripheral pathogenesis and induces similar lesions than CpHV-1 in goats. Different degrees of protection against genital HHV-2 challenge has been demonstrated after intranasal immunisation with adenovirus vectors expressing HHV-2 gB (Gallichan and Rosenthal, 1998), live attenuated HHV-2 strains (Parr and Parr, 1999), or plasmid DNA encoding HHV-2 gB (Kuklin et al., 1997). To date no treatment is available to prevent the disease induced by HHV-2. CpHV-1 infection of goats could be a useful model to assess vaccine prototypes against HHV-2 infection as already done for the development of antiviral therapy (Tempesta et al., 2007a).

The mechanism inducing the genital protection observed after intranasal immunisation was investigated in mice following genital HHV-2 challenge. In vaginal secretions of immunised mice, neutralising antibodies are mainly IgG that is different than in upper respiratory tract where protective antibodies are secretory IgA. The importance of these latter for protective immunity in the upper respiratory tract led to the hypothesis that a strong IgA response is needed to raise an optimal immune protection in the female genital tract. Thus, it was demonstrated that the intranasal immunisation, more than immunisation at any other IgA inductive site, has the potential to induce better protection against genital infections because of its ability to induce IgA responses there (Parr and Parr, 1994; 1997; Murphy et al., 1999). Recently, an inactivated CpHV-1 vaccine in

association with the heat-labile enterotoxin of *Escherichia coli* LTK63 has been demonstrated to induce a strong protection against CpHV-1 genital infection. In this experiment, virus-specific mucosal IgA showed a progressive increasing level starting from the first immunisation to reach a peak after the second immunisation, with a booster post-challenge (Tempesta et al., 2007b). In regard to this result, the likely explanation of the genital protection observed in the current study would be a consequence of the induction of a specific IgA response in goat vaginal secretions.

The key of the vaccination described in third and fourth studies was the antigenic and genetic relationship between BoHV-1 and CpHV-1. Such heterologous vaccination through the species barrier with an already licensed vaccine meets the "cascade" principle and the concept of "minor use - minor species" recently pointed out by the working party of immunological products of the European agency for the evaluation of medicinal products (Committee for Veterinary Medicinal Products, 2004). A minor species includes a wide range of animals other than cattle, horses, pigs, chickens, turkeys, dogs and cats which are classified as major species. Despite its importance in agriculture, goat is considered as a "minor species" and drugs to treat it are included in the category of "minor use" drugs. There are few approved drugs available for "minor use", but efforts to increase their number are being pursued on two fronts: through new legislation and through research partnerships. The protection of goats against CpHV-1 described in the present work illustrates these objectives. Nevertheless, the treatment of minor species is of an important animal health concern. However, the biosafety issue of such vaccination has to be assessed. As for example, the massive use of a live attenuated gE-negative BoHV-1 vaccine raises the question of the risk of recombination between the vaccinal strain, wild type BoHV-1 strains and related viruses. About cross-infection of goats with BoHV-1, the risk of recombination between BoHV-1 and CpHV-1 appears limited because no recombinant virus was detected in vitro between these two viruses (Meurens, 2004). The major requirements of the European Pharmacopoeia for registration of vaccines, a significant reduction of clinical signs and virus shedding, is met and the live attenuated gE-negative BoHV-1 vaccine could be used in goats illustrating the concept "minor use - minor species".

In conclusion, this work contributed to the knowledge of ruminant alphaherpesviruses related to BoHV-1. Further molecular studies should investigate the ruminant alphaherpesvirus history and evolution. The isolation of CvHV-1 in a free ranging red deer and the serological detection of CpHV-1 in goats demonstrated that each BoHV-1 related virus is closely associated with its specific host. However, a continuous attention has to be paid because ruminant hosts are still potential reservoirs of BoHV-1. In this context, the distribution of ruminant alphaherpesviruses in Europe has to be studied in more details especially in wild ruminant populations. The use of BoHV-1 gB blocking ELISA should be currently the best serological tool to detect such infections as for example CvHV-2 in reindeer and BuHV-1 in buffaloes. Nevertheless, it is still of interest to study the antigenic relationship in this viral cluster in order to develop a serological method able to clearly discriminate ruminant alphaherpesvirus infections.

CHAPTER SIX

Summary/Résumé/Sommario

Viral diversity and heterologous protection in the cluster of ruminant alphaherpesviruses related to bovine herpesvirus 1

Diversité virale et protection hétérologue dans le groupe des alphaherpèsvirus de ruminants apparentés à l'herpèsvirus bovin 1

Diversità virale e protezione eterologa nel gruppo di alphaherpesvirus dei ruminanti correlati a bovine herpesvirus 1

Introduction

Herpesviruses have mainly co-evolved with their hosts for millions of years. Consequently, different related host species may have been infected by various genetically related herpesviruses. Illustrating this concept, several ruminant alphaherpesviruses have been shown to form a cluster of viruses closely related to bovine herpesvirus 1 (BoHV-1). This latter virus, prototype of this cluster, is a major pathogen in cattle associated with various clinical manifestations including infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV). IBR is a disease of major economic concern in many parts of the world and especially in Europe, both in European countries where this viral infection has been eradicated and in those where the control of IBR is currently or will be undertaken. The massive use of vaccination allowed the reduction of the number of clinical IBR cases.

However, the existence of alphaherpesviruses closely related to BoHV-1 has to be taken into account in IBR control. The following viruses can be distinguished: bovine herpesvirus 5 (BoHV-5) responsible for severe meningo-encephalitis in calves, bubaline herpesvirus 1 (BuHV-1) inducing a subclinical genital infection in water buffalo (*Bubalus bubalis*), caprine herpesvirus 1 (CpHV-1) causing systemic disease in young kids and abortion in adult goats, cervid herpesvirus 1 (CvHV-1) responsible of severe ocular syndrome in red deer (*Cervus elaphus*), cervid herpesvirus 2 (CvHV-2) and elk herpesvirus 1 (ElkHV-1), which induce a subclinical genital infection respectively in reindeer (*Rangifer tarandus*) and elk (*Cervus canadensis*). Ruminant alphaherpesviruses related to BoHV-1 are antigenically and genetically closely related to each other. Their common biological properties allow heterologous infection and therefore the crossing of the species barrier. Thus, experimental cross-infection studies show that BoHV-1 is able to infect buffalo, goats, red deer, reindeer and sheep. Inversely, bovines are susceptible to BoHV-5, CpHV-1, CvHV-1 and CvHV-2.

Consequently, the main objective of the present work is dedicated to afford a better knowledge of the interaction between alphaherpesviruses and their ruminant hosts. To meet the objective, two approaches have been developed: the study of the viral diversity aiming to extend both epidemiological and virological data about ruminant alphaherpesviruses related to BoHV-1 and the study of the heterologous protection aiming to protect a minor ruminant species against infections with BoHV-1 related alphaherpesviruses.

Results

Illustrating the issues associated to the distribution of ruminant alphaherpesviruses related to BoHV-1, an original situation has been described in Belgium. In 2001 and 2002, 28.9% of red deer have been detected seropositive to BoHV-1. In absence of contact between cattle and red deer, it has been suggested that a virus related to BoHV-1 was spreading in the Belgian red deer population. Nasal and genital swabs have been collected in hunted red deer and have been inoculated onto bovine kidney cells in order to isolate the viral agent responsible of the seropositivity of Belgian red deer. A viral isolate was detected from nasal sample of a male fawn hunted in the Anlier forest. The analysis of a 443 bp sequence of glycoprotein B demonstrated that the viral isolate was related to ruminant alphaherpesviruses and especially CvHV-1. This red deer alphaherpesvirus was then characterised by comparison with the other ruminant alphaherpesviruses. The viral isolate was antigenically distinct from the other related viruses although it presumably possesses some common epitopes with CvHV-1 and CvHV-2 because a weak reaction was detected in immunofluorescence. The analysis of BamHI and BstEII restriction profiles showed ruminant alphaherpesviruses and especially the viral isolate can be distinguished

by their genomic profile. A phylogenetic analysis of these related viruses has been also undertaken. Partial sequence data from UL27 encoding the glycoprotein B (gB) and US8 encoding the glycoprotein E (gE) have been sequenced. This analysis has confirmed that the viral isolate and related viruses constituted a consistent cluster within the subfamily of alphaherpesviruses. BoHV-5 and BuHV-1 clustered together and are the most closely related to BoHV-1 1.1, BoHV-1 1.2. CpHV-1 is the most diverging ruminant alphaherpesvirus. CvHV-1 is more related to BoHV-1 than ElkHV-1 and CvHV-2 which is the most closely related to CpHV-1. The Anlier isolate clustered with CvHV-1 and the most closely related virus is ElkHV-1. The viral isolate is indeed a different CvHV-1 strain than the CvHV-1 Banffshire 82 strain. Taken together, these data report the first isolation of a ruminant alphaherpesvirus in wild fauna, which has been named CvHV-1 Anlier.

This isolation demonstrates that a ruminant can be identified as positive to BoHV-1 while it is infected with another alphaherpesvirus. In France, cattle purchased from herds so-called "IBR-free status" are sporadically identified as seropositive to BoHV-1. Among other hypotheses, the likely explanation for this "false" seropositivity would be a cross-infection with a related alphaherpesvirus. The most "plausible" virus leading to such diagnosis misinterpretation is CpHV-1 infecting goat. In this context, an epidemiologic study has been performed in order to determine if a CpHV-1 infection was present in France. The analysis of 2,548 serums in BoHV-1 gB blocking ELISA revealed that a ruminant alphaherpesvirus infection was spreading in Corse-du-Sud while no goat was detected positive in Dordogne and Vendée. Taking into account the results obtained in Dordogne and Vendée, a specificity of 100% has been measured for the BoHV-1 gB blocking ELISA. By testing serums from goats experimentally infected with CpHV-1, the sensibility has been calculated to 93.5%. The analysis in BoHV-1 gE blocking ELISA showed that 22.6% of gB-seropositive serums were also gE-seropositive. The BoHV-1 gE blocking ELISA was therefore not able to differentiate between BoHV-1 and CpHV-1 infections. However, the analysis of serums in cross seroneutralisation has strongly identified antibodies against CpHV-1 in gB-positive serums. The presence of CpHV-1 in Corse-du-Sud associated to high prevalence (61.9%) in all analysed flocks extends the number of countries infected with CpHV-1. Moreover, the difference observed between Corse-du-Sud and Dordogne and Vendée suggests that CpHV-1 is more prevalent in Mediterranean regions or countries than in Central or northern Europe.

CpHV-1 therefore appears as the BoHV-1 related virus which has the highest economic concern in Europe. Goat is a species defined as minor and the veterinary medicine industry has no commercial interest toward vaccine development protecting this species against CpHV-1. As BoHV-1 and CpHV-1 are antigenically and genetically related, a live attenuated BoHV-1 vaccine carrying a deletion in the gene encoding gE could protect goats against CpHV-1 infection. The vaccine safety has been assessed by intranasal inoculation of either BoHV-1 virulent strain or gE deleted BoHV-1 vaccine in two groups of two goats. The length of viral excretion and the peak viral titre have been decreased in the immunised group. In order to assess the vaccine efficacy, a group of goats was immunised twice two weeks a part with the vaccine while a control group was kept uninoculated. Four weeks later, immunised and control goats have been intranasally challenged with CpHV-1. A decrease of the peak viral titre was observed in immunised goats. However, the viral excretion lengthened two days more than in control group. It is concluded that this live attenuated gE-negative BoHV-1 vaccine induces a partial cross-protection against a CpHV-1 nasal infection in goats.

In regards to the results obtained in this preliminary experiment, the vaccine has been assessed against a CpHV-1 genital infection which is the natural route of infection in goats. To reach this goal, a group of goats was immunised twice three weeks a part with the gE-negative BoHV-1 vaccine followed by a subsequent CpHV-1 intravaginal challenge. To analyse the safety and

the efficacy of this marker vaccine, two groups of goats served as controls: one was immunised with a virulent CpHV-1 and one was kept uninoculated until the challenge. The vaccine did not induce any undesirable local or systemic reaction and goats did not excrete gE-negative BoHV-1. After challenge, a significant reduction in disease severity was observed in immunised goats. Moreover, goats immunised with either gE-negative BoHV-1 or CpHV-1 exhibited a significant reduction in the length and the peak of viral excretion. Antibodies neutralising both BoHV-1 and CpHV-1 were raised in immunised goats. These data show that the intranasal application of a live attenuated gE-negative BoHV-1 vaccine is able to afford a clinical protection and a reduction of virus excretion in goats challenged by a CpHV-1 genital infection.

Conclusions

This study has been performed in order to afford a better knowledge of the interaction between alphaherpesviruses and their ruminant hosts. The antigenic, genetic and genomic relationships existing between BoHV-1 and ruminant alphaherpesviruses have been analysed. It has been demonstrated that these viruses constitute a strong cluster within the subfamily of alphaherpesviruses. The exact knowledge of the evolutionary relationships among these viruses request complete genome sequencing but this analysis is complicated by the large size of herpesvirus genome. Another possibility would be to compare different regions distributed along the genome of each virus as demonstrated by the study of the BoHV-4 history evolution (Dewals et al., 2006). Such analysis could bring interesting data about recombination and transmission events that occurred in the past between herpesviruses and their ruminant species. Nevertheless, the present work gives a contribution to the knowledge of the natural history of the cluster of ruminant alphaherpesviruses related to BoHV-1. Thus, the first isolation of CvHV-1 in a free-ranging red deer linked to the knowledge of the evolution of red deer populations in Europe allowed an interesting hypothesis about the origin of this virus. In regards of the observed prevalence and the presence of red deer over large area, this virus could be responsible of an endemic infection spreading in Europe. This virus may have been introduced after European red deer importation to constitute the initial stocks of Scottish farms. Consequently, CvHV-1 Anlier strain could be the ancestor of the Scottish CvHV-1 Banffshire 82 strain. However, molecular epidemiologic study of several isolates of the two strains is required to firmly assess this hypothesis.

This isolation has demonstrated that misinterpretation of the serologic status of ruminant can be observed; the Belgian red deer was not infected with BoHV-1 but with its own herpesvirus. These difficulties of interpretation are encountered in France in bovines free of IBR and detected later as positive in serology. As BoHV-5 and ElkHV-1 have not been identified in Europe, that there is a very few number of buffalo farms in France, and that CvHV-1 and CvHV-2 transmission to bovine would be exceptional, the only virus that could be responsible of misdiagnosis in France is CpHV-1. Indeed, goats and cattle can be in close contact in some field situations. The results obtained in the second study did not support this hypothesis. Indeed, CpHV-1 infection is either absent or at a very low prevalence in continental France. Contrarily, a very high seroprevalence of CpHV-1 was measured in the Corse-du-Sud department. In Corsica, the contact between cattle and goats is very limited because flocks and herds are not mixed. Therefore, the transmission of CpHV-1 from goats to cattle would be only exceptional, such cross-infection being very rare, and a BoHV-1 infection in goats is not likely to occur. As the current sampling is restricted to Dordogne and Vendée, a particular attention to CpHV-1 has to be paid in continental France and especially in the Mediterranean region. Such monitoring can be performed with the BoHV-1 gB blocking ELISA which has been validated in the current study.

The CpHV-1 infection is therefore extended to the entire Mediterranean region and appears to be the most relevant ruminant alphaherpesvirus infection besides BoHV-1. In this context, it is necessary to protect goats to reduce the CpHV-1 circulation in the caprine population and economic concerns caused by this infection. The preliminary assessment of the live attenuated BoHV-1 vaccine carrying a deletion in the gene encoding gE has shown that goats could respond to such vaccination. As goat is completely susceptible to BoHV-1, this species could also be used as an experimental model to study the in vivo BoHV-1 properties. A partial efficacy against CpHV-1 nasal infection has been observed. Moreover, the gE-negative BoHV-1 vaccine has been assessed against CpHV-1 genital infection which is the natural route of infection in goats. The intranasal use of a gE-negative BoHV-1 vaccine enabled a clinical protection and a significant reduction of the viral excretion in goats infected genitally with CpHV-1. These results demonstrate that nasal mucosa can serve as an efficient site for the induction of a specific protective response in the genital tract. In humans, several mechanisms are proposed to explain such immune induction involving antibody secreting cells and leading to specific IgA and IgG responses. Such mechanisms could be involved in the observed response in vaccinated goats but were not analysed in the current study. The biosafety of such cross protection induced with BoHV-1 vaccine against CpHV-1 has also to be considered. Indeed, viruses can be established in a latent state. In bovine, experiments have shown that gE-negative viruses are less reactivated and reexcreted than wild viruses. Consequently, the risk of reactivation and reexcretion in goats is therefore very low. Otherwise, the emergence of recombinant viruses between CpHV-1 and BoHV-1 after cross infection in goats has to be discussed. Among the cluster of ruminant alphaherpesviruses related to BoHV-1, only two recombinant viruses between BoHV-1 and BoHV-5 were isolated, and no recombinant between BoHV-1 and less closely related CpHV-1 was detected in vitro. Consequently, the cross vaccination described here is likely to be completely safe. Moreover, the use of already licensed vaccine is interesting knowing that the development of new vaccines to protect minor species is of a weak interest for the pharmaceutical industry.

Introduction

Les herpèsvirus ont principalement co-évolué avec leurs hôtes depuis des millions d'années. Par conséquent, différentes espèces hôtes apparentées ont pu être infectées avec des herpèsvirus génétiquement apparentés. Illustrant ce concept, il a été démontré que plusieurs alphaherpèsvirus de ruminants forment un groupe de virus proches de l'herpèsvirus bovin 1 (BoHV-1). Ce dernier virus, prototype du groupe, est un pathogène majeur du bétail associé à différentes manifestations cliniques comprenant la rhinotrachéite infectieuse bovine (IBR) et la vulvovaginite infectieuse pustuleuse (IPV). L'IBR est une maladie à répercussion économique mondiale et plus spécialement en Europe, dont plusieurs états membres ont éradiqué cette infection virale ou entrepris un programme de contrôle de l'IBR. L'utilisation massive de la vaccination a permis une réduction significative du nombre de cas cliniques d'IBR.

Cependant, l'existence d'alphaherpèsvirus étroitement apparentés au BoHV-1 doit être prise en considération dans les programmes de contrôle de l'IBR. On distingue l'herpèsvirus bovin 5 (BoHV-5) responsable de méningo-encéphalites mortelles chez le veau, l'herpèsvirus du buffle (BuHV-1) qui induit une infection génitale subclinique chez le buffle d'eau (*Bubalus bubalis*), l'herpèsvirus caprin 1 (CpHV-1) causant une maladie généralisée chez le chevreau et des affections génitales chez la chèvre, l'herpèsvirus du cerf (CvHV-1) responsable d'une grave maladie oculaire chez le cerf (*Cervus elaphus*), les herpèsvirus du renne (CvHV-2) et du wapiti (ElkHV-1), qui induisent une infection génitale subclinique respectivement chez le renne (*Rangifer tarandus*) et chez le wapiti (*Cervus canadensis*). Les alphaherpèsvirus de ruminants apparentés au BoHV-1 sont antigéniquement et génétiquement proches entre eux. Leurs propriétés biologiques communes leur permet d'infecter des espèces hétérologues et donc de traverser la barrière d'espèce. Ainsi, des expériences d'infections croisées ont montré que le BoHV-1 était capable d'infecter le buffle, la chèvre, le cerf et le renne, ainsi que le mouton. Inversement, les bovins sont sensibles aux BoHV-5, CpHV-1, CvHV-1 et CvHV-2.

Par conséquent, l'objectif principal de ce travail est d'apporter une meilleure connaissance de l'interaction entre les alphaherpèsvirus et leurs hôtes ruminants. Afin d'atteindre cet objectif, deux approches ont été développées : l'étude de la diversité virale ayant pour but d'étendre les données épidémiologiques et virologiques concernant les alphaherpèsvirus de ruminants apparentés au BoHV-1, et l'étude de la vaccination hétérologue ayant pour but de protéger les espèces de ruminants considérées comme mineures contre l'infection par les alphaherpèsvirus apparentés au BoHV-1.

Résultats

Illustrant la problématique liée à la distribution des alphaherpèsvirus de ruminants apparentés au BoHV-1, une situation originale a été décrite en Belgique. En 2001 et 2002, 28,9% de cerfs ont été détectés séropositifs envers le BoHV-1. En l'absence de contact entre le bétail et les cerfs, il a été suggéré qu'un virus apparenté au BoHV-1 circulait dans la population belge de cerfs. Des écouvillons nasaux et génitaux ont été prélevés sur des cerfs tués sur chasse et ont été inoculés à des cellules rénales bovines afin d'isoler l'agent viral responsable de la séropositivité des cerfs belges. Un isolat viral a été détecté à partir du prélèvement nasal d'un faon male tué dans la forêt d'Anlier. L'analyse d'une séquence de 443 pb de la glycoprotéine B a montré que l'isolat viral était apparenté au groupe des alphaherpèsvirus de ruminants et plus spécialement au CvHV-1. Cet alphaherpèsvirus de cerf fut alors caractérisé par rapport aux autres alphaherpèsvirus de ruminants. L'isolat viral était différent des autres virus apparentés au niveau antigénique bien qu'il

possède certainement des épitopes en commun avec le CvHV-1 et le CvHV-2 car une faible réaction est détectée en immunofluorescence. L'analyse des profils de restriction par BamHI et BstEII a montré que les alphaherpèsvirus de ruminants et plus spécialement l'isolat viral pouvaient être différenciés par leurs profils génomiques. Une analyse phylogénétique des différents virus a également été entreprise. A cette fin, une région du gène UL27 codant la glycoprotéine B (gB) et une région du gène US8 codant la glycoprotéine E (gE) ont été séquencées. Cette analyse a confirmé que l'isolat viral et les virus apparentés formaient un groupe consistant au sein de la sous-famille des alphaherpèsvirus. Le BoHV-5 et le BuHV-1 se groupent ensemble et sont les virus les plus proches des sous-types 1 et 2 du BoHV-1. Le CpHV-1 est le virus le plus divergent au sein des alphaherpèsvirus de ruminants. Le CvHV-1 est plus apparenté au BoHV-1 que l'ElkHV-1 et le CvHV-2, ce dernier étant le plus proche du CpHV-1. L'isolat viral forme un groupe avec le CvHV-1 et est en fait une souche de CvHV-1 différente de la souche de référence CvHV-1 Banffshire 82. L'ensemble de ces résultats décrit le premier isolement d'un alphaherpèsvirus de ruminant en faune sauvage, qui a été dénommé CvHV-1 Anlier.

Cet isolement démontre qu'un ruminant peut être identifié comme positif envers le BoHV-1 alors qu'il est infecté par un autre alphaherpèsvirus. En France, certains bovins issus d'élevages possédant une appellation « indemnes d'IBR » sont sporadiquement révélés positifs à l'achat. Une explication de cette fausse séropositivité serait une infection croisée avec un alphaherpèsvirus apparenté. Le virus le plus plausible causant ce type d'erreur de diagnostic est le CpHV-1 infectant la chèvre. Dans ce cadre, une enquête épidémiologique a été réalisée pour déterminer si une infection à CpHV-1 était présente en France. L'analyse de 2548 sérums par un ELISA de blocage BoHV-1 gB a révélé qu'une infection à alphaherpèsvirus de ruminant circulait en Corse-du-Sud alors qu'aucune chèvre n'était détectée positive en Dordogne et en Vendée. Sur base des résultats obtenus en Dordogne et Vendée, une spécificité de 100% a été mesurée pour l'ELISA de blocage BoHV-1 gB. En testant des sérums de chèvres expérimentalement infectées avec le CpHV-1, la sensibilité du test a été calculée à 93,5%. L'analyse par un ELISA de blocage BoHV-1 gE a montré que 22,6% des sérums gB-positifs étaient également gE-positifs. L'ELISA gE n'était donc pas capable de différencier les infections à BoHV-1 et à CpHV-1. Cependant, une analyse de ces sérums par séroneutralisation croisée a formellement identifié la présence d'anticorps dirigés contre le CpHV-1 dans les sérums positifs envers gB. La présence du CpHV-1 en Corse-du-Sud, supportée par une prévalence élevée (61,9%) dans toutes les chèvreries analysées, étend le nombre de pays infectés par le CpHV-1. De plus, la différence observée entre la Corse-du-Sud et la Dordogne et la Vendée suggère que le CpHV-1 est plus prévalent dans les régions ou pays méditerranéens qu'en Europe centrale ou du nord.

Le CpHV-1 apparaît donc comme le virus apparenté au BoHV-1 qui a la plus grande importance épidémiologique en Europe. La chèvre est une espèce qualifiée de mineure et l'industrie du médicament vétérinaire ne montre pas d'intérêt à développer un vaccin protégeant cette espèce contre le CpHV-1. Comme le BoHV-1 et le CpHV-1 sont antigéniquement et génétiquement apparentés, un vaccin vivant atténué porteur d'une délétion dans le gène codant la gE du BoHV-1 pourrait protéger la chèvre contre l'infection à CpHV-1. L'innocuité du vaccin a été évaluée par l'inoculation intranasale d'une souche sauvage de BoHV-1 ou du vaccin BoHV-1 délété de la gE dans deux groupes de chèvres. La durée d'excrétion virale et le pic du titre viral ont été réduits dans le groupe vacciné. Afin d'évaluer l'efficacité du vaccin, un groupe de chèvres a été immunisé à deux semaines d'intervalle avec le vaccin alors qu'un groupe, servant de contrôle, n'a pas été inoculé. Quatre semaines plus tard, les chèvres immunisées et contrôles ont été soumis à une infection nasale par le CpHV-1. Une diminution du pic du titre viral de l'ordre de deux $\log_{10}$ a été observée chez les chèvres immunisées. Cependant, l'excrétion virale a duré deux jours de plus

qu'au sein du groupe contrôle. Il en ressort donc que ce vaccin vivant délété de la gE du BoHV-1 induit une protection croisée partielle contre l'infection nasale de la chèvre par le CpHV-1.

Sur base des résultats issus de cette expérience préliminaire, le vaccin a été testé contre une infection génitale par le CpHV-1, qui est la voie naturelle d'infection chez la chèvre. A cette fin, un groupe de chèvres a été immunisé deux fois à trois semaines d'intervalle avec le vaccin BoHV-1 gE-négatif suivi par une épreuve virulente avec le CpHV-1 inoculé par voie intravaginale. Pour analyser l'innocuité et l'efficacité du vaccin, deux groupes de chèvres ont servi de contrôle: un groupe immunisé par voie intranasale avec le CpHV-1 et un groupe non inoculé jusqu'à l'épreuve virulente. Le vaccin n'a pas induit de réactions locale ou généralisée indésirables et les chèvres n'ont pas excrété le virus vaccinal BoHV-1 gE-négatif. Après l'épreuve virulente, une diminution significative des signes cliniques a été observée chez les chèvres vaccinées. De plus, les chèvres immunisées avec le vaccin ou le CpHV-1 ont montré une diminution de la durée et du pic d'excrétion virale. Des anticorps neutralisant à la fois le BoHV-1 et le CpHV-1 ont également été détectés. L'ensemble de ces résultats montre que l'application intranasale de ce vaccin vivant atténué délété de la gE du BoHV-1 apporte une protection clinique et une diminution de l'excrétion virale chez les chèvres infectées par le CpHV-1 par la voie génitale.

Conclusions

Ce travail a été réalisé afin d'obtenir une meilleure connaissance des interactions entre les alphaherpèsvirus et leurs hôtes ruminants. Les relations antigéniques, génétiques et génomiques existant entre le BoHV-1 et les alphaherpèsvirus de ruminants ont été analysées. Il a été démontré notamment que ces virus formaient un groupe consistant au sein de la sous-famille des alphaherpèsvirus. La relation évolutive exacte entre ces virus apparentés requiert certainement un séquençage complet du génome. Cette analyse est compliquée à mettre en œuvre car les herpèsvirus possèdent un génome de grande taille. Cependant, la comparaison de différentes régions au niveau du génome pourrait être envisagée et permettrait d'obtenir des informations intéressantes concernant les événements de recombinaison et de transmission qui se sont produits entre ces virus. Toutefois, les données obtenues ont permis de contribuer à la connaissance de l'histoire naturelle de ce groupe d'alphaherpèsvirus de ruminants. Ainsi, le premier isolement de CvHV-1 chez un cerf sauvage associé à la connaissance de l'évolution des populations de cerfs en Europe permet d'émettre une hypothèse intéressante à propos de l'origine de ce virus. En effet, en tenant compte des prévalences observées dans les pays de l'Est et la présence de cerfs sur de grandes étendues en Europe, le CvHV-1 Anlier pourrait être responsable d'une infection endémique circulant de l'Europe de l'Est vers l'Europe occidentale. Ce virus aurait pu être introduit après importation de cerfs européens afin de constituer les stocks initiaux dans les fermes écossaises. Par conséquent, le CvHV-1 Anlier pourrait être l'ancêtre du CvHV-1 Banffshire 82 isolé en Ecosse. Cependant, une étude d'épidémiologie moléculaire de plusieurs isolats est requise pour confirmer cette hypothèse.

Cet isolement a montré que des erreurs d'interprétation quant au statut sérologique des ruminants peuvent survenir, le cerf belge n'était pas infecté par le BoHV-1 mais bien par son herpèsvirus spécifique. Ces difficultés d'interprétation sont reproduites en France chez des bovins certifiés indemnes d'IBR et détectés par la suite positifs en sérologie. Etant donné que le BoHV-5 et l'ElkHV-1 n'ont pas été identifiés en Europe, que les fermes de buffles sensibles au BuHV-1 sont extrêmement rares en France, et que la transmission du CvHV-1 ou du CvHV-2 chez le bovin serait exceptionnelle, le seul virus apparenté au BoHV-1 pouvant être responsable d'erreur de diagnostic est le CpHV-1, principalement dû au fait que les chèvres et le bétail pourraient être en contact sur

le terrain. Les résultats obtenus lors de l'analyse séroépidémiologique de l'infection à CpHV-1 en France ne supportent pas cette hypothèse. En effet, l'infection à CpHV-1 soit n'est pas présente, soit est présente à une très faible prévalence en France continentale. Par contre, des prévalences élevées ont été mesurées en Corse-du-Sud. Cependant, en Corse, le contact entre les chèvres et les bovins apparaît très limité car les élevages ne sont pas mélangés. La transmission du CpHV-1 au bovin serait très certainement exceptionnelle. Etant donné que l'échantillonnage présent était restreint à la Dordogne et la Vendée, il faut rester vigilant vis à vis de cette infection en France continentale et plus spécialement dans la région méditerranéenne, le CpHV-1 ayant été identifié en Corse. Ce monitoring peut être réalisé à l'aide de l'ELISA de blocage BoHV-1 gB qui a été validé pour la détection du CpHV-1 lors de cette étude.

L'infection du CpHV-1 est donc étendue à l'ensemble de la région méditerranéenne et apparaît être l'infection à alphaherpèsvirus de ruminants épidémiologiquement la plus importante à côté du BoHV-1. Dans ce contexte, il est nécessaire de protéger les chèvres afin de limiter la circulation du CpHV-1 au sein de la population caprine et les répercussions économiques provoquées par cette infection. L'évaluation préliminaire du vaccin vivant atténué porteur d'une délétion dans le gène codant la gE a montré que la chèvre était réceptive à ce type de vaccination. Etant donné que la chèvre est complètement sensible au BoHV-1, cette espèce pourrait être utilisée comme modèle expérimental de moindre coût pour l'étude des propriétés *in vivo* du BoHV-1. Une efficacité partielle contre l'infection nasale par le CpHV-1 a été observée. De plus, le vaccin BoHV-1 gE-négatif a été évalué contre l'infection génitale par le CpHV-1 qui est la voie naturelle d'infection chez la chèvre. L'administration intranasale de ce vaccin vivant atténué a induit une protection clinique et une diminution significative de l'excrétion virale chez les chèvres infectées par voie génitale avec le CpHV-1. Ceci montre que la muqueuse nasale peut être un site efficace pour l'induction d'une réponse protectrice spécifique dans le tractus génital. Chez l'homme, plusieurs mécanismes sont proposés pour expliquer cette induction d'immunité impliquant des cellules sécrétrices d'anticorps spécifiques et conduisant à des réponses IgA et IgG. Ces mécanismes pourraient intervenir dans la réponse observée chez les chèvres vaccinées mais n'ont pas été investigué lors de ce travail. La protection croisée induite par un vaccin BoHV-1 contre une infection par le CpHV-1 pose aussi des questions de biosécurité. Il faut considérer un éventuel établissement du virus sous forme latente. Des expériences chez les bovins ont montré que les virus BoHV-1 gE-négatif sont moins réactivés et réexcrétés que les virus BoHV-1 sauvages. Par conséquent, le risque de réactivation et de réexcrétion chez la chèvre doit être très faible. La question de l'émergence de recombinants entre le CpHV-1 et le BoHV-1 après infection croisée chez la chèvre doit également être posée. Des expériences de co-infection entre les différents alphaherpèsvirus de ruminants ont permis l'isolement in vitro de recombinants entre le BoHV-1 et le BoHV-5 mais aucun recombinants entre le BoHV-1 et le CpHV-1 n'ont été détectés. Cette vaccination croisée apparaît donc être complètement sûre. De plus, l'utilisation d'un vaccin déjà commercialisé est intéressante sachant que le développement de nouveaux vaccins pour protéger les espèces mineures est de faible intérêt pour l'industrie pharmaceutique.

Introduzione

I virus erpetici si sono per la maggior parte co-evoluti con i loro ospiti nell'arco di milioni di anni. Di conseguenza, specie ospiti correlate possono essere infettate da diversi herpesvirus geneticamente correlati. E' stato osservato che diversi alphaherpesvirus dei ruminanti hanno formato un gruppo di virus strettamente correlati a bovine herpesvirus 1 (BoHV-1). Quest'ultimo, che rappresenta il virus prototipo di questo gruppo, è uno dei patogeni più importanti del bovino, associato a diverse manifestazioni cliniche tra cui la rinotracheite infettiva bovina (IBR), e la vulvovaginite pustolosa infettiva (IPV). L'IBR è una malattia economicamente molto importante in diverse parti del mondo e soprattutto in Europa sia in quei paesi dove l'infezione è stata eradicata sia in quelli in cui sono in corso o stanno per essere intrapresi piani di controllo. Il massiccio uso delle vaccinazioni ha permesso di ridurre il numero dei casi clinici di IBR.

Tuttavia, nell'ottica del controllo dell'IBR deve essere tenuta in conto l'esistenza di alphaherpesvirus strettamente correlati a BoHV-1. Sono da prendere in considerazione bovine herpesvirus 5 (BoHV-5), responsabile di meningoencefalite nei vitelli, bubaline herpesvirus 1 (BuHV-1) che causa un'infezione genitale a decorso subclinico nel bufalo d'acqua (*Bubalus bubalis*), caprine herpesvirus 1 (CpHV-1) che causa un'infezione sistemica nei capretti ed aborto nelle capre adulte, cervid herpesvirus 1 (CvHV-1) responsabile di una grave sindrome oculare nel cervo (*Cervus elaphus*), cervid herpesvirus 2 (CvHV-2) ed elk herpesvirus 1 (ElkHV-1) che inducono un'infezione genitale a decorso subclinico rispettivamente nella renna (*Rangifer tarandus*) e nell'alce (*Cervus canadensis*). Gli alphaherpesvirus dei ruminanti correlati a BoHV-1 sono antigenicamente e geneticamente correlati tra loro. Le proprietà biologiche che li accomunano permettono loro di infettare specie eterologhe e quindi di superare le barriere di specie. In tal senso, studi sperimentali di infezione crociata hanno dimostrato che BoHV-1 è in grado di infettare i bufali. All'inverso i bovini sono recettivi all'infezione da BoHV-5, CpHV-1, CvHV-1 and CvHV-2.

Di conseguenza, l'obiettivo principale del presente lavoro è quello di dare un contributo per una più approfondita conoscenza dell'interazione tra gli alphavirus e i loro ospiti ruminanti. Sono stati utilizzati due approcci: lo studio della diversità dei virus allo scopo di fornire ulteriori dati virologici ed epidemiologici riguardo gli alphaherpesvirus dei ruminanti correlati a BoHV-1 e lo studio della protezione eterologa al fine di proteggere le specie ruminanti minori nei confronti delle infezioni da herpesvirus correlati a BoHV-1.

Risultati

Una situazione particolare è stata descritta in Belgio che può illustrare le problematiche associate alla distribuzione degli alphaherpesvirus dei ruminanti correlati a BoHV-1. Nel 2001 e 2002, il 28.9% dei cervi è risultato sierologicamente positivo nei confronti di BoHV-1. In considerazione della mancanza di contatto tra bovini e cervi è stato ipotizzato che un virus correlato a BoHV-1 stesse diffondendo nella popolazione cervina del Belgio. Tamponi nasali e genitali prelevati da cervi cacciati sono stati inoculati in colture di cellule renali di bovino al fine di isolare il virus responsabile della sieropositività dei cervi belgi. Un virus è stato isolato da un campione nasale di un daino maschio cacciato nella foresta di Anlier. L'analisi di una sequenza di 442 bp della glicoproteina B ha dimostrato che il virus isolato era correlato agli alphaherpesvirus dei ruminanti, soprattutto a CvHV-1. L'herpesvirus del cervo è stato poi caratterizzato sulla base del confronto con gli altri herpesvirus dei ruminanti. Il virus era antigenicamente distinto dagli altri virus correlati benché presumibilmente condividesse alcuni epitopi con CvHV-1 e CvHV-2 come evidenziato da una debole reattività al test di immunofluorescenza. L'analisi di restrizione con gli

enzimi BamHI e BstEII ha permesso la differenziazione dei diversi alphaherpesvirus dei ruminanti, soprattutto del virus isolato, sulla base dei loro profili genomici. E' stata effettuata l'analisi filogenetica di questi virus correlati. In quest'ottica sono stati parzialmente sequenziati il segmento UL27 che codifica la glicoproteina B (gB) e il segmento US8 che codifica la glicoproteina E (gE). Tale analisi ha confermato che sia il virus isolato che i virus correlati costituiscono un gruppo omogeneo all'interno della sottofamiglia degli alphaherpesvirus. BoHV-5 and BuHV-1 fanno parte dello stesso gruppo e sono gli alphaherpesvirus maggiormente correlati a BoHV-1 1.1 e BoHV-1 1.2. CpHV-1 è l'alphaherpesvirus dei ruminanti che maggiormente si discosta. CvHV-1 è più correlato a BoHV-1 rispetto a ElkHV-1 e CvHV-2 che è il virus più strettamente correlato a CpHV-1. L'isolato di Anlier è raggruppato con CvHV-1 ed il virus più strettamente correlato è ElKHV-1. Il virus isolato è un ceppo CvHV-1 diverso dal ceppo CvHV-1 Banffshire 82. Nell'insieme, questi dati descrivono il primo isolamento di un alphaherpesvirus dei ruminanti nella fauna selvatica, denominato poi ceppo Anlier di CvHV-1.

Questo isolamento dimostra che un bovino può essere identificato come postivo nei confronti di BoHV-1 mentre in realtà è infetto con un altro alphaherpesvirus. In Francia, bovini acquistati da allevamenti denominati "indenni da IBR" sono sporadicamente identificati come sieropositivi nei confronti di BoHV-1. Tra le varie ipotesi che possono essere formulate, la spiegazione più probabile per questa "falsa" sieropositività potrebbe essere un' infezione crociata con un alphaherpesvirus correlato. Il virus maggiormente imputato di queste erronee interpretazioni diagnostiche è CpHV-1 che infetta le capre. In questo contesto è stato effettuato uno studio epidemiologico per valutare se in Francia è presente l'infezione da CpHV-1. L'analisi di 2,548 sieri mediante un test gB blocking Elisa per BoHV-1 ha rivelato che un'infezione da alphaherpesvirus dei ruminanti si stava diffondendo nella Corsica del Sud mentre era completamente assente in Dordogne e Vendée. Tenendo conto dei risultati ottenuti in Dordogne e Vendée il test BoHV-1 gB blocking ELISA ha dimostrato una specificità del 100%. La sensibilità è stata calcolata del 93.5% quando sono stati testati i sieri di capre sperimentalmente infettate con CpHV-1. Il test BoHV-1 gE blocking Elisa ha dimostrato che il 22.6% dei sieri gB positivi era anche gE positivi. Il test BoHV-1 gE blocking ELISA non è stato in grado, quindi, di differenziare le infezioni da CpHV-1 e BoHV-1. Tuttavia, l'analisi dei sieri mediante il test di sieroneutralizzazione crociata ha chiaramente identificato gli anticorpi indotti da CpHV-1 nei sieri gB positivi. La presenza di CpHV-1 nella Corsica del Sud, insieme all'alta sieroprevalenza riscontrata (61.9%) in tutte le capre analizzate allunga la lista dei paesi in cui è presente l'infezione da CpHV-1. In più, la differenza evidenziata tra la Corsica del Sud e la Dordogne e la Vendée suggerisce che CpHV-1 ha una prevalenza maggiore nelle regioni o nei paesi che si affacciano sul Mediterraneo piuttosto che nell'Europa Centrale o del Nord.

CpHV-1 quindi sembra essere il virus correlato a BoHV-1 con il maggiore impatto economico in Europa. Le capre sono considerate una specie minore e l'industria farmaceutica veterinaria è scarsamente interessata allo sviluppo di vaccini per la profilassi dell'infezione da CpHV-1. Dal momento che BoHV-1 e CpHV-1 sono geneticamente e antigenicamente correlati, è ipotizzabile che un vaccino attenuato costituito da un ceppo di BoHV-1 deleto per il gene codificante la gE potrebbe indurre protezione delle capre nei confronti dell'infezione da CpHV-1. La sicurezza del vaccino è stata accertata tramite inoculazione intranasale di due gruppi di due capre, con un ceppo di BoHV-1 virulento e un vaccino BoHV-1 deleto per la gE. Nel gruppo di animali immunizzati è stata osservata la diminuzione sia della durata dell'escrezione virale che del picco del titolo virale escreto. Al fine di verificare l'efficacia del vaccino, un gruppo di capre è stato immunizzato con il vaccino due volte a distanza di due settimane mentre un gruppo è stato tenuto come controllo e non vaccinato. Quattro settimane dopo, sia il gruppo immunizzato che quello di controllo sono stati infettati per via intranasale con CpHV-1. Nelle capre immunizzate è stata

osservata una diminuzione di due log_{10} del picco del titolo virale escreto. Tuttavia, l'escrezione virale è durata due giorni in più rispetto al gruppo di controllo. In conclusione il vaccino attenuato contenente BoHV-1 gE negativo induce una parziale protezione crociata nei confronti dell'infezione nasale da CpHV-1.

Facendo riferimento ai risultati ottenuti in questo esperimento preliminare, il vaccino è stato valutato nei confronti dell'infezione genitale da CpHV-1 che rappresenta la via naturale d'infezione nelle capre. A tale scopo, un gruppo di capre è stato immunizzato due volte a tre settimane di distanza con un vaccino attenuato contenente BoHV-1 deleto per la gE, e successivamente è stato infettato per via genitale con CpHV-1. Al fine di analizzare la sicurezza e l'efficacia di questo vaccino marker, due gruppi di capre sono stati utilizzati come controllo: un gruppo è stato immunizzato con ceppo virulento di CpHV-1 e l'altro non è stato inoculato fino al momento del challenge. Il vaccino non ha indotto alcun effetto avverso né locale né generale e le capre non hanno eliminato BoHV-1 gE negativo. Dopo il challenge, nelle capre immunizzate è stata osservata una diminuzione della gravità della malattia. Inoltre sia le capre immunizzate con BoHV-1 gE negativo che quelle immunizzate con CpHV-1 hanno avuto una significativa riduzione della durata dell'escrezione virale e del piccolo del titolo virale eliminato. Le capre immunizzate hanno prodotto anticorpi neutralizzanti nei confronti di BoHV-1 e CpHV-1. Questi dati dimostrano che la somministrazione intranasale del vaccino attenuato con BoHV-1 gE negativo è in grado di offrire protezione clinica e riduzione dell'escrezione del virus nelle capre infettate con CpHV-1 per via genitale.

Conclusioni

Questo studio è stato condotto con lo scopo di contribuire ad una migliore conoscenza dell'interazione tra gli alphaherpesvirus e i loro ospiti ruminanti. Sono state analizzate le relazioni antigeniche, genetiche e genomiche esistenti tra BoHV-1 e gli alphaherpesvirus dei ruminanti. E' stato dimostrato che questi virus costituiscono un gruppo molto forte all'interno della sottofamiglia degli alphaherpesvirus. La perfetta conoscenza delle relazioni evolutive esistenti tra questi virus richiede il sequenziamento completo del genoma ma questa analisi è complicata dalla grandezza del genoma degli herpesvirus. Un'altra possibilità potrebbe essere offerta dal confronto di differenti regioni distribuite nel genoma di ciascun virus come dimostrato dallo studio dell'evoluzione di BoHV-4. Tale analisi potrebbe fornire dati interessanti riguardo i fenomeni di ricombinazione e di trasmissione che sono accaduti nel passato tra gli herpesvirus e i loro ospiti ruminanti. Ciononostante, il presente lavoro offre un contributo alla conoscenza della storia naturale del gruppo di alphaherpesvirus dei ruminanti correlati a BoHV-1. Così, il primo isolamento di CvHV-1 in un cervo selvatico, unito alla conoscenza dell'evoluzione della popolazione cervina in Europa, ha permesso di formulare un'interessante ipotesi circa l'origine di questo virus. In considerazione della prevalenza osservata e della presenza dei cervi su una vasta area, questo virus potrebbe essere responsabile di un'infezione endemica che si sta diffondendo n Europa. Questo virus potrebbe essere stato introdotto in seguito all'importazione di cervi in Europa per costituire il primo nucleo di animali nelle fattorie scozzesi. Di conseguenza, il ceppo Anlier di CvHV-1 potrebbe rappresentare il progenitore del ceppo scozzese Banffshire 82 di CvHV-1. Tuttavia, è necessario uno studio epidemiologico molecolare di diversi isolati dei due ceppi per confermare definitivamente questa ipotesi.

Questo isolamento ha dimostrato che può esistere una confusione riguardo lo stato sierologico dei ruminanti; il cervo belga non era infetto con BoHV-1 ma con il suo proprio omologo herpesvirus. Tali difficoltà di interpretazione si sono verificate in Francia in bovini indenni da IBR

riscontrati successivamente sierologicamente positivi. Dal momento che BoHV-5 e ElkHV-1 non sono stati identificati in Europa, che in Francia è presente un numero molto baso di allevamenti di bufali e che la trasmissione di CvHV-1 e CvHV-2 al bovino dovrebbe essere eccezionale, l'unico virus che potrebbe essere responsabile della confusione diagnostica in Francia è CpHV-1 poiché capre e bovini possono essere a stretto contatto in alcune situazioni di campo. I risultati ottenuti nel secondo studio non supportano questa ipotesi. Invero, l'infezione da CpHV-1 è assente o a prevalenza molto bassa nella Francia continentale. Al contrario, una sieroprevalenza molto alta di infezione da CpHV-1 è stata riscontrata nella Corsica del Sud. In Corsica, il contatto tra bovini e capre è molto limitato perché capre e bovini non sono allevati in promiscuità. Quindi, la trasmissione di CpHV-1 da capre a bovini dovrebbe essere eccezionale essendo l'infezione crociata molto rara e l'infezione da BoHV-1 nelle capre difficile da osservare. Poiché il campionamento è stato ristretto ai compartimenti della Dordogne e della Vendée, è auspicabile che lo studio epidemiologico su CpHV-1 sia esteso alla Francia continentale e soprattutto alla regione mediterranea. Tale monitoraggio potrebbe essere condotto con il test BoHV-1 gB blocking ELISA che è stato validato nel presente lavoro.

L'infezione da CpHV-1 si è quindi estesa a tutta la regione mediterranea e sembra essere l'infezione da alphaherpesvirus dei ruminanti più importante dopo quella da BoHV-1. In questo contesto è necessario proteggere le capre per ridurre la circolazione di CpHV-1 nella popolazione caprina e l'impatto economico dell'infezione. La valutazione preliminare del vaccino vivo attenuato costituito da BoHV-1 gE negativo, ha dimostrato che le capre sono "recettive" a tale vaccinazione. Poiché le capre sono totalmente sensibili a BoHV-1, questa specie potrebbe essere utilizzata come un modello sperimentale per studiare le caratteristiche di BoHV-1 in vivo. E' stata osservata una parziale efficacia del vaccino nei confronti dell'infezione nasale da CpHV-1. Inoltre, il vaccino BoHV-1 gE negativo è stato valutato nei confronti dell'infezione genitale da CpHV-1 che è la via naturale d'infezione nelle capre. L'uso della via intranasale per la somministrazione di un vaccino BoHV-1 gE negativo, ha determinato la protezione clinica e una significativa riduzione dell'escrezione virale nelle capre infettate per via genitale con CpHV-1. Questi risultati dimostrano che la mucosa nasale può rappresentare un sito efficiente per l'induzione di una risposta immune specifica nel tratto genitale. Nell'uomo, diversi meccanismi sono stati proposti per spiegare tale induzione immunitaria che coinvolge le cellule secernenti anticorpi e che conduce ad una risposta specifica rappresentata dalla produzione di IgA e IgG. Tali meccanismi potrebbero essere coinvolti nella risposta che è stata osservata nelle capre vaccinate ma non sono stati analizzanti nel presente lavoro. Deve essere presa in considerazione la biosicurezza di tale protezione crociata indotta con un vaccino costituito da BoHV-1 nei confronti di CpHV-1. Invero, i virus possono stabilire delle infezioni latenti. Gli esperimenti hanno dimostrato che nei bovini i virus gE negativi sono riattivabili meno facilmente ed escreti in misura minore rispetto ai virus selvaggi. Di conseguenza, il rischio di riattivazione e re-escrezione nelle capre è molto basso. In caso contrario, dovrebbe essere discussa anche l'insorgenza di virus ricombinanti tra CpHV-1 e BoHV-1 dopo infezione crociata. Tra i gruppi di alphaherpesvirus dei ruminanti correlati a BoHV-1, solo due virus ricombinanti tra BoHV-1 e BoHV-5 sono stati isolati e nessun ricombinante tra BoHV-1 e il virus meno strettamente correlato CpHV-1 è stato svelato in vitro. Di conseguenza la vaccinazione crociata, qui descritta è verosimilmente sicura. Inoltre l'uso di un vaccino già registrato è interessante dal punto di vista commerciale in considerazione dello scarsa propensione delle industrie farmaceutiche a sviluppare nuovi vaccini per proteggere le specie minori.

CHAPTER SEVEN

References

Ackermann M., Metzler A.E., McDonaugh H., Bruckner L., Muller H.K., Kihm U., Stellen nichtbovine paarhufer ein IBR-Virus-reservoir dar? I. BHV-1 und CapHV-1 infektions und reaktivierungsversuche an ziegen, virustyp-spezifitat der humoralen antikorper und charakterisierung der viralen antigene, Schweiz. Arch. Tierheilkd. 128 (1986) 557-573.

Aguirre A.A., Hansen D.E., Starkey E.E., McLean R.G., Serologic survey of wild cervids for potential disease agents in selected national parks in the United States, Prev. Vet. Med. 21 (2005) 313–322.

Alegre M., Nanni M., Fondevila N., Development of a multiplex polymerase chain reaction for the differentiation of bovine herpesvirus-1 and -5, J. Vet. Med. B Infect. Dis. Vet. Public Health 48 (2001) 613-621.

Al-Mubarak A., Chowdhury S., In the absence of glycoprotein I (gI), gE determines bovine herpesvirus type 5 neuroinvasiveness and neurovirulence, J. Neurovirol. 10 (2004a) 233-243.

Al-Mubarak A., Zhou Y., Chowdhury S.I., A glycine-rich bovine herpesvirus 5 (BHV-5) gE-specific epitope within the ectodomain is important for BHV-5 neurovirulence, J. Virol. 78 (2004b) 4806-4816.

Bagust T.J., Comparison of the biological, biophysical and antigenic properties of four strains of infectious bovine rhinotracheitis herpesvirus, J. Comp. Pathol. 82 (1972a) 365-374.

Bagust T.J., Clark L., Pathogenesis of meningo-encephalitis produced in calves by infectious bovine rhinotracheitis herpesvirus, J. Comp. Pathol. 82 (1972b) 375-383.

Baranowski E., Dubuisson J., van Drunen Littel-van den Hurk S., Babiuk A.L., Michel A., Pastoret P.-P., Thiry E., Synthesis and processing of bovine herpesvirus-1 glycoprotein H, Virology 206 (1995) 651-654.

Baranowski E., Keil G., Lyaku J., Rijsewijk F.A., van Oirschot J.T., Pastoret P.-P., Thiry E., Structural and functional analysis of bovine herpesvirus 1 minor glycoproteins, Vet. Microbiol. 53 (1996) 91-101.

Bartha A., Hajdu G., Aldasy P., Paczolay G., Occurrence of encephalitis caused by infectious bovine rhinotracheitis virus in calves in hungary, Acta Vet. Acad. Sci. Hung. 19 (1969) 145-151.

Beer M., Konig P., Schielke G., Trapp S., Diagnostic markers in the prevention of bovine herpesvirus type 1: possibilities and limitations, Berl. Munch. Tierarztl. Wochenschr. 116 (2003) 183-191.

Belak K., Kucsera L., Ros C., Kulcsar G., Makranszki L., Soos T., Belak S., Studies on the pathogenicity of bovine herpesvirus type 5 in sheep, Comp. Immunol. Microbiol. Infect. Dis. 22 (1999) 207-220.

Belknap E.B., Collins J.K., Ayers V.K., Schultheiss P.C., Experimental infection of neonatal calves with neurovirulent bovine herpesvirus type 1.3, Vet. Pathol. 31 (1994) 358-365.

Bergquist C., Johansson E.L., Lagergard T., Holmgren J., Rudin A., Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina, Infect. Immun. 65 (1997) 2676-2684.

Berrios P.E., McKercher D.G., Knight H.D., Pathogenicity of a caprine herpesvirus, Am. J. Vet. Res. 36 (1975) 1763-1769.

Bosch J.C., Kaashoek M.J., Kroese A.H., van Oirschot J.T., An attenuated bovine herpesvirus 1 marker vaccine induces a better protection than two inactivated marker vaccines, Vet. Microbiol. 52 (1996) 223-234.

Brake F., Studdert M.J., Molecular epidemiology and pathogenesis of ruminant herpesviruses including bovine, buffalo and caprine herpesviruses l and bovine encephalitis herpesvirus, Aust. Vet. J. 62 (1985) 331-334.

Brandtzaeg P., Baekkevold E.S., Farstad I.N., Jahnsen F.L., Johansen F.E., Nilsen E.M., Yamanaka T., Regional specialization in the mucosal immune system: what happens in the microcompartments?, Immunol. Today 20 (1999) 141-151.

Bratanich A.C., Sardi S.I., Smitsaart E.N., Schudel A.A., Comparative studies of BHV-1 variants by in vivo-in vitro tests, Zentralbl. Veterinarmed. B 38 (1991) 41-48.

Bulach D.M., Studdert M.J., Comparative genome mapping of bovine encephalitis herpesvirus, bovine herpesvirus 1, and buffalo herpesvirus, Arch. Virol. 113 (1990) 17-34.

Buonavoglia C., Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Conserva A., Corrente M., Reactivation of caprine herpesvirus 1 in latently infected goats, Comp. Immunol. Microbiol. Infect. Dis. 19 (1996) 275-281.

Camero M., Bellacicco A.L., Tarsitano E., Decaro N., Martella V., Tempesta M. Buonavoglia C., Intravaginal administration of an inactivated vaccine prevents lesions induced by caprine herpesvirus-1 in goats, Vaccine 25 (2007) 1658-1661.

Caron L., Flores E.F., Weiblen R., Scherer C.F., Irigoyen L.F., Roehe P.M., Odeon A., Sur J.H., Latent infection by bovine herpesvirus type-5 in experimentally infected rabbits: virus reactivation, shedding and recrudescence of neurological disease, Vet. Microbiol. 84 (2002) 285-295.

Carrillo B.J., Ambrogi A., Schudel A.A., Vazquez M., Dahme E., Pospischil A., Meningoencephalitis caused by IBR virus in calves in Argentina, Zentralbl. Veterinarmed. B 30 (1983) 327-332.

Cascio K.E., Belknap E.B., Schultheiss P.C., Ames A.D., Collins J.K., Encephalitis induced by bovine herpesvirus 5 and protection by prior vaccination or infection with bovine herpesvirus 1, J. Vet. Diagn. Invest. 11 (1999) 134-139.

Chenier S., Montpetit C., Helie P., Caprine herpesvirus-1 abortion storm in a goat herd in Quebec, Can. Vet. J. 45 (2004) 241-243.

Chowdhury S.I., Lee B.J., Ozkul A., Weiss M.L., Bovine herpesvirus 5 glycoprotein E is important for neuroinvasiveness and neurovirulence in the olfactory pathway of the rabbit, J. Virol. 74 (2000a) 2094-2106.

Chowdhury S.I., Lee B.J., Onderci M., Weiss M.L., Mosier D., Neurovirulence of glycoprotein C(gC)-deleted bovine herpesvirus type-5 (BHV-5) and BHV-5 expressing BHV-1 gC in a rabbit seizure model, J. Neurovirol. 6 (2000b) 284-295.

Collins J.K., Ayers V.K., Whetstone C.A., van Drunen Littel-van den Hurk S., Antigenic differences between the major glycoproteins of bovine herpesvirus type 1.1 and bovine encephalitis herpesvirus type 1.3, J. Gen. Virol. 74 (1993) 1509-1517.

Committee for Veterinary Medicinal Products, Position paper regarding availability of products for minor uses and minor species (MUMS), in: European Medicine Agency (Ed.), EMEA/CVMP/477/03/final, Luxembourg, 2004, pp. 1-13.

d'Arce R.C., Almeida R.S., Silva T.C., Franco A.C., Spilki F., Roehe P.M., Arns C.W., Restriction endonuclease and monoclonal antibody analysis of Brazilian isolates of bovine herpesviruses types 1 and 5, Vet. Microbiol. 88 (2002) 315-324.

Davis S.S., Nasal vaccines, Adv. Drug Deliv. Rev. 51 (2001) 21-42.

Davison A.J., Evolution of the herpesviruses, Vet. Microbiol. 86 (2002) 69-88.

De Carlo E., Re G.N., Letteriello R., Del Vecchio V., Giordanelli M.P., Magnino S., Fabbi M., Bazzocchi C., Bandi C., Galiero G., Molecular characterisation of a field strain of bubaline herpesvirus isolated from buffaloes (*Bubalus bubalis*) after pharmacological reactivation, Vet. Rec. 154 (2004) 171-174.

Delhon G., Moraes M.P., Lu Z., Afonso C.L., Flores E.F., Weiblen R., Kutish G.F., Rock D.L., Genome of bovine herpesvirus 5, J. Virol. 77 (2003) 10339-10347.

Deregt D., Jordan L.T., van Drunen Littel-van den Hurk S., Masri S.A., Tessaro S.V., Gilbert S.A., Antigenic and molecular characterization of a herpesvirus isolated from a North American elk, Am. J. Vet. Res. 61 (2000) 1614-1618.

Deregt D., Tessaro S.V., Gilbert S.A., Serological evidence of latency in cattle experimentally infected with elk herpesvirus, Vet. Rec. 156 (2005a) 610-611.

Deregt D., Gilbert S.A., Campbell I., Burton K.M., Reid H.W., Hurk S.V., Penniket C., Baxi M.K., Phylogeny and antigenic relationships of three cervid herpesviruses, Virus Res. 114 (2005b) 140-148.

Dewals B., Thirion M., Markine-Goriaynoff N., Gillet L., de Fays K., Minner F., Daix V., Sharp P.M., Vanderplasschen A., Evolution of Bovine herpesvirus 4: recombination and transmission between African buffalo and cattle, J. Gen. Virol. 87 (2006) 1509-1519.

de Wit J.J., Hage J.J., Brinkhof J., Westenbrink F., A comparative study of serological tests for use in the bovine herpesvirus 1 eradication programme in The Netherlands, Vet. Microbiol. 61 (1998) 153-163.

Dietrich R.A., Respiratory viruses, in: Dietrich R.A. (Ed.), Alaskan wild life diseases, University of Alaska, Fairbanks, 1981, pp. 28-29.

d'Offay J.M., Mock R.E., Fulton R.W., Isolation and characterization of encephalitic bovine herpesvirus type 1 isolates from cattle in North America, Am. J. Vet. Res. 54 (1993) 534-539.

Egyed L., Brocchi E., Rusvai M., Bartha A., Study of bovine herpesvirus type 1 strains with monoclonal antibodies, Acta Vet. Hung. 40 (1992) 225-230.

Ek-Kommonen C., Veijalainen P., Rantala M., Neuvonen E., Neutralizing antibodies to bovine herpesvirus 1 in reindeer, Acta Vet. Scand. 23 (1982) 565-569.

Ek-Kommonen C., Pelkonen S., Nettleton P.F., Isolation of a herpesvirus serologically related to bovine herpesvirus 1 from a reindeer (Rangifer tarandus), Acta Vet. Scand. 27 (1986) 299-301.

El Azhary M.A., Roy R.S., Frechette J.L., Serological evidence of IBR and BVD infection in caribou (Rangifer tarandus), Vet. Rec. 105 (1979) 336.

Engels M., Steck F., Wyler R., Comparison of the genomes of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis virus strains by restriction endonuclease analysis, Arch. Virol. 6 (1981) 169-174.

Engels M., Gelderblom H., Darai G., Ludwig H., Goat herpesviruses: biological and physicochemical properties, J. Gen. Virol. 64 (1983) 2237-2247.

Engels M., Giuliani C., Wild P., Beck T.M., Loepfe E., Wyler R., The genome of bovine herpesvirus 1 (BHV-1) strains exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization, Virus Res. 6 (1986) 57-73.

Engels M., Loepfe E., Wild P., Schraner E., Wyler R., The genome of caprine herpesvirus 1: genome structure and relatedness to bovine herpesvirus 1, J. Gen. Virol. 68 (1987) 2019-2023.

Engels M., Palatini M., Metzler A.E., Probst U., Kihm U., Ackermann M., Interactions of bovine and caprine herpesviruses with the natural and the foreign hosts, Vet. Microbiol. 33 (1992) 69-78.

Engels M., Ackermann M., Pathogenesis of ruminant herpesvirus infections, Vet. Microbiol. 53 (1996) 3-15.

Engels M., Thiry E., L'infection de la chèvre par l'herpèsvirus caprin de type 1, Point Vét. 31 (2000) 37-42.

Eriksson K., Quiding-Jarbrink M., Osek J., Moller A., Bjork S., Holmgren J., Czerkinsky C., Specific-antibody-secreting cells in the rectums and genital tracts of nonhuman primates following vaccination, Infect. Immun. 66 (1998) 5889-5896.

European Food Safety Authority, Definition of a BoHV-1-free animal and a BoHV-1-free holding, and the procedures to verify and maintain this status, EFSA Journal 311 (2005) 1-65.

Favoreel H.W., Van de Walle G.R., Nauwynck H.J., Pensaert M.B., Virus complement evasion strategies, J. Gen. Virol. 84 (2003) 1-15.

Fehler F., Herrmann J.M., Saalmuller A., Mettenleiter T.C., Keil G.M., Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant, J. Virol. 66 (1992) 831-839.

Felsenstein J., PHYLIP--Phylogeny Inference Package, (Version 3.2), Cladistics 5 (1989) 164-166.

Feulner P.G., Bielfeldt W., Zachos F.E., Bradvarovic J., Eckert I., Hartl G.B., Mitochondrial DNA and microsatellite analyses of the genetic status of the presumed subspecies Cervus elaphus montanus (Carpathian red deer), Heredity 93 (2004) 299-306.

Frantz A.C., Pourtois J.T., Heuertz M., Schley L., Flamand M.C., Krier A., Bertouille S., Chaumont F., Burke T., Genetic structure and assignment tests demonstrate illegal translocation of red deer (Cervus elaphus) into a continuous population, Mol. Ecol. 15 (2006) 3191-3203.

French E.L., A specific virus encephalitis in calves: isolation and characterization of the causal agent, Aust. Vet. J. 38 (1962) 216-221.

Friedli K., Metzler A.E., Reactivity of monoclonal antibodies to proteins of a neurotropic bovine herpesvirus 1 (BHV-1) strain and to proteins of representative BHV-1 strains, Arch. Virol. 94 (1987) 109-122.

Gallichan W.S., Rosenthal K.L., Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization, J. Infect. Dis. 177 (1998) 1155-1161.

Gompels U.A., Craxton M.A., Honess R.W., Conservation of glycoprotein H (gH) in herpesviruses: nucleotide sequence of the gH gene from herpesvirus saimiri, J. Gen. Virol. 69 (1988) 2819-2829.

Grewal A.S., Wells R., Vulvovaginitis of goats due to a herpesvirus, Aust. Vet. J. 63 (1986) 79-82.

Griffin A.M., The nucleotide sequence of the glycoprotein gB gene of infectious laryngotracheitis virus: analysis and evolutionary relationship to the homologous gene from other herpesviruses, J. Gen. Virol. 72 (1991) 393-398.

Guercio A., Greco G., Lanizzoto G., Di Marco V., Todaro M., Valutazione della diffusione di anticorpi anti Herpes Virus della capra in allevamenti caprini della Sicilia, Atti. SIPAOC 12 (1998) 138-142.

Hage J.J., Vellema P., Schukken Y.H., Barkema H.W., Rijsewijk F.A., van Oirschot J.T., Wentink G.H., Sheep do not have a major role in bovine herpesvirus 1 transmission, Vet. Microbiol. 57 (1997) 41-54.

Hall W.T., Simmons G.C., French E.L., Snowdon W.A., Asdell M., The pathogenesis of encephalitis caused by the infectious bovine rhinotracheitis virus, Aust. Vet. J. 42 (1966) 229-237.

Hamilton W.J., The industry, in: Alexander T.L., Buxton D. (Eds), Management and diseases of deer, The Veterinary Deer Society, London, 1994, pp. 7-13.

Hartl G.B., Zachos F., Nadlinger K., Genetic diversity in European red deer (*Cervus elaphus* L.); anthropogenic influences on natural populations, C. R. Biologies 326 (2003) S37-S42.

Horner G.W., Hunter R., Day A.M., An outbreak of vulvovaginitis in goats caused by a caprine herpesvirus, N. Z. Vet. J. 30 (1982) 152.

Howell D.C., Power considerations in terms of sample size, Statistical Methods for Psychology, 4th ed., Duxbury, 1997, pp. 226.

Hubner S.O., Oliveira A.P., Franco A.C., Esteves P.A., Silva A.D., Spilki F.R., Rijsewijk F.A., Roehe P.M., Experimental infection of calves with gI, gE, US9 negative bovine herpesvirus type 5, Comp. Immunol. Microbiol. Infect. Dis. 28 (2005) 187-196.

Hugot S., Bouche R., Regard sur les pratiques de l'élevage extensif de la chèvre corse : préalable à la mise en place d'un schéma de sélection, Options Méditerranéennes 38 (1999) 137-143.

Hutchinson L., Goldsmith K., Snoddy D., Ghosh H., Graham F.L., Johnson D.C., Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion, J. Virol. 66 (1992) 5603-5609.

Hyllseth B., Stuen S., Rimstad E., Dahle H.K., Tyler J.C., Serologiske undersokelser over herpesvirus infecksjoner hos reain fra Finnmark og Svalbard, Norsk Veterinaertidsskrift 105 (1993) 733-736.

Inglis D.M., Bowie J.M., Allan M.J., Nettleton P.F., Ocular disease in red deer calves associated with a herpesvirus infection, Vet. Rec. 113 (1983) 182-183.

International Committee on Taxonomy of viruses, in: ICTVdB Index of Viruses, Appendix 00.031 herpesviridae [on line] (2002) http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_herpe.htm [consulted 5 May 2005].

Isa G., Schelp C., Truyen U., Comparative studies with three different bovine blood sample BHV-1 ELISA tests: indirect ELISA and bG-blocking-ELISA, Berl. Munch. Tierarztl. Wochenschr. 116 (2003) 192-196.

Jacobs L., Kimman T.G., Epitope-specific antibody response against glycoprotein E of pseudorabies virus, Clin. Diagn. Lab. Immunol. 1 (1994) 500-505.

Johansen F.E., Baekkevold E.S., Carlsen H.S., Farstad I.N., Soler D., Brandtzaeg P., Regional induction of adhesion molecules and chemokine receptors explains disparate homing of human B cells to systemic and mucosal effector sites: dispersion from tonsils, Blood 106 (2005) 593-600.

Johansson E.L., Rask C., Fredriksson M., Eriksson K., Czerkinsky C., Holmgren J., Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates, Infect. Immun. 66 (1998) 514-520.

Johansson E.L., Rudin A., Wassen L., Holmgren J., Distribution of lymphocytes and adhesion molecules in human cervix and vagina, Immunology 96 (1999) 272-277.

Johansson E.L., Wassen L., Holmgren J., Jertborn M., Rudin A., Nasal and vaginal vaccinations have differential effects on antibody responses in vaginal and cervical secretions in humans, Infect. Immun. 69 (2001) 7481-7486.

Johnston L., Simmons G., McGavin M., A viral meningo-encephalitis in calves, Aust. Vet. J. 38 (1962) 207-215.

Jones C., Herpes simplex virus type 1 and bovine herpesvirus 1 latency, Clin. Microbiol. Rev. 16 (2003) 79-95.

Jordan L.T., Rettie W.J., Tessaro S.V., Evidence of herpesvirus infection in Woodland Caribou in Saskatchewan, J. Wildl. Dis. 39 (2003) 216-220.

Kendrick J.W., Gillespie J.H., Mc Entee K., Infectious pustular vulvovaginitis of cattle, Cornell Vet. 48 (1958) 458-495.

Kaashoek M.J., Moerman A., Madic J., Rijsewijk F.A., Quak J., Gielkens A.L., van Oirschot J.T., A conventionally attenuated glycoprotein E-negative strain of bovine herpesvirus type 1 is an efficacious and safe vaccine, Vaccine 12 (1994) 439-444.

Kaashoek M.J., Moerman A., Madic J., Weerdmeester K., Maris-Veldhuis M., Rijsewijk F.A., van Oirschot J.T., An inactivated vaccine based on a glycoprotein E-negative strain of bovine herpesvirus 1 induces protective immunity and allows serological differentiation, Vaccine 13 (1995) 342-346.

Kaashoek M.J., van Oirschot J.T., Early immunity induced by a live gE-negative bovine herpesvirus 1 marker vaccine, Vet. Microbiol. 53 (1996) 191-197.

Kaashoek M.J., Rijsewijk F.A., Oirschot J.T., Persistence of antibodies against bovine herpesvirus 1 and virus reactivation two to three years after infection, Vet. Microbiol. 53 (1996) 103-110.

Kalman D., Egyed L., PCR detection of bovine herpesviruses from nonbovine ruminants in Hungary, J. Wildl. Dis. 41 (2005) 482-488.

Kao M., Leiskau T., Koptopoulos G., Papadopoulos O., Horner G.W., Hyllseth B. et al., Goat herpesvirus infections: a survey on specific antibodies in different countries, in: Pastoret P-P., Thiry E., Saliki J.T. (Eds), Immunity to herpesvirus infections of domestic animals, Report EUR 9737 EN, Luxembourg, 1985, pp. 93-97.

Keuser V., Thiry E., Conséquences de l'infection des cervidés par des alpha-herpèsvirus apparentés au virus de l'IBR, Point Vét. 31 (2000) 219-223.

Keuser V., Gogev S., Schynts F., Thiry E., Demonstration of generalized infection with caprine herpesvirus 1 diagnosed in an aborted caprine fetus by PCR, Vet. Res. Commun. 26 (2002) 221-226.

Keuser V., Infection de la chèvre par l'herpèsvirus caprin 1: diagnostic différentiel et caractérisation de la glycoprotéine D, Doctorate thesis, Liège, Belgium, 2003.

Keuser V., Schynts F., Detry B., Collard A., Robert B., Vanderplasschen A., Pastoret P.-P., Thiry E., Improved antigenic methods for differential diagnosis of bovine, caprine, and cervine alphaherpesviruses related to bovine herpesvirus 1, J. Clin. Microbiol. 42 (2004a) 1228-1235.

Keuser V., Espejo-Serrano J., Schynts F., Georgin J.P., Thiry E., Isolation of caprine herpesvirus type 1 in Spain, Vet. Rec. 154 (2004b) 395-399.

Keuser V., Detry B., Thiry J., de Fays K., Schynts F., Pastoret P.-P., Vanderplasschen A., Thiry E., Characterization of caprine herpesvirus 1 glycoprotein D gene and its translation product, Virus Res. 115 (2006) 112-121.

Kokles R., Dedek J., Loepelmann H., Serologische Untersuchungen auf Infektionen mit dem Virus der infektiösen bovinen Rhinotracheitis/infektiöse Vulvovaginitis und dem Parainfluenza-3-virus bei Rot-, Reh-, Dam- und Muffelwild, Monatsh. Veterinärmed. 43 (1988) 60-63.

Konig P., Giesow K., Keil G.M., Glycoprotein M of bovine herpesvirus 1 (BHV-1) is nonessential for replication in cell culture and is involved in inhibition of bovine respiratory syncytial virus F protein induced syncytium formation in recombinant BHV-1 infected cells, Vet. Microbiol. 86 (2002) 37-49.

Koppers-Lalic D., Reits E.A.J., Ressing M.E., Lipinska A.D., Abele R., Koch J., Rezende M.M., Admiraal P., van Leeuwen D., Bienkowska-Szewczyk K., Mettenleiter T.C., Rijsewijk F.A.M., Tampe R., Neefjes J., Wiertz E.J.H.J., Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing, Proc. Natl. Acad. Sci. USA 102 (2005) 5144-5149.

Koptopoulos G., Papanastasopoulou M., Papadopoulos O., Ludwig H., The epizootiology of caprine herpesvirus (BHV-6) infections in goat populations in Greece, Comp. Immunol. Microbiol. Infect. Dis. 11 (1988) 199-205.

Kramps J.A., Magdalena J., Quak J., Weerdmeester K., Kaashoek M.J., Maris-Veldhuis M.A., Rijsewijk F.A., Keil G., van Oirschot J.T., A simple, specific, and highly sensitive blocking enzyme-linked immunosorbent assay for detection of antibodies to bovine herpesvirus 1, J. Clin. Microbiol. 32 (1994) 2175-2181.

Kramps J.A., Banks M., Beer M., Kerkhofs P., Perrin M., Wellenberg G.J., Oirschot J.T., Evaluation of tests for antibodies against bovine herpesvirus 1 performed in national reference laboratories in Europe, Vet. Microbiol. 102 (2004) 169-181.

Kuehn R., Haller H., Schroeder W., Rottmann O., Genetic roots of the red deer (Cervus elaphus) population in Eastern Switzerland, J. Hered. 95 (2004) 136-143.

Kuklin N., Daheshia M., Karem K., Manickan E., Rouse B.T., Induction of mucosal immunity against herpes simplex virus by plasmid DNA immunization. J. Virol. 71 (1997) 3138-3145.

Lee B.J., Weiss M.L., Mosier D., Chowdhury S.I., Spread of bovine herpesvirus type 5 (BHV-5) in the rabbit brain after intranasal inoculation, J. Neurovirol. 5 (1999) 474-484.

Lehmkuhl H.D., Cutlip R.C., Protection from parainfluenza-3 virus and persistence of infectious bovine rhinotracheitis virus in sheep vaccinated with modified live IBR-PI-3 vaccine, Can. J. Comp. Med. 49 (1985) 58-62.

Lemaire M., Schynts F., Meyer G., Thiry E., Antibody response to glycoprotein E after bovine herpesvirus type 1 infection in passively immunised, glycoprotein E-negative calves, Vet. Rec. 144 (1999) 172-176.

Lemaire M., Hanon E., Schynts F., Meyer G., Thiry E., Specific passive immunity reduces the excretion of glycoprotein E-negative bovine herpesvirus type 1 vaccine strain in calves, Vaccine 19 (2000) 1013-1017.

Lemaire M., Schynts F., Meyer G., Georgin J.-P., Baranowski E., Gabriel A., Ros C., Belak S., Thiry E., Latency and reactivation of a glycoprotein E negative bovine herpesvirus type 1 vaccine: influence of virus load and effect of specific maternal antibodies, Vaccine 19 (2001) 4795-4804.

Liebermann H.D., Tabbaa J., Dedek H., Loepelmann H., Stubbe J., Selbitz H.J., Serologische Untersuchungen auf ausgewählte Virusinfektionen bei Wildwiederkäuern in der DDR, Monatsh. Veterinärmed. 44 (1989) 380-382.

Lillehaug A., Vikoren T., Larsen I.L., Akerstedt J., Tharaldsen J., Handeland K., Antibodies to ruminant alpha-herpesviruses and pestiviruses in Norwegian cervids, J. Wildl. Dis. 39 (2003) 779-786.

Liman A., Engels M., Meyer G., Ackermann M., Glycoprotein C of bovine herpesvirus 5 (BHV-5) confers a distinct heparin-binding phenotype to BHV-1, Arch. Virol. 145 (2000) 2047-2059.

Limbourg B., Kerkhofs P., Massard C., Michelet S., Saegerman C., Thiry E., Avantages et inconvénients d'un plan de lutte contre la rhinotrachéite infectieuse bovine en Belgique, Ann. Med. Vet. 147 (2002) 57-69.

Littell R.C., Henry P.R., Ammerman C.B., Statistical analysis of repeated measures data using SAS procedures, J. Anim. Sci. 76 (1998) 1216-1231.

Lowe V.P.M., Gardiner A.S., A re-examination of the sub-species of Red deer (Cervus elaphus) with particular reference to the stocks in Britain, J. Zool. Lond. 174 (1974) 185-201.

Lyaku J.R., Nettleton P.F., Mardsen H., A comparison of serological relationships among five ruminant alphaherpesviruses by ELISA, Arch. Virol. 124 (1992) 333-341.

Lyaku J.R., Vilcek S., Nettleton P.F., Marsden H.S., The distinction of serologically related ruminant alphaherpesviruses by the polymerase chain reaction (PCR) and restriction endonuclease analysis, Vet. Microbiol. 48 (1996) 135-142.

Madin S.H., McKercher D.G., York C.J., Isolation of the infectious bovine rhinotracheitis virus, Science 124 (1956) 721-722.

Mars M.H., De Jong M.C., van Oirschot J.T., A gE-negative bovine herpesvirus 1 vaccine strain is not re-excreted nor transmitted in an experimental cattle population after corticosteroid treatments, Vaccine 18 (2000) 1975-1981.

Mars M.H., De Jong M.C., Franken P., van Oirschot J.T., Efficacy of a live glycoprotein E-negative bovine herpesvirus 1 vaccine in cattle in the field, Vaccine 19 (2001) 1924-1930.

Martin W.B., Castrucci G., Frigeri F., Ferrari M., A serological comparison of some animal herpesviruses, Comp. Immunol. Microbiol. Infect. Dis. 13 (1990) 75-84.

Mestecky J., Abraham R., Ogra P.L., Common mucosal immune system and strategies for the development of vaccines effective at the mucosal surfaces, in: Ogra P.L., Mestecky J., Lamm M.E., Strober W., McGhee J.R., Bienenstock J. (Eds), Handbook of Mucosal Immunology, Academic Press, San Diego, 1994, pp. 357.

Mettler F., Engels M., Wild P., Bivetti A., Herpesvirus-infektion bei Zieklein in der Schweiz, Schweiz. Arch. Tierheilkd. 121 (1979) 655-662.

Metzler A.E., Schudel A.A., Engels M., Bovine herpesvirus 1: molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease, Arch. Virol. 87 (1986) 205-217.

Meurens F., Schynts F., Keil G.M., Muylkens B., Vanderplasschen A., Gallego P., Thiry E., Superinfection prevents recombination of the alphaherpesvirus bovine herpesvirus 1, J. Virol. 78 (2004a) 3872-3879.

Meurens F., Keil G.M., Muylkens B., Gogev S., Schynts F., Negro S., Wiggers L., Thiry E., Interspecific recombination between two ruminant alphaherpesviruses, bovine herpesviruses 1 and 5, J. Virol. 78 (2004b) 9828-9836.

Meurens F., Influence du délai de surinfection et de la parenté génétique sur la recombinaison de l'herpèsvirus bovin 1, Doctorate thesis, Liège, Belgium, 2004.

Meurens F., Whale J., Brownlie R., Dybvig T., Thompson D.R., Gerdts V., Expression of mucosal chemokines TECK/CCL25 and MEC/CCL28 during fetal development of the ovine mucosal immune system, Immunology 120 (2007) 544-555.

Meyer G., Vilcek C., Paces V., O'Hara M.K., Pastoret P.-P., Thiry E., Schwyzer M., Sequence analysis of the bovine herpesvirus type 1 genes homologous to the DNA polymerase (UL30), the major DNA-binding protein (UL29) and the ICP 18.5 assembly protein (UL28) genes of herpes simplex virus, Arch. Virol. 142 (1997) 89-102.

Meyer G., Hanon E., Georlette D., Pastoret P.-P., Thiry E., Bovine herpesvirus type 1 glycoprotein H is essential for penetration and propagation in cell culture, J. Gen. Virol. 79 (1998) 1983-1987.

Meyer G., Bare O., Thiry E., Identification and characterization of bovine herpesvirus type 5 glycoprotein H gene and gene products, J. Gen. Virol. 80 (1999) 2849-2859.

Meyer G., d'Offay J., Thiry E., Les encéphalites à herpèsvirus bovins, Point Vét. 31 (2000) 417-424.

Meyer G., Lemaire M., Ros C., Belak K., Gabriel A., Cassart D., Coignoul F., Belak S., Thiry E., Comparative pathogenesis of acute and latent infections of calves with bovine herpesvirus types 1 and 5, Arch. Virol. 146 (2001) 633-652.

Meyer G., Thiry J., Thiry E., Bovine herpesvirus 5 infection, in: Lefèvre P.C., Blancou J., Chermette R. (Eds), Infectious and Parasitic Diseases of Livestock, Lavoisier Editions, Provigny, France, 2007, in press.

Miller J.M., Van der Maaten M.J., Reproductive tract lesions in heifers after intrauterine inoculation with infectious bovine rhinotracheitis virus, Am. J. Vet. Res. 45 (1984) 790-794.

Mollema L., Rijsewijk F.A.M., Nodelijk G., de Jong M.C.M., Quantification of the transmission of bovine herpesvirus 1 among red deer (Cervus elaphus) under experimental conditions, Vet. Microbiol. 111 (2005) 25-34.

Motha J., Jenner J., Serological relatedness of cervine herpesvirus-1 and bovine herpesvirus-1 and the prevalence of cervine herpesvirus-1 infection in farmed deer in New Zealand, New Z. Vet. J. 49 (2001) 162-163.

Muller T., Kramer M., Beier D., Untersuchungen zum Vorkommen von Antikorpern gegen ausgewahlte bovine und ovide Viruserkrankungen bei Reh-(Capreolus capreolus), Rot- (Cervus elaphus), Dam-(Dama dama) und Muffelwild (Ovis musimon) im Bundesland Brandenburg, Zeitschrift fur Jagdwissenschaft 43 (1997) 166-175.

Muluneh A., Liebermann H., Occurrence of caprine herpesvirus (BHV-6) infection in goat population of the GDR, Arch. Exp. Veterinarmed. 44 (1990) 254-257.

Murphy B.R., Mucosal immunity to viruses, in: Ogra P.L., Mestecky J., Lamm M.E., Strober W., Bienenstock J., McGhee J.R. (Eds), Mucosal Immunology, 2nd ed, Academic Press, San Diego, 1999, pp. 695.

Muylkens B., Meurens F., Schynts F., Farnir F., Pourchet A., Bardiau M., Gogev S., Thiry J., Cuisenaire A., Vanderplasschen A., Thiry E. Intraspecific bovine herpesvirus 1 recombinants carrying glycoprotein E deletion as a vaccine marker are virulent in cattle, J. Gen. Virol. 87 (2006) 2149-2154.

Muylkens B., Thiry J., Kirten P., Schynts F., Thiry E., Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis, Vet. Res. 38 (2007) 181-209.

Nettleton P.F., Sinclair J.A., Herring J.A., Inglis D.M., Fletcher T.J., Ross H.M., Bonniwell M.A., Prevalence of herpesvirus infection in British red deer and investigations of further disease outbreaks, Vet. Rec. 118 (1986) 267-270.

Nettleton P.F., Thiry E., Reid H.W., Pastoret P.-P., Herpesvirus infections in cervidae, Rev. Sci. Tech. Off. Int. Epiz. 7 (1988a) 977-988.

Nettleton P.F., Ek-Kommonen C., Tanskanen R., Reid H.W., Sinclair J.A., Herring J.A., Studies in the epidemiology and pathogenesis of alphaherpesviruses from red deer (cervus elaphus) and reindeer (rangifer tarandus), The management and Health of Farmed deer (1988b) 143-147.

Niethammer G., Die Einbürgerung von Säugetieren und Vögeln in Europa. Ergebnisse und Aussichten. Unter Mitarbeit von Niethammer J. und Szijj J., Paul Parey, 1963, Hamburg, Berlin.

Nixon P., Edwards S., White H., Serological comparisons of antigenically related herpesviruses in cattle, red deer and goats, Vet. Res. Commun. 12 (1988) 355-362.

Nussey D.H., Pemberton J., Donald A., Kruuk L.E., Genetic consequences of human management in an introduced island population of red deer (Cervus elaphus), Heredity 97 (2006) 56-65.

Oldoni I., Weiblen R., Inkelmann M.A., Flores E.F., Production and characterization of monoclonal antibodies to a Brazilian bovine herpesvirus type 5, Braz. J. Med. Biol. Res. 37 (2004) 213-221.

Pagamjav O., Sakata T., Matsumura T., Yamaguchi T., Fukushi H., Natural recombinant between equine herpesviruses 1 and 4 in the ICP4 gene, Microbiol. Immunol. 49 (2005) 167-179.

Papanastasopoulou M., Koptopoulos G., Lekkas S., Papadopoulos O., Ludwig H., An experimental study on the pathogenicity of the caprine herpesvirus type 1 (CHV-1), Comp. Immunol. Microbiol. Infect. Dis. 14 (1991) 47-53.

Parr M.B., Parr E.L., Mucosal immunity in the female and male reproductive tracts, in: : Ogra P.L., Mestecky J., Lamm M.E., Strober W., McGhee J.R., Bienenstock J. (Eds) Handbook of Mucosal Immunology, 1st ed., Academic Press, San Diego, 1994, pp. 677.

Parr E.L., Parr M.B., Immunoglobulin G is the main protective antibody in mouse vaginal secretions after vaginal immunization with attenuated herpes simplex virus type 2, J. Virol. 71 (1997) 8109-8115.

Parr M.B., Parr E.L., Immune responses and protection against vaginal infection after nasal or vaginal immunization with attenuated herpes simplex virus type-2, Immunology 98 (1999) 639-645.

Pastoret P.-P., Thiry E., Brochier B., Derboven G., Bovid herpesvirus 1 infection of cattle: pathogenesis, latency, consequences of latency, Ann. Rech. Vét. 13 (1982) 221-235.

Perez S.E., Bretschneider G., Leunda M.R., Osorio E.A., Flores E.F., Odeon A.C., Primary infection, latency, and reactivation of bovine herpesvirus type 5 in the bovine nervous system, Vet. Pathol. 39 (2002) 437-444.

Perez S.E., Vagnozzi A., Sur J.H., Odriozola E., Campero C.M., Odeon A.C., Análisis retrospectivo de casos con diagnóstico de necrosis cerebrocortical y su relación com herpesvirus bovino tipo 5, Rev. Argent. Microbiol. 35 (2003) 69-73.

Perry L.L., Feilzer K., Portis J.L., Caldwell H.D., Distinct homing pathways direct T lymphocytes to the genital and intestinal mucosae in Chlamydia-infected mice, J. Immunol. 160 (1998) 2905-2914.

Petit E., Etude sur la procédure « résultants aberrants » I.B.R. proposée par l'A.C.E.R.S.A., Epidémiol. Santé Anim. 42 (2002) 133-150.

Pidone C.L., Galosi C.M., Echeverria M.G., Nosetto E.O., Etcheverrigaray M.E., Restriction endonuclease analysis of BHV-1 and BHV-5 strains isolated in Argentina, Zentralbl. Veterinarmed. B 46 (1999) 453-456.

Pirak M., Thiry E., Brochier B., Pastoret P.-P., Infection expérimentale de la chèvre par le virus de la rhinotrachéite infectieuse bovine (Bovine herpesvirus type 1) et tentative de réactivation virale, Rec. Méd. Vét. (1983) 1103-1106.

Plebani G.F., Engels M., Metzler A.E., Wyler R., Caprine herpesvirus in Switzerland: distribution, incidence and latency of infection, Schweiz. Arch. Tierheilkd. 125 (1983) 395-411 (in German).

Pospisil Z., Vyvlecka R., Cihal P., Lany P., Zendulkova D., Detection of herpesvirus serum antibodies in red deer (Cervus elaphus) imported into the Czech Republic, Vet. Med. 41 (1996) 279-282.

Pratelli A., Greco G., Dall'Ara P., Engels M., Tempesta M., Buonavoglia C., Restriction endonuclease analysis of the genome of two Italian caprine herpesvirus 1 strains, Arch. Virol. 145 (2000) 845-851.

Rebhun W.C., Jenkins D.H., Riis R.C., Dill S.G., Dubovi E.J., Torres A., An epizootic of blindness and encephalitis associated with a herpesvirus indistinguishable from equine herpesvirus I in a herd of alpacas and llamas, J. Am. Vet. Med. Assoc. 192 (1988) 953-956.

Reid H.W., Nettleton P.F., Pow I., Sinclair J.A., Experimental infection of red deer (Cervus elaphus) and cattle with a herpesvirus isolated from red deer, Vet. Rec. 119 (1986) 156-158.

Rimstad E., Krona R., Hyllseth B., Comparison of herpesviruses isolated from reindeer, goats, and cattle by restriction endonuclease analysis, Arch. Virol. 123 (1992) 389-397.

Roizman B., Knipe M.D., Herpes simplex virus and their replication, in: Knipe D.M., Howley P.M. (Eds), Fields Virology, 4th ed., Lippincott Williams and Wilkins publishers, Philadelphia, 2001, pp. 2399-2461.

Roizman B., Pellett P.E., The family *Herpesviridae*: A brief introduction, in: Knipe D.M., Howley P.M. (Eds), Fields Virology, 4th ed., Lippincott Williams and Wilkins publishers, Philadelphia, 2001, pp. 2381-2398.

Ronsholt L., Christensen L.S., Bitsch V., Latent herpesvirus infection in red deer: characterization of a specific deer herpesvirus including comparison of genomic restriction fragment patterns, Acta. Vet. Scand. 28 (1987) 23-31.

Roop C., Hutchinson L., Johnson D.C., A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H, J. Virol. 67 (1993) 2285-2297.

Roperto F., Pratelli A., Guarino G., Ambrosio V., Tempesta M., Galati P., Iovane G., Buonavoglia C., Natural caprine herpesvirus 1 (CpHV-1) infection in kids, J. Comp. Pathol. 122 (2000) 298-302.

Ros C., Belak S., Studies of genetic relationships between bovine, caprine, cervine, and rangiferine alphaherpesviruses and improved molecular methods for virus detection and identification, J. Clin. Microbiol. 37 (1999a) 1247-1253.

Ros C., Riquelme M.E., Forslund K.O., Belak S., Improved detection of five closely related ruminant alphaherpesviruses by specific amplification of viral genomic sequences, J. Virol. Methods 83 (1999b) 55-65.

Ros C., Belak S., Characterization of the glycoprotein B gene from ruminant alphaherpesviruses, Virus Genes 24 (2002) 99-105.

Saito J.K., Gribble D.H., Berrios P.E., Knight H.D., Mc Kercher D.G., A new herpesvirus isolate from goats: Preliminary report, Am. J. Vet. Res. 35 (1974) 847-848.

Salvador S.C., Lemos R.A.A., Riet-Correa F., Osorio A.L.A.R., Meningoencefalite em bovinos causada por bovino-5 no Mato Grosso do Sul and São Paulo, Pesqui. Vet. Bras. 18 (1998) 76-83.

Schudel A.A., Carrillo B.J., Wyler R., Metzler A.E., Infection of calves with antigenic variants of bovine herpesvirus 1 (BHV-1) and neurological disease, J. Vet. Med. B 33 (1986) 303-310.

Schwyzer M., Ackermann M., Molecular virology of ruminant herpesviruses, Vet. Microbiol. 53 (1996) 17-29.

Schynts F., Baranowski E., Lemaire M., Thiry E., A specific PCR to differentiate between gE negative vaccine and wildtype bovine herpesvirus type 1 strains, Vet. Microbiol. 66 (1999) 187-195.

Schynts F., McVoy M.A., Meurens F., Detry B., Epstein A.L., Thiry E., The structures of bovine herpesvirus 1 virion and concatemeric DNA: implications for cleavage and packaging of herpesvirus genomes, Virology 314 (2003a) 326-335.

Schynts F., Meurens F., Detry B., Vanderplasschen A., Thiry E., Rise and survival of bovine herpesvirus 1 recombinants after primary infection and reactivation from latency, J. Virol. 77 (2003b) 12535-12542.

Silva A.M., Weiblen R., Irigoyen L.F., Roehe P.M., Sur H.J., Osorio F.A., Flores E.F. Experimental infection of sheep with bovine herpesvirus type-5 (BHV-5): acute and latent infection, Vet. Microbiol. 66 (1999) 89-99.

Six A., Banks M., Engels M., Bascunana C.R., Ackermann M., Latency and reactivation of bovine herpesvirus 1 (BHV-1) in goats and of caprine herpesvirus 1 (CapHV-1) in calves, Arch. Virol. 146 (2001) 1325-1335.

Spilki F.R., Silva A.D., Hubner S., Esteves P.A., Franco A.C., Driemeier D., Roehea P.M., Partial protection induced by a BHV-1 recombinant vaccine against challenge with BHV-5., Ann. NY Acad. Sci. 1026 (2004) 247-250.

StataCorp, Stata Statistical Software: Release 8, Stata Corporation, College Station, Texas, 2003.

St George T.D., Philpott M., Isolation of infectious bovine rhinotracheitis virus from the prepuce of water buffalo bulls in Australia, Aust. Vet. J. 48 (1972) 126.

Tarigan S., Webb R.F., Kirkl D., Caprine herpesvirus from balanoposthitis, Aust. Vet. J. 64 (1987) 321.

Tempesta M., Cavalli A., Voigt V., Buonavoglia D., Presenza di anticorpi per caprine herpesvirus 1 (CapHV.1) in allevamenti caprini dell'Italia meridionale, Atti. SIPAOC 11 (1994) 121-122.

Tempesta M., Buonavoglia D., Sagazio P., Pratelli A., Buonavoglia C., Natural reactivation of caprine herpesvirus 1 in latently infected goats, Vet. Rec. 143 (1998) 200.

Tempesta M., Pratelli A., Corrente M., Buonavoglia C., A preliminary study on the pathogenicity of a strain of caprine herpesvirus-1, Comp. Immunol. Microbiol. Infect. Dis. 22 (1999a) 137-143.

Tempesta M., Pratelli A., Greco G., Martella V., Buonavoglia C., Detection of caprine herpesvirus 1 in sacral ganglia of latently infected goats by PCR, J. Clin. Microbiol. 37 (1999b) 1598-1599.

Tempesta M., Pratelli A., Normanno G., Camero M., Buonavoglia D. Greco G., Buonavoglia C., Experimental intravaginal infection of goats with caprine herpesvirus 1, J. Vet. Med. B Infect. Dis. Vet. Public Health 47 (2000) 197-201.

Tempesta M., Camero M., Greco G., Pratelli A., Martella V., Buonavoglia C., A classical inactivated vaccine induces protection against caprine herpesvirus 1 infection in goats, Vaccine 19 (2001) 3860-3864.

Tempesta M., Greco G., Pratelli A., Buonavoglia D., Camero M., Martella V., Buonavoglia C., Reactivation of caprine herpesvirus 1 in experimentally infected goats, Vet. Rec. 150 (2002) 116-117.

Tempesta M., Camero M., Sciorsci R.L., Greco G., Minoia R., Martella V., Pratelli A., Buonavoglia C., Experimental infection of goats at different stages of pregnancy with caprine herpesvirus 1, Comp. Immunol. Microbiol. Infect. Dis. 27 (2004a) 25-32.

Tempesta M., Tarsitano E., Camero M., et al.; Short sequence repeats (SSR) in the glycoprotein C gene of caprine herpesvirus type 1 (CpHV.1), Proceedings of the 2nd European congress of virology, Madrid, 2004b, pp. 11-14.

Tempesta M., Decaro N., Elia G., et al., Development of a real time PCR for the detection and quantitation of caprine herpesirus type 1 DNA, in: Nauwynck, H., Favoreel, H. (Eds), Proc. 2nd ESVV Veterinary Herpesirus Symposium, Ghent, 2005.

Tempesta M., Camero M., Bellacicco A.L., Tarsitano E., Crescenzo G., Thiry J., Martella V., Decaro N., Elia G., Neyts J., Thiry E., Buonavoglia C., Potent inhibition of genital herpesvirus infection in goats by cidofovir, Antivir. Ther. 12 (2007a) 977-979.

Tempesta M., Camero M., Bellacicco A.L., Tarsitano E., Lorusso A., Martella V., Decaro N., Del Giudice G., Cassone A., Quaranta A., Buonavoglia C., Caprine herpesvirus 1 vaccine with the LTK63 mutant as a mucosal adjuvant induces strong protection against genital infection in goats, Vaccine (2007b) doi:10.1016/j.vaccine. 2007.09.004.

Thiry E., Lemaire M., Infection de ruminants par des herpèsvirus hétérologues, Point Vét. 207 (2001) 20-25.

Thiry E., Vercouter M., Dubuisson J., Barrat J., Sepulchre C., Gerardy C., Meersschaert C., Collin B., Blancou J., Pastoret P.-P., Serological survey of herpesvirus infections in wild ruminants of France and Belgium, J. Wildl. Dis. 24 (1988) 268-273.

Thiry E., Lemaire M., Schynts F., Meyer G., Dispas M., Gogev S., Les conséquences de l'infection des bovins par le virus de la rhinotrachéite infectieuse bovine, Point Vét. 199 (1999) 279-285.

Thiry E., Ackermann M., Banks M., Belak K., Belak S., Campbell I., Ek-Kommonen C., Engels M., Meyer G., Reid H., Ros C., Six A., Risk evaluation of cross-infection of cattle with ruminant alphaherpesviruses related to bovine herpesvirus type 1, in: Körber R. (Ed.), 3. Internationales Symposium zur BHV-1-/BVD-Bekämpfung, Stendal, 2001, pp. 99-104.

Thiry E., Meurens F., Muylkens B., McVoy M., Gogev S., Thiry J., Vanderplasschen A., Epstein A., Keil G., Schynts F., Recombination in alphaherpesviruses, Rev. Med. Virol. 15 (2005) 89-103.

Thiry E., Muylkens B., Meurens F., Gogev S., Thiry J., Vanderplasschen A., Schynts F., Recombination in the alphaherpesvirus bovine herpesvirus 1, Vet. Microbiol. 113 (2006a) 171-177.

Thiry E., Lemaire M., Thiry J., Muylkens B., Certification: comment s'assurer de l'introduction d'un animal indemne dans un troupeau ? Exemple de l'IBR, Journées Nationales des Groupements Techniques Vétérinaires (2007a) 925-930.

Thiry J., Keuser V., Muylkens B., Meurens F., Gogev S., Vanderplasschen A., Thiry E., Ruminant alphaherpesviruses related to bovine herpesvirus 1, Vet. Res. 37 (2006b) 169-190.

Thiry J., Tempesta M., Camero M., Tarsitano E., Bellacicco A.L., Thiry E., Buonavoglia C., A live attenuated glycoprotein E negative bovine herpesvirus 1 vaccine induces a partial cross-protection against caprine herpesvirus 1 infection in goats, Vet. Microbiol. 113 (2006c) 303-308.

Thiry J., Keuser V., Schynts F., Chartier C., Tempesta M., Espejo-Serrano J., Saegerman C., Thiry E. Evaluation de la prévalence sérologique de l'infection à herpèsvirus caprin 1 dans le sud-ouest de l'Europe, Epidémiol. Et Santé Anim. 49 (2006d) 55-58.

Thiry J., Widén F., Grégoire F., Linden A., Belák S., Thiry E., Isolation and characterisation of a ruminant alphaherpesvirus closely related to bovine herpesvirus 1 in a free-ranging red deer, BMC Vet. Res. (2007b) 3:26.

Thiry J., Engels M., Tempesta M., Thiry E., Caprine herpesvirus 1 infection, in: Lefèvre P.C., Blancou J., Chermette R. (Eds), Infectious and Parasitic Diseases of Livestock, Lavoisier Editions, Provigny, France, 2007c, in press.

Thiry J., Saegerman C., Chartier C., Mercier P., Keuser V., Thiry E., Serological evidence of caprine herpesvirus 1 infection in Mediterranean France, Vet. Microbiol. (2007d) doi:10.1016/j.vetmic.2007.10.009.

Thiry J., Tempesta M., Camero M., Tarsitano E., Muylkens B., Meurens F., Thiry E., Buonavoglia C., Clinical protection against caprine herpesvirus 1 genital infection by intranasal administration of a live attenuated glycoprotein E negative bovine herpesvirus 1 vaccine, submitted.

Tikoo S.K., Campos M., Babiuk L.A., Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control, Adv. Virus. Res. 45 (1995) 191-223.

Tisdall D.J., Rowe S.M., Isolation and characterisation of cervine herpesvirus-1 from red deer semen, New Z. Vet. J. 64 (2001) 111-114.

Tolari F., White H., Nixon P., Isolation and reactivation of bovid herpesvirus 1 in goats, Microbiologica 13 (1990) 67-71.

Toma B., Dufour B., Sanaa M., Bénet J.J., Shaw A., Moutou F., Louza A., Epidémiologie appliquée à la lutte collective contre les maladies transmissibles majeures, Association pour l'étude de l'épidémiologie des maladies animales, Maisons-Alfort, 2001, pp. 49-78.

Tyborowska J., Bienkowska-Szewczyk K., Rychlowski M., Van Oirschot J.T., Rijsewijk F.A., The extracellular part of glycoprotein E of bovine herpesvirus 1 is sufficient for complex formation with glycoprotein I but not for cell-to-cell spread, Arch. Virol. 145 (2000) 333-351

Uzal F.A., Woods L., Stillian M., Nordhausen R., Read D.H., Van Kampen H., Odani J., Hietala S., Hurley E.J., Vickers M.L., Gard S.M., Abortion and ulcerative posthitis associated with caprine herpesvirus-1 infection in goats in California, J. Vet. Diagn. Invest. 16 (2004) 478-484.

Van der Lugt J.J., Randles J.L., Systemic herpesvirus infection in neonatal goats, J. S. Afr. Vet. Assoc. 64 (1993) 169-171.

Vanderplasschen A., Bublot M., Pastoret P.-P., Thiry E., Restriction maps of the DNA of cervid herpesvirus 1 and cervid herpesvirus 2, two viruses related to bovine herpesvirus 1, Arch. Virol. 128 (1993) 379-388.

Vannier P., Capua I., Le Potier M.F., Mackay D.K., Muylkens B., Parida S., Paton D.J., Thiry E., Marker vaccines and the impact of their use on diagnosis and prophylactic measures, Rev. Sci. Tech. 26 (2007) 351-372.

van Oirschot J.T., Kaashoek M.J., Maris-Veldhuis M.A., Rijsewijk F.A., Strains of bovine herpesvirus 1 that do not express an epitope on glycoprotein E in cell culture still induce antibodies that can be detected in a gE-blocking ELISA, Vet. Microbiol. 65 (1999) 103-113.

Vilcek S., Detection of the bovine herpesvirus-1 (BHV-1) genome by PCR, J. Virol. Methods 41 (1993) 245-247.

Vogel F.S., Caron L., Flores E.F., Weiblen R., Winkelmann E.R., Mayer S.V., Bastos R.G., Distribution of bovine herpesvirus type 5 DNA in the central nervous systems of latently, experimentally infected calves, J. Clin. Microbiol. 41 (2003) 4512-4520.

Vossen M.T., Westerhout E.M., Soderberg-Naucler C., Wiertz E.J., Viral immune evasion: a masterpiece of evolution, Immunogenetics 54 (2002) 527-542.

Wafula J.S., Mushi E.Z., Wamwayi H., Reaction of goats to infection with infectious bovine rhinotracheitis virus, Res. Vet. Sci. 39 (1985) 84-86.

Waldvogel A., Engels M., Wild P., Stunzi H., Wyler R., Caprine herpesvirus infection in Switzerland: some aspects of its pathogenicity, Zentralbl. Veterinarmed. B 28 (1981) 612-623.

Weiblen R., de Barros C.S., Canabarro T.F., Flores I.E., Bovine meningoencephalitis from IBR virus, Vet. Rec. 24 (1989) 666-667.

Wellenberg G.J., Mars M.H., van Oirschot J.T., Antibodies against bovine herpesvirus (BHV) 5 may be differentiated from antibodies against BHV1 in a BHV1 glycoprotein E blocking ELISA, Vet. Microbiol. 78 (2001) 79-84.

Whetstone C.A., Evermann J.F., Characterization of bovine herpesviruses isolated from six sheep and four goats by restriction endonuclease analysis and radioimmunoprecipitation, Am. J. Vet. Res. 49 (1988) 781-785.

Williams N.M., Vickers M.L., Tramontin R.R., Petrites-Murphy M.B., Allen G.P., Multiple abortions associated with caprine herpesvirus infection in a goat herd, J. Am. Vet. Med. Assoc. 211 (1997) 89-91.

Wittmann G., Aujeszky's disease in ruminants, in: Wittmann G. (Ed.), Herpesvirus diseases of cattle, horses, and pigs, Developments in veterinary virology, Kluwer academic publishers, Boston, 1989, pp. 163-175.