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RESPIRATORY VIRUSES IN ALPINE CHAMOIS

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ABSTRACT

In the heterogeneous ecosystem of the Alps an interdisciplinary approach is necessary to prevent, survey and control wildlife diseases in order to ensure the biological integrity, the environmental conservation and so the biodiversity. In this contest the matter of livestock-wildlife interface is of particular importance for the presence of grazing domestic herds and the increase of wild ruminants populations, that lead to novel cohabitation situations with a possible "spill-over" of diseases from livestock or *vice versa*. Livestock-wildlife interfaces are dynamic and bidirectional and pathogens could be transmitted freely within and between the species. Mountain ungulates appear as a good biological model to study inter-species transmission and in particular, respiratory infections of wild ruminants. Chamois has already been subjected in the past to demographic decreases due to respiratory viruses' circulation. In this study a total of 394 chamois sera hunted in two different areas of North Western Italian Alps were analysed by virus-neutralization test to detect antibody against Bovine Respiratory Syncytial virus (BRSV), Bovine Viral Diarrhea virus (BVDV) and Mammalian Orthoreovirus (MRV). Seroprevalence of viruses and statistical analysis of antibody titres suggest that infection of pestivirus in chamois populations is sporadic as a spill-over from livestock; BRSV has a high adaptation level in wildlife and can be considered endemic in this two areas; high MRV seroprevalence has been observed and confirms the spread of MRV, that has been identified in a previous study in three chamois lungs. Furthermore, in this study PCR and phylogenetic analysis showed that chamois MRV strains belong to serotype 3 and are closely related to Italian dog and Italian bats strains.

INTRODUCTION

Events like the habitat destruction and pollution, the introduction of exotic species and the extinction of native species, are able to change the life on the Earth. In this background an interdisciplinary approach is necessary to prevent, survey and control wildlife diseases in order to ensure the biological integrity, the environmental conservation and so the biodiversity. The actual concept of "One Medicine, One Health" underlines that humans, livestock, wildlife and environment are strictly related, above all in a century in which zoonosis emerge and reemerge (Cutler et al., 2010). In fact, the 12 Manhattan principles (WHO, 2004) exhort to recognize the essential link among human being, livestock and wildlife and the threat of diseases, the food safety and the necessary biodiversity in a good ecosystem (Cutler et al., 2010).

Focusing attention on livestock-wildlife interface the matter is of particular importance in marginal areas for the presence of grazing domestic herds and the increase of wild ruminants populations, partly due to human manipulation, such as introduction or reinforcing, that lead to novel cohabitation situations (Hudson et al., 2002) with a possible "spill-over" of diseases from domestic species to wild ones (Foreyt et al., 1982; Callan et al., 1991; Hudson et al., 2002; Frolich et al., 2002). In particular, wildlife-livestock interfaces are dynamic and bidirectional and pathogens could be transmitted freely within and between the two species (Bengis et al., 2002) as they come into direct and, above all, indirect contact in a communal environment, through use of shared pasture and water and via vectors (Wiethoelter et al., 2015). The bighorn sheep (*Ovis canadensis*) die-off in Southern Colorado in 2007/2008 winter season is an example of the impact livestock strains may have on wildlife. In fact, Authors suggest that

pneumonia in affected bighorns may have been caused by a combination of pathogens including one pathogenic Pasteurellaceae strain of cattle origin (Wolfe et al., 2010).

On the basis of the classification of the emerging infectious diseases (EIDs) of free-living animals published by Daszak et al. (2000), we have to consider the potential sharing of pathogens among wild, domestic and human populations.

Much of the interest in disease ecology and wildlife health has been prompted by emergence, or resurgence, of many parasites that move between livestock, wildlife and humans. Emergence and re-emergence are most commonly associated with ecological changes, and specific risk factors are related to the type of pathogen, route of transmission and host range. Even if the biological determinants of host range remain not completely understood, most pathogens can infect multiple hosts, and the majority of emerging human diseases are zoonotic. Surveillance is a key defence against emerging pathogens, but will often need to be integrated across human, domestic animal and wildlife populations (Woolhouse, 2002). Therefore, it derives that wildlife diseases may have an important role in both the natural ecosystem and the human health (WHO 2002). In fact, pathogens spread and maintenance in wildlife and spill-over/spill-back between wildlife and livestock have been reported as precursors of emerging diseases in humans (Wolfe et al., 2007; Jones et al., 2008; Morse et al., 2012). Spill-over epizootic outbreaks represent a serious threat both to wildlife and to sympatric population of susceptible livestock (spill-back). For example, in USA Brucellosis was probably introduced with cattle and in Yellowstone National Park, in particular, the presence of the disease in elk and bison is a potential threat to cattle grazing at the park boundaries (Dobson and Meagher, 1996).

Mountain ungulates appear as a good biological model to study inter-species transmission and to develop a method that could be adapted to other situations. In fact, domestic and wild-living ungulates are competitors for food, which results in pasture sharing and, thus, to the transmission of micro- and macro-parasites (Richomme et al., 2006). However, their spatial behavior may be variable according to the species considering environmental conditions such as natural barriers, and human management of wild and domestic herds; in mountain pastures, these conditions can be compared (Richomme et al., 2006).

Focusing attention on the Alps, the environment is probably one of the most valuable ecosystems, with a tricky balance (Zunino, 2003). In the past, human activities (like forestry and farming) modified mountain slopes, subtracting land to forest, creating the typical heterogeneity of this territory. This aspect characterized the environmental biodiversity, but, during last 30 years, the contraction of mountain activities in favor of less inaccessible territories, led to the depopulation of the Alps (Zanzi, 2004) and the loss of the heterogeneity, in fact the forest started to appropriate the areas which had been stolen (Briand et al., 1989; Chemini and Giannelle, 1999).

ALPINE CHAMOIS

The chamois belongs to the order Artiodactyla, family Bovidae, subfamily Caprinae, tribe Rupicaprini and genus *Rupicapra* (Linnaeus, 1758). Moreover, a revision by Masini and Lovari (1988) describes two species that differ in morphologic, biometric, some behavioral, and genetic issues: one species is represented by the northern chamois, *Rupicapra rupicapra*, with seven subspecies (the alpine one is the most widespread, *Rupicapra rupicapra rupicapra*) (Mustoni et al., 2002); and the other species is the southern chamois, *Rupicapra pyrenaica*, with three subspecies.

Certainly, the chamois is the most spread mountainous wild ungulate, in fact, in Europe and Near East there are more than 36.000 *R. pyrenaica* individuals and 250.000-350.000 *R. rupicapra* individuals (Giacometti et al., 1997; Corlatti et al., 2011). The Alpine Chamois population in the Italian Alps can be estimated at ca. 137,000 individuals, with an 11% increase with respect to the previous datum, due mainly to the raise of the western Alps populations (+15% vs. +7% in the eastern Alps) (Carnevali et al., 2009).

The ideal habitat for this species is represented by steep and rocky slopes, the adaptation to this inhospitable territory has an anti-predatory meaning. Chamois can take advantage of different vegetation types; in fact, he also frequents coniferous and deciduous rich undergrowth, glades and canyons, alder or rhododendrons bushes with some larch trees, pinewoods and grasslands (Mustoni et al., 2002). In summer, females and the yearlings usually remain above the woods, while the adult males, which tend to be more solitary and dispersed throughout the territory, occupy lower altitudes. In autumn, at the beginning of the reproduction season, males join the female groups and occupy grasslands. During winter, the chamois retreat toward rocky zones situated

below the limits of the woods, i.e. on the very steep slopes and windy crests with mainly southern exposures (Mustoni et al., 2002; Tosi and Pedrotti, 2003).

Alpine chamois shows a moderate sexual dimorphism, in fact, unlike deer, both male and female have horns and in juvenile classes there is no difference in body weight (Rughetti and Festa-Bianchet, 2010). In female chamois, the first reproduction often occurs at 2 years of age, but it depend on the population density; while in male the sexual maturity is reached at 5 years old (Loison et al., 1999b). Following a gestation period of about six months, females usually give birth to a single kid, rarely two, in May or early June (Ruckstuhl et al., 1999). Kids start following mother almost immediately after birth (Ruckstuhl et al., 1994). Only females give parental care, thus establishing a strong bond with their kids by using the follower-tactic (Lent, 1974) to reduce predation risk, the kid follows the mother by staying near her, in case of danger moves among mother's legs (Ruckstuhl et al., 1994). Weaning occurs at about six months of age of kids, i.e. November-early December, corresponding with the mating season, but suckling could be observed in January (Ruckstuhl et al., 1999).

Natural mortality is high during the first year of life (30-50% of kids in a population with a good density), females (max 21-24 years) live on the average more than male (max 15-18 years). Different survivals lead to a population structure in favor of females. This mechanism means that the annual growth of the population tends to decrease approaching to the biotic density (a mean of 6-10 heads/100ha) (Capurro et al., 1993; Mustoni et al., 2002).

RESPIRATORY DISEASES IN WILD RUMINANTS

Some studies demonstrate that parasites can change the outcome of inter-specific interactions and can thus play keystone roles in determining species coexistence and biodiversity (Holt et al., 2006; Hatcher et al., 2006). There is also good evidence demonstrating how both micro- and macro-parasites significantly influence birth and death rates (Hudson et al., 2002), although in absence of evident clinical symptoms (Telfer et al., 2002; Burthe et al., 2008). Furthermore, we have to consider that different parasites infect and even co-exist within the same host (Petney et al., 1998). In particular, respiratory tract of wild ruminants is a good example of this "co-infection"; in fact, it is demonstrated that it could be infected and infested by different agents together (Miller et al., 2011; Miller et al., 2012); such as macro-parasites that can cause the so called "lungworms bronchopneumonia" (*Protostrongylus* spp., *Muellerius capillaris*) (Nocture et al., 1998; Panayotova-Pencheva et al., 2010). Also, bacteria (*Mannheimia haemolytica*, *Pasteurella multocida, Biberstenia trehalosi*) are commonly a component of respiratory diseases and they can be either primary or opportunistic infections (Wolfe et al., 2010; Besser et al., 2012). *Mycoplasma ovipneumoniae* and respiratory viruses, especially Parainfluence-3 (PI-3), Respiratory Syncytial Virus (RSV) and Pestivirus (Border Disease Virus - BDV), should be added to list of potential pathogens (Aune et al., 1998; Weiser et al., 2003; Rudolph et al., 2007; Besser et al., 2008; Marco et al., 2009; Marco et al., 2011). Both in North America and Europe, severe outbreaks of pneumonia have been studied in the last 30 years.

Pneumonia has played a significant role in the drastic decline of the bighorn sheep population in North America (Miller, 2001), population in which the

seroprevalence (42%) of Bovine RSV (BRSV) is studied since 1985 (Dunbar et al., 1985). Bacterial pathogens commonly detected in pneumonic bighorn lungs include *Mannheimia (Pasteurella) haemolytica, Bibersteinia (Pasteurella) trehalosi, Pasteurella multocida,* and *Mycoplasma ovipneumoniae* (Miller 2001; Besser et al., 2008; Wolfe et al., 2010). Between 2002 and 2006 concurrent outbreaks have occurred bringing high mortality in different geographic areas of Montana, in all samples *Biberstenia trehalosi* and seropositivity against PI3 and RSV were found (Miller et al., 2011). These results highlighted the importance of identifying the causes and defining appropriate intervention strategies (Hodo, 2010).

In Europe, chamois is the species in which the greatest impact was observed (Citterio et al., 2003; Marco et al., 2008; Fernandez-Sirera et al., 2012; Posautz et al., 2014). One of the most serious pneumonia outbreaks occurred in chamois of Italian Alps during autumn-winter 2000-2001, with a mortality rate until 80% that interested all age classes (Citterio et al., 2003). Most of the chamois hunted or found dead had pulmonary lesions of acute catarrhal pneumonia and acute or chronic fibrinous lobar pneumonia. Serological monitoring before and after this outbreak showed an increase in seroprevalence (up to 95.8%) and antibody titres against Bovine RSV (BRSV), but all samples were seronegative against Bovine Viral Diarrhoea Virus (BVDV), Infectious Bovine Rhinotracheitis (IBR) and PI3. Bacteriological analysis showed the presence of *Mannheimia haemolytica* and *Aerococcus viridans*; most of the lungs were also infested with lungworms (Protostrongylidae). Moreover, this population, in addition to being infested by ticks, showed macroscopic skin lesions due to *Dermatophylus congolensis*, confirmed by histological examinations; this bacteria is considered a warning

about the poor health condition of the animals (Citterio et al., 2003). Roe deer hunted or found dead during the same hunting seasons were examined. Lung pathology in this species was rare; 3% of the shot deer had visible parasitic lesions, but bronchopneumonia or lobar pneumonia were never either observed in the shot deer or in the deer found dead. All roe deer serologically tested were seronegative against BVDV, IBR and PI3 and had a low seroprevalence (8.3% - 23.5%) against BRSV (Citterio et al., 2003).

Between 2001 and 2006, an important outbreak with high mortality involved pyrenean chamois, causing respiratory symptoms and alopecia (Marco et al., 2008). A new pestivirus was isolated, a high virulence strain in chamois, BDV-4 (Marco et al., 2009). The immunosuppressive effect in association with the high virulence of this virus led to high mortality, up to 85%, in several areas. During the same period, in the same area, 57 mouflon, 15 red deer (*Cervus elaphus*), 21 roe deer (*Capreolus capreolus*) and 3 fallow deer (*Dama dama*) were tested for the detection of pestivirus antibodies, only one mouflon resulted seropositive (Marco et al., 2009). A retrospective study was performed in archived sera and spleen of 74 Pyrenean chamois and in archived sera of 28 mouflon (*Ovis musimon*), 56 red deer, 43 roe deer and 29 fallow deer from the Pyrenees between the years 1990 and 2000. Thirty-six of 74 (48.6%) sera of Pyrenean chamois, one of mouflon and one of red deer were positive by an ELISA antibody test (Marco et al., 2011).

However, seroprevalence of pestiviruses has been reported in more than 40 species of free-ranging wild ruminants, but there are only a few confirmed cases of isolation of pestivirus from disease and no mortality outbreaks have been reported (Van Campen et al., 2001). In the European Cervids, the most frequently found seropositive species is the red deer, with seroprevalence

ranging from 0.4% to 5.9%, while seroprevalence in roe deer ranging from negative to 12.3 %, and in fallow deer from negative to 58% (Baradel et al., 1988; Giovannini et al., 1988; Frolich, 1995; Schmitt & Wittowski, 1999; Nielsen et al., 2000; Lillehaug et al., 2003; Krametter et al., 2004; Olde Riekerink et al., 2005; Bregoli et al., 2006; Gaffuri et al., 2006; Boadella et al., 2010).

Although RSV and pestiviruses have been reported in chamois populations, their impact on population dynamics has not yet been clarified. Pestiviruses are not strictly host-specific, in fact, several studies have shown interspecies transmission, and for example, Classical Swine Fever Virus (CSFV) causes a contagious haemorrhagic disease in pigs and in wild boars and BVDV specific antibodies have been reported in captive and free- living animals as Cervids and Bovids (Vilcek & Nettleton, 2006). Concerning chamois, there's high seroprevalence in Spain and France (Pioz et al., 2007; Marco et al., 2011), low seroprevalence in Italian North-Western Alps (Olde Riekerink et al., 2005) and in Central Alps (Citterio et al., 2003), while in Austria (Krametter et al., 2004) chamois populations are seronegative. BRSV seroprevalence observed in several wild ruminants populations suggests an endemic frequency of infection (Citterio et al., 2003; Armaroli et al., 2006; Gaffuri et al., 2006; Boyce et al., 2011). In domestic ruminants there are two strains, one is the Bovine and Caprine RSV, the other is the Ovine RSV (Alansari et al., 1999), showing serological cross-reaction. Overall, available data suggest that respiratory viruses have different adaptation levels to wild ruminants, in particular to chamois. Pestiviruses seem to have a lower adaptation level, based on sporadic frequency or absence in some areas and on the singular virulence profile during outbreaks. While the endemic spread of RSV suggests a higher adaptation level to the host species.

RESPIRATORY SYNCYTIAL VIRUS

Respiratory syncytial virus (RSV) is a pneumovirus of the family *Paramixoviridae* and it is highly prevalent in cattle, with a significant economic impact as the most important viral cause of bovine respiratory disease worldwide (Bunt et al., 2005; Meyer et al., 2008). Pneumoviruses include pneumonia virus of mice (PVM), bovine (B)RSV, ovine (O)RSV and caprine (C)RSV. Although the viruses are structurally and antigenically related, BRSV and CRSV are the most closely related (Alansari et al., 1994; Lehmkhul et al., 1980; Smith et al., 1979; Trudel et al., 1989).

Although bovines are the natural host of BRSV, an epidemiological role of other species, such as ovine, caprine, chamois or camelids, is possible in certain circumstances (Dunbar et al., 1985; Rivera et al., 1987; Van Der Poel et al., 1995; Sausker et al., 2002; Citterio et al., 2003).

BRSV is also genetically and antigenically closely related to Human RSV (HRSV), which is a major cause of respiratory disease in young children, and the epidemiology and pathogenesis of infection with these viruses are similar **(**Van Der Poel et al., 1994**).**

It is an enveloped, non-segmented, negative-stranded RNA virus (Stott et al., 1985), the virion is very pleomorphic, with a shape roughly rounded and a diameter between 150 and 35 nm (Trudel et al., 1989). The envelope containing three virally encoded transmembrane surface glycoproteins, which are organised separately into spikes on the surface of the virion (Collins et al., 2001). The genome is characterised by the existence of two non-structural proteins, NS1 and NS2, and a transcriptional overlap between M2 and L that lead to the synthesis of M2-1 and M2-2 proteins (Valarcher et al., 2007).

The virus causes regular winter outbreaks of respiratory disease in cattle (Stott et al., 1980). The seroprevalence in domestic herds could be very high (Baker et al., 1986; Caldow et al., 1988; Elvander et al., 1996; Luzzago et al., 2010), but the frequency of infections is related to the density population in an area and the age of the animals (Elvander, 1996). The infection is associated with high morbidity (60-80%) and mortality up to 20% (Valarcher et al., 2007). The transmission is mainly by direct contact between infected and healthy animals or by aerosol (Mars et al., 1999), but also humans act a role as passive vectors of virus (Hall et al., 1980). Moreover, some data indicate that the virus may persist in infected animals (Thomas et al., 1980; De Jong et al., 1996).

Calves, mainly, demonstrate severe clinical signs (Kimman et al., 1988; Stott et al., 1980), but sometimes also adults (Elvander, 1996); this situation can be explained by the level of specific immunity following frequent exposure to the virus (Valarcher et al., 2007). BRSV infection may be asymptomatic or cause cough with seromucoid nasal and ocular discharge, in slight cases, or depression, anorexia, hyperthermia, polypnea, abdominal dyspnoea, if bronchopneumonia or bronchiolitis are present (Verhoeff et al., 1984). In more severe cases pulmonary emphysema, oedema and subcutaneous emphysema might occur (Belknap, 1993; Bryson, 1993). Macroscopic lesions are typical of broncho-interstitial pneumonia with lung areas consolidated and with mucopurulent discharge in bronchus and small bronchi (Baker et al., 1986). Microscopic lesions are characterised by a proliferative and exudative bronchiolitis, alveolar collapse and peribronchiolar infiltration by mononuclear cells (Thomas et al., 1984); giant cells and syncytia may be present in bronchi lumen and epithelium (Viuff et al., 2002).

PESTIVIRUS

The family *Flaviviridae* comprises the genera *Flavivirus*, *Pestivirus* and *Hepacivirus*, the members of which, although similar in genomic organization and physicochemical properties, are genetically distinct and biologically quite different (Fenner, 2001). The genus *Pestivirus* comprises viruses that are major pathogens for both domestic and wild ungulates (Vilcek and Nettleton, 2006) and which can cross species barriers infecting several hosts (Rossi et al., 2005; Passler et al., 2009). These viruses are border disease virus (BDV) of small domestic ruminants, that segregates into at least eight phylogenetic groups (Giammarioli et al., 2011), bovine viral diarrhea virus 1 and 2 (BVDV-1, BVDV-2) of cattle and classical swine fever virus (CSFV) of domestic and wild pigs (Vilcek et al., 2014). Genetic changes of viruses can lead to alterations of virulence and adaptation to new hosts, as recently observed with the Bungowannah pestivirus causing myocarditis with high mortality in pigs (Kirkland et al., 2007; Finlaison et al., 2009) and the emergence of ovine pestiviruses more closely related to CSFV than to ruminant pestiviruses in Tunisia and Spain (Hurtado et al., 2003; Thabti et al., 2005). Pestiviruses are enveloped RNA viruses containing single-stranded, positive-sense RNA genomes (Becher et al., 2003), virions are spherical 40-60 nm in particles (Meyers and Thiel, 1996).

These viruses can infect different species and lead to important economically losses worldwide (Houe, 1999), in particular BVDV and BDV can cross species barriers and infect many hosts within the order of *Artiodactyla* (Becher et al., 1999; Becher et al., 1997; Doyle and Heuschele, 1983; Hamblin and Hedger, 1979; Nettleton 1990; Pellerin et al., 1994). They causes important reproductive problems, such as abortion, stillbirth, decrease of fertility, and have an

immunosuppressive effects that may increase other opportunistic infections (Nettleton, 2000). Moreover, there may be diarrhea, thrombocytopenia, and, frequently, unapparent courses (Baker 1987; Thiel et al., 1996). Small ruminants, in particular sheep, can show neurological signs, body malformation and poor growth rate of lambs (Nettleton, 2000). The major problem is the unapparent course, because may lead to a trans placental infection and so the birth of persistently infected (PI) animals that keep viral circulation (Letellier and Kerkhofs, 2003).

The virus was detected in blood feeding flies fed on PI calves (Tarry et al., 1991), so the transmission by flies has been suggested (Rehbinder et al., 1992).

Also in wildlife, Pestiviruses have been widely described and isolated from camelids (Evermann, 2006), cervids (Frolich and Hofmann, 1995), and in a great number of Bovidae (Vilcek and Nettleton, 2006). A recent outbreak with high mortality of a new Pestivirus has been largely studied Pyrenean chamois (*Rupicapra pyrenaica*) in Spain and France (Marco et al., 2007; Pioz et al., 2007). A novel strain was isolated from chamois found dead and the results of the studies show the adaptation of a BDV strain (BDV-4) in chamois and the wide spread in the population (Marco et al., 2008; Marco et al., 2009).

In mountainous areas, the risk is the interaction between domestic and wild ruminants because of sharing summer grazing, so both direct contact and contamination of pasture could spread the infection (Richomme et al., 2006). In fact, pestiviruses can be eliminated by different body fluids, including nasal discharge, urine, milk, semen, saliva, tears and fetal fluids (Meyling et al., 1990), while feces are a poor source of virus (Brownlie et al., 1987).

BVDV

BVDV is highly mutable and endemic in cattle (Hamers et al., 2001). This virus is divided into non-cytopathogenic (ncp) and cytopathogenic (cp) biotypes based on effects on cultured cells rather than in the infected host. Cp biotypes induce apoptosis in cultured cells (Gamlen et al., 2010), while ncp biotypes do not. Ncp strain is the responsible of the birth of persistently infected (PI) animals when the infection occurs before approximately 125 days of gestation (Grooms, 2004). These animals develop immunotolerance to the virus, so they have no antibodies against BVDV, and exhibit presence of virus throughout the body (skin, semen, secretions, milk, blood) (Thurmond, 2005; Marley et al., 2009; VanderLey et al., 2011). PI calves exhibit a 50% greater death rate in the first year of life than uninfected calves (Duffell and Harkness, 1985). PI calves may develop fatal mucosal disease (MD), from death animals both an ncp and a cp biotype can be isolated (Bolin, 1995; Brownlie et al., 1984) that are antigenically very similar (Brownlie, 1990). While immunocompetent animals infected with BVDV develop acute infection, clearing the virus from the body within 7–21 days and develop lifelong antibodies (Nettleton et al., 1995). In both types of infection, clinical signs vary between asymptomatic through mild transient signs to severe acute disease with signs from enteric, hematopoietic, reproductive, or respiratory systems.

BDV

BDV infects small ruminants and causes border disease worldwide. Several BDV strains have been proved to infect pigs and cattle under experimental or nature conditions (Cabezon et al., 2010; Sharp et al., 1986). This virus is the causative agent of barren ewes, abortions, stillbirths, births of persistently infected lambs with tremors, ataxia, hairy fleece, brain malformations and poor

growth, in case of congenital infection (Nettleton et al., 1998). While the clinical manifestations in acutely infected healthy sheep are mild or unapparent. However, an unusually virulent BDV isolate, Aveyron strain, was reported in sheep from the Aveyron region (France) in 1984 and was associated with an outbreak of disease with high mortality (Chappuis et al., 1986).

Based on recent reports, BDV isolates have been divided into seven genotypes at least, and widely distributed in different countries, such as many European countries, Australia, New Zealand, Canada, the United States, India, Turkey, and Japan (Oguzoglu et al., 2009; Strong et al., 2010).

Some cross-reactivity of BDV toward BVDV-1 NADL strain was observed (Becher et al., 2003).

MAMMALIAN ORTHOREOVIRUS

Reoviruses (respiratory enteric orphan viruses) represent a large and diverse group of non-envelope viruses and they are taxonomically classified into 15 genera in the family *Reoviridae* (Chapell et al., 2005). This family can be divided into two subfamilies: the *Sedoreovirinae* and *Spinareovirinae* with six and nine genera respectively (Day, 2009). *Orthoreovirus* is one genus within the first subfamily and consists of five species: *Mammalian orthoreovirus* (MRV), *Avian orthoreovirus* (ARV), *Reptilian orthoreovirus* (RRV), *Baboon orthoreovirus* (BRV) and *Pteropine orthoreovirus* (PRV). The genus is also divided into two subgroups, fusogenetic and non-fusogenetic orthoreoviruses, based on the ability to cause formation of large multinucleated syncytial cells as part of the cytopathic effect (Duncan, 1999).

Orthoreoviruses are non-enveloped viruses with a segmented dsRNA genome (Day, 2009), each viral particle contains 10 genome segments, which are designed as large (L1, L2, L3), medium (M1, M2, M3) and small (S1, S2, S3, S4) on the basis of the electrophoretic mobility (Nibert and Schiff, 2001). The virions have an average size of 70-80 nm with a typical icosahedral, double-layered protein capsid structure (Nibert and Schiff, 2001).

MRV is the prototype species being the first orthoreovirus isolated from humans in 1950 (Sabin, 1959) and the only non-fusogenetic species. It has four major serotypes differentiated by the anti-sera capacity to neutralize viral infectivity and inhibit haemaglutination (HA): type 1 Lang (T1L), type 2 Jones (T2J), type 3 Dearing (T3D) and type 4 Ndelle (T4N) (Day, 2009; Attoui et al., 2001). Neutralization and HA activities are restricted to a single gene segment, S1 that encodes for σ1 (Weiner and Fields, 1977). The σ1 protein is located on the outer capsid of the virion and it is responsible for viral attachment on cellular receptors

and determines the serotype (Bassel-Duby et al., 1986). Analysis of the S1 gene has shown a strict correlation between sequence similarity and viral serotype (Dermody et al., 1990; Duncan et al., 1990; Nibert et al., 1990). The other genome segments show no correlation to viral serotype, suggesting that MRV have evolved independently of serotype (Leary et al., 2002).

MRV have a wide geographic distribution and can virtually infected all mammals, including humans, which are responsible for either symptomatic or asymptomatic infections (Tyler et al., 2001).

Orthoreoviruses are resistant to lipid solvents and are stable over a wide range of pH and proteolytic enzymes increase their infectivity (Fenner, 2001).

MRV, especially type 3, cause an experimental disease syndrome in neonatal laboratory mice that is characterized by jaundice, diarrhea, runting, oily hair, and neurologic signs (e.g., ataxia). Natural infections in laboratory mice are invariably subclinical. Laboratory mice, rats, hamsters, guinea pigs and rabbits, and many other mammals as well, may be infected with any of the serotypes of reovirus (Gauvin et al., 2013). The greatest significance of reoviruses in laboratory animals is the cost of surveillance and prevention, which are questionable endeavors. These viruses have also been implicated as causes of respiratory and enteric disease in horses, cattle, sheep, swine, and dogs (Jackson and Muldoon, 1973; Tyler et al., 1990; Decaro et al., 2005; Narayanappa et al., 2015); however, as with mice, their true pathogenic significance is highly conjectural. Infection of primates and humans has been associated with meningitis (Tyler et al., 2004; Jiang et al., 2006; Kumar et al., 2014).

Studies on reoviral entry into the intestinal tract have provided a possible clue for viral (and possibly microbial) entry in the respiratory tract (Schiff and Fields, 1990). They have served as useful models for the study of viral pathogenesis,

including studies on how viruses interact with the intestinal tract, central nervous system, myocardium, liver (Tyler et al., 1990), and more recently, the lungs (Morin et al., 1994). In fact, reoviruses enter bronchus associated lymphoid tissue (BALT) through pulmonary M cells and spread from the airways to regional lymph nodes (Morin et al., 1994), and then they directly infect the alveolar epithelium following experimental inoculation, causing severe pneumonia. The host response was characterized by an infiltration of leukocytes into the pulmonary alveoli, viral replication in type I alveolar epithelial cells, and type II alveolar cell hyperplasia (Morin et al., 1995).

Recent MRVs isolations in Italian microbats (Lelli et al., 2013), without symptoms neither macroscopic lesions, closely related to T3/porcine/Sichguan/SC-A/2006 isolated in China and others strains isolated from dogs in Italy (Decaro et al., 2005), underline the apparent lack of species barriers (Lelli et al., 2013). Moreover, MRVs of bat origin isolated in Malaysia from adult human patients with acute respiratory diseases (Chua et al., 2007; Chua et al., 2011) put on alert on the possibility of a zoonotic implication of these viruses.

WILDLIFE MANAGEMENT AND HUNTING ACTIVITY

The principle of a correct wildlife health control and management is based on the detection of diseases as soon as possible to prevent secondary cases and limit the spread of pathogens by applying appropriate strategies and techniques (Vallat, 2008). Starting from this point of view, it is necessary to understand the importance of surveillance, monitoring and surveys. *Surveillance* is the systematic collection, analysis and interpretation of animal health data and the spread of information to the stakeholders and it can be reactive or proactive. *Monitoring* is addressed in measuring epidemiological parameters, such as prevalence and incidence, related to a defined disease. *Surveys* are specific activities to identify and understand a specific problem and they are limited in space and time (Guberti et al., 2014). The first step is the proactive surveillance that analyses the whole population or a selective part of it by sampling. Sampling wild population we have to consider that the domestic animal approach can't be applied. So, in this framework, hunters and gamekeepers are the most useful stakeholders thanks to their knowledge of the territory and of the animals living in. For this reason, hunting activity can be one important means to implement the sample size.

On a national level, law n.157/92 is the base for wildlife management and conservation, including hunting activity. All mammals and birds, stably or temporarily, living in free-range condition in the national territory are considered "wildlife" (art. 2.1), and they are unavailable heritage of the State (art. 1.1). Private citizen, of at least 18 years old, can ask for a hunting permission and take part to the selective cull (art. 12.1) even in that areas identified as "Alpine territory", i.e. the territory in which typical alpine animals and vegetation are well represented (art. 11.1). This area is considered a part from the rest of the

national territory and is divided in hunting districts called “Comprensori Alpini” respecting local habits and traditions (art. 14.4), established by the provinces (art. 10.7). All the alpine regions have the autonomy to manage hunting activity, respecting the law mentioned above (art. 9.1); provinces are quite independent to manage hunting in their Comprensorio (art. 10.7).

In Piemonte, hunting is regulated also by the regional law 5/2012 and by the actuations of the abrogated L.R. 70/96. In particular, hunting wild ruminants on the Piemonte Alps is permitted by Guide Lines for the organization and realization of hunting plans of bovids and cervids, approved by D.G.R. 94-3804/2012. The number of wild ungulates each hunter can shot is decided on the basis of the annual census and of the trend of the previous hunting season, trying to respect the natural biology and ecology of the species and the natural ratio between age and sex classes.

Then the individual provinces have some autonomy in deciding the hunting method and the hunting days during the week (a part from Tuesday and Friday that are for “hunting silence” all over the national territory).

MATERIALS AND METHODS

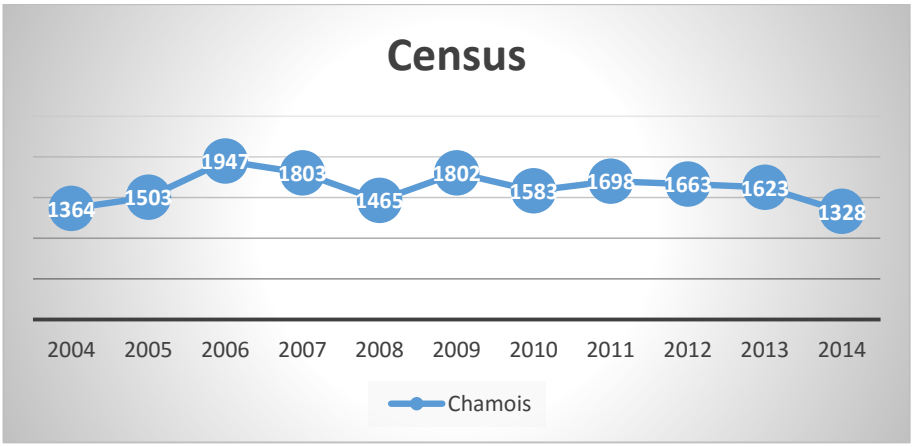
STUDY AREA

The PhD project was carried out during three hunting seasons (2012-2013-2014) in two neighboring alpine areas: Comprensorio Alpino di Caccia Verbania Cusio Ossola 2 - Ossola Nord (VCO2) and Comprensorio Alpino di Caccia Vercelli 1 – Valle del Sesia (VC1) respectively.

VCO2

This area is located in Verbania province (North-West Italian Alps - 445 Km E, 5107 Km N), with an extent of 72.740 ha, of which 49.275 ha are open for hunting. The territory includes three Comunità Montane (Antigorio-Divedro-Formazza, Vigezzo and Ossola), three principal valleys (Val Vigezzo, Valle Antigorio, Val Formazza) and three secondary valleys (Valle Devero, Valle Isorno, Val d'Ossola). The agricultural, forestry and farming territory amounts to 48.452 ha without considering unproductive surfaces, waters and urbanized areas (Carlini et al., 2014).

There are stable populations of chamois (*Rupicapra r. rupicapra*) (species useful surface area of 32.736 ha), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and ibex (*Capra ibex*). Wild boar is widespread in the south territories. Bovids and cervids hunting has the aim to reach the maximum possible cull in the shortest time, so starting from the end of September the culling ends in a maximum of ten hunting days. Wild boar is hunted either with selective cull method that hound method. Each hunter can shoot a maximum of six wild ruminants and five wild boars per year. Furthermore, some domestic herds occupy alpine pastures during summer season, but before reaching grazing areas, at the beginning and at the end of the season, they all stay together some days in the municipality of Premia.



Graph 1. Population trend derived from annual census.

This is a quite healthy population, an outbreak of keratoconjunctivitis is reported in 2012 and a severe outbreak of respiratory disease during 2014 hunting season. Another aspect reported is the decrease of yearlings mean weight since 1996 to 2009 (Viganò, 2009). Moreover, there is an historical series of serological data since 2006.

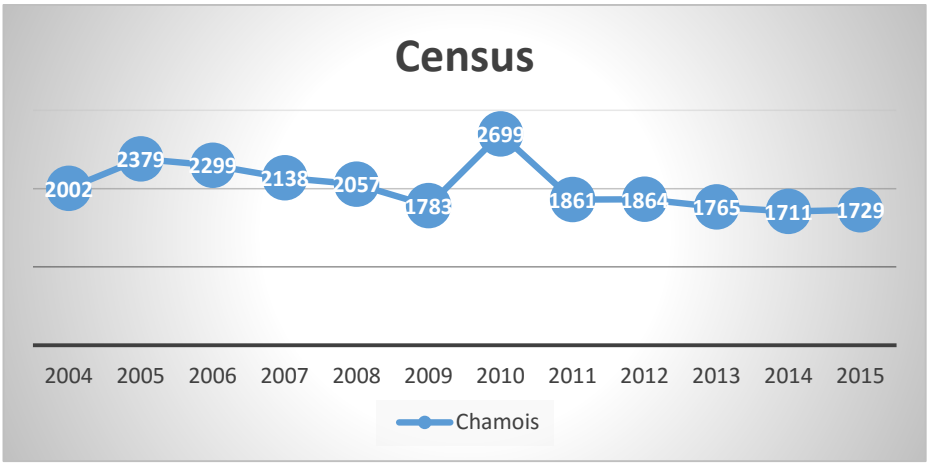
VC1

This area is located in Vercelli province, with an extent of 77.668 ha, of which 51.182 ha are used for hunting, and includes three geographically different district, "Alta Valsesia", "Media Valsesia" and "Prealpi Biellesi e Valsesiane" (De Biaggi et al., 1990). The agricultural, forestry and farming territory amounts to 46.315, 25 ha without considering unproductive surfaces, waters and urbanized areas (Bevilacqua et al., 2014).

Climatic, geological and anthropogenic factors, in particular high rainfall during summer season and soil acidity, have affected the development of a uniform vegetation of chestnut tree (*Castanea sativa*) than other Alpine Western valleys (Perrone, 2009).

There are stable populations of chamois (species useful surface area of 48.792,11 ha), roe deer, red deer, mouflon and ibex. Wild boar is widespread, in particular

in pre-alpine territories. Bovids and cervids hunting is based on the selective cull method which provides the assignment of determined species, age class and sex to each hunter based on previous census, hunters can shot their heads in about two and half months. Each hunter can shoot one wild ruminant per species and twenty wild boars per year. Furthermore, several flocks are present in this territory, in particular the province is famous for wool production.



Graph 2. Population trend derived from annual census.

A part from annual census, no previous data is available for this population, the creation of a dataset began in 2013.

SAMPLING

Blood samples

Hunters collected post-mortem-blood samples via jugular or heart clot from their own bag and leaded up to the Control Centre where serum is obtained by centrifugation and stored at -20°C until further processing. Because of their quality, such as haemolysis or bacteriological contamination, not all sera could be used for further examination.

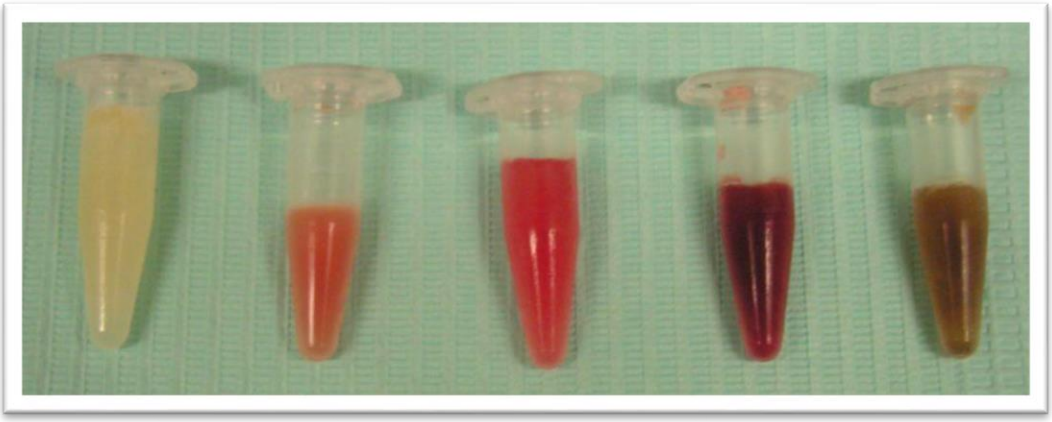


Photo 1. From left to right: example of different degrees of haemolysis, starting from a serum obtained *in vivo,* up to a serum obtained from a blood sample contaminated with other fluids.

Tissues and Organs samples

Lungs of yearlings or adults with macroscopic lesions, other than verminous pneumonia, were selected for sampling. Lungs were collected by means of swabs in universal transport medium (UTM Kit, Copan) for virus preservation, that were stocked at +4° C for one night and subsequently transferred at -80° C to the laboratory facilities until further processing. Samples of lung tissue were also collected and stored at different conditions: directly stored at -80°C for the subsequent homogenization and inoculation of cell cultures, frozen at -80° C in OCT in order to section the tissue with cryostat for direct and indirect immunofluorescence, fixed in formalin for histopathological and immunohistochemical examinations and a part in Trizol® for PCR. Moreover, pulmonary swabs with Amies transport medium for bacteriological survey have been collected.

In VC1, also samples of skin were collected and fixed in formalin for histopathological examinations because of a suspect dermatophylosis.

MACROSCOPIC EXAMINATION

A macroscopic examination of each carcass is conducted at the Control Centre. Soon after the shoot, hunters have to eviscerate the hunted animal removing at least the gastrointestinal tract. For this reason, at the Control Centre is not possible to check all the organs, in some cases there is no organ.

SEROLOGICAL SCREENING

Virus neutralization test

According to the method of O.I.E. (1996), partially modified, the sera were tested by virus-neutralization (VN) test against BVDV strain NADL (ATCC VR-534), BRSV 375 and MRV Type 3 strain Abney (ATCC VR-232). VN was performed onto Madin Darby Bovine Kidney (MDBK ATCC CCL-22) cells, maintained in minimum essential medium (MEM) with 1% of L-glutamine 200mM, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2.5 µg/ml of fungizone and 10% of fetal bovine serum (FBS). The plates were incubated at 37°C with 5% of CO_2 for 96 hours (BVDV), 7 days (BRSV) and 72 hours (MRV).

VIRAL ISOLATION IN CELL CULTURE

The bronchopulmonary swabs tubes were vortexed and the transport medium was transferred in sterile tubes and centrifuged at 3.300 RPM for 10 minutes at 2-8° C. The supernatant was inoculated in 24 well plates in subconfluent monolayers of MDBK cells, maintained in MEM supplemented as previously described. The plates were incubated at 37°C with 5% of CO_2 and after a 1-2 hours adsorption period the cell culture were rinsed and maintenance medium was added. The cell cultures were observed daily for cytopathic effect (CPE) for 6 days. Two blind passages have been made if no CPE was observed, the cell

cultures were scraped and vigorously mixed with culture medium and used for the inoculation of fresh monolayers.

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

Specimens of affected lungs were fixed in 10% buffered formalin, included in paraffin-wax and sectioned at 4 mm. Sections of each sample were stained with hematoxylin-eosin and Periodic acid Schiff reaction. Serial sections were also processed with avidin biotin complex method for detection of BVDV and BDV using the monoclonal antibody 15c5 (Corapi et al., 1990; Arnal et al., 2004).

POLIMERASE CHAIN REACTION AND SEQUENCING

Viral RNA was extracted from lysates of infected cells using TRIZOL® LS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was resuspended in 10 µl of DPEC water. The eluted RNA was used for retrotranscription using QIAamp One-For-All Nucleic Acid kit (Qiagen, Mississauga, Ont) and subject to PCR for detection of S1 genomic fragment of MRV (Decaro et al., 2005).

For each sample, the amplicons of the expected sizes were purified and sent for outsource sequencing with the same forward and reverse primers used for the amplification.

PHYLOGENETIC ANALYSIS

The chamois sequences of L1 obtained in a previous study (Luzzago et al., 2011) and S1 segments were aligned with MRV representative reference strains and other sequences downloaded from GenBank and used to build the phylogenetic trees. Only strains for which the complete genome or both L1 and S1 segments were available in Genbank were included. Sequences were aligned using Clustal X; manual editing was performed with Bioedit software version 7.0 (freely

available at http://www.mbio.ncsu.edu/bioedit/bioedit.html). Phylogeny was estimated by the neighbor-joining algorithm (NJ) using MEGA version 5 (Tamura et al., 2011). The robustness of the branching was evaluated by bootstrapping using 1000 replications.

BACTERIOLOGICAL EXAMINATION

Swabs with Amies were maintained refrigerated (+4°C) until processing within 24 hours. Swabs ere passed on blood-agar plates and incubated at 37°C ± 2°C for 24 and 48 hours. After incubation was carried out the isolation and the identification of bacterial colonies.

STATISTICAL ANALYSIS

Data collected on the spreadsheet were analysed with statistical software (IBM® SPSS®, Versione 20). For statistical purposes, the distribution of the frequencies of different variables (age and sex classes, date and place of culling) were compared with One-Way ANOVA test. Significance was accepted for p values<0.05. As regards the calculation of prevalence, with their confidence intervals, it is used the online program Winepi (http://www.winepi.net/).

RESULTS

SEROLOGICAL SCREENING AND STATISTICAL ANALYSIS

In three years PhD, a total of 207 sera have been collected in the two study areas. In VCO2, the study could be implemented thanks to the serological screening and the storage of sera of previous years. So on the whole, 1528 chamois has been checked at the Control Centre, and 284 blood samples have been collected. Regarding BRSV and BVDV, during 2007-2009 sera were analysed as a part of a previous monitoring project (Viganò, 2009; Besozzi, 2012), sera collected and stocked during 2010-2011 were analysed retrospectively together with those collected since 2012. Regarding MRV3, sera were tested retrospectively after the isolation of virus from three chamois lungs sampled in 2009 hunting season. In VC1, 413 chamois has been brought to the Control Centre and from 109 blood samples have been collected. Table 1 shows number of chamois hunted and sampled in different years.

	VCO2		VC1	
YEAR	N. HUNTED	N.SAMPLED	N. HUNTED	N.SAMPLED
2007	209	28		
2008	175	39		
2009	202	65		
2010	201	37		
2011	136	17		
2012	197	29		
2013	217	52	211	60
2014	191	17	202	49

Table 1. Number of hunted and sampled chamois during hunting seasons in the two study areas.

Table 2, 3 and 4 show number of tested and seropositive chamois and seroprevalence during years of study in VCO2.

BRSV			
YEAR	N. TESTED	N. POSITIVE	% SEROPREVALENCE (C.I. 95%)
2007	25	19	76.00 (59.37-92.63)
2008	35	27	77.14 (63.40-90.89)
2009	60	34	56.67 (44.34-68.99)
2010	28	17	60.71 (42.78-78.64)
2011	14	3	21.43 (0.02-42.83)
2012	24	8	33.33 (14.61-52.06)
2013	48	19	39.58 (25.95-53.21)
2014	17	5	29.41 (7.89-50.93)

Table 2. Number of tested and positive chamois and % of seroprevalence for BRSV during hunting seasons in VCO2.

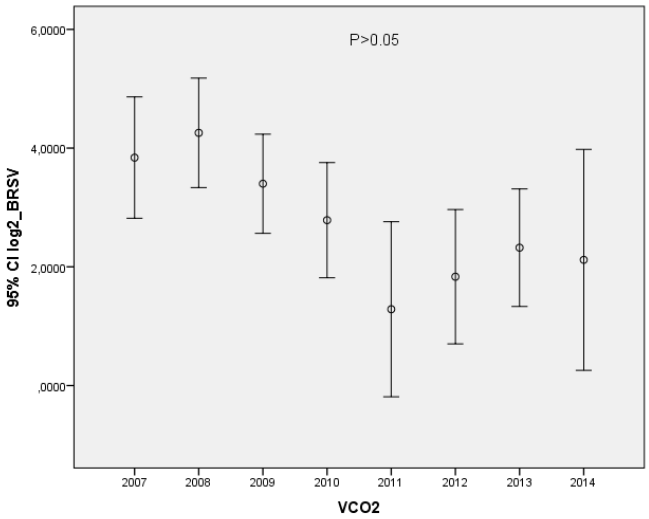
BVDV			
YEAR	N. TESTED	N. POSITIVE	% SEROPREVALENCE (C.I. 95%)
2007	28	0	0.00 (0.00-10.09)
2008	38	1	2.63 (0.00-7.65)
2009	55	3	5.45 (0.00-11.36)
2010	30	3	10.00 (0.00-20.63)
2011	14	1	7.14 (0.00-20.58)
2012	28	6	21.43 (6.36-36.50)
2013	43	4	9.30 (0.74-17.87)
2014	15	1	6.67 (0.00-19.22)

Table 3. Number of tested and positive chamois and % of seroprevalence for BVDV during hunting seasons in VCO2.

MRV3			
YEAR	N. TESTED	N. POSITIVE	% SEROPREVALENCE (C.I. 95%)
2008	16	9	56.25 (32.08-80.42)
2009	37	23	62.16 (46.70-77.63)
2010	19	8	42.11 (20.04-64.17)
2011	12	9	75.00 (50.59-99.41)
2012	18	10	55.56 (32.72-78.39)

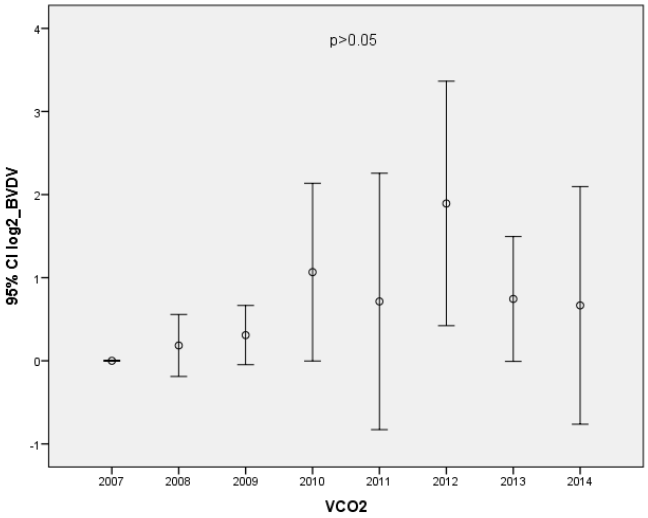
Table 4. Number of tested and positive chamois and % of seroprevalence for MRV during hunting seasons in VCO2.

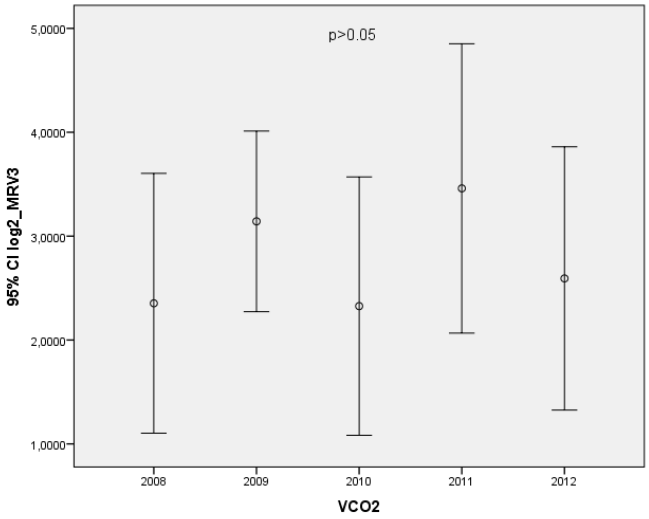
Seroprevalence of BRSV decreases during years from 76% in 2007 to 29% in 2014; while seroprevalence of BVDV and MRV is stable at low (BVDV) and mildly high (MRV) rates. In graphs below (Graphs 3, 4, 5), no significant differences between years are shown in antibody titres for three viruses studied in VCO2.



Graph 3. Error bar graph. Trend of antibody titres of BRSV in VCO2.

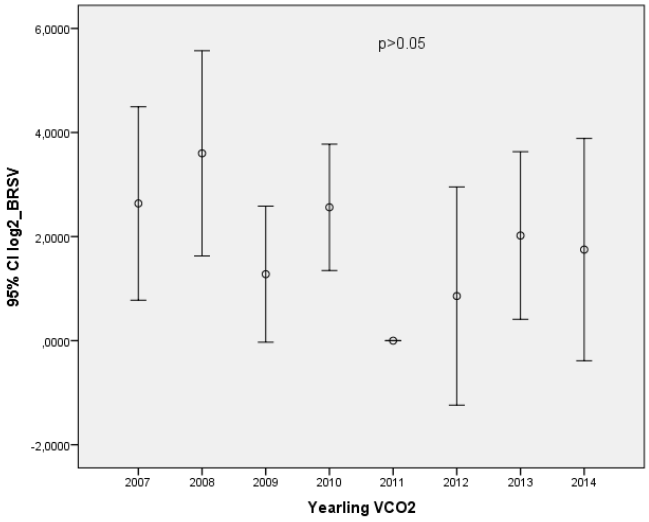
Graph 4. Error bar graph. Trend of antibody titres of BVDV in VCO2.





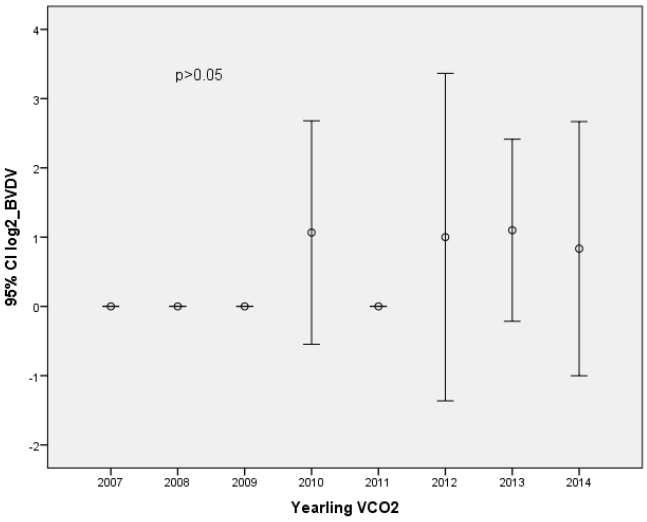
Graph 5. Error bar graph. Trend of antibody titres of MRV in VCO2.

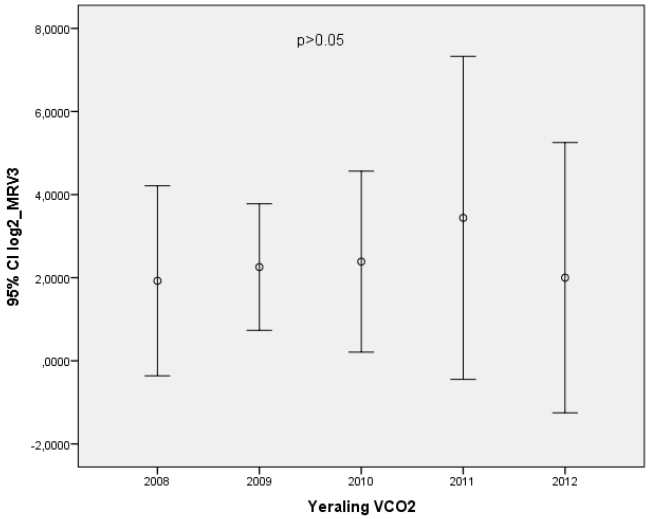
The next six graphs show the trends of antibody titres of the viruses in VCO2 during years of study in yearlings and in adults showing no significant statistical differences. Although, it can be underlined that for BRSV antibody titres have a downtrend during years above all in yearlings (Graph 6); while concerning BVDV, yearlings became to be seropositive only since 2010 hunting season (Graph 7). Regarding MRV, antibody titres are steady during year both in yearlings and adults (Graph 8 and 11).



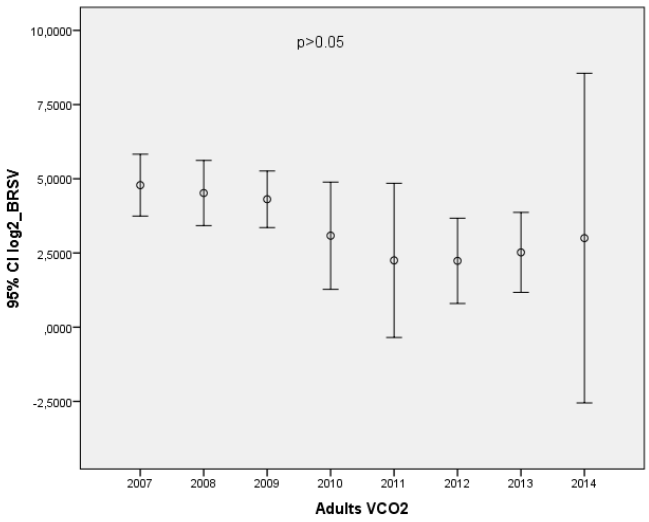
Graph 6. Error bar graph. Trend of yearlings antibody titres of BRSV in VCO2.

Graph 7. Error bar graph. Trend of yearlings antibody titres of BVDV in VCO2.

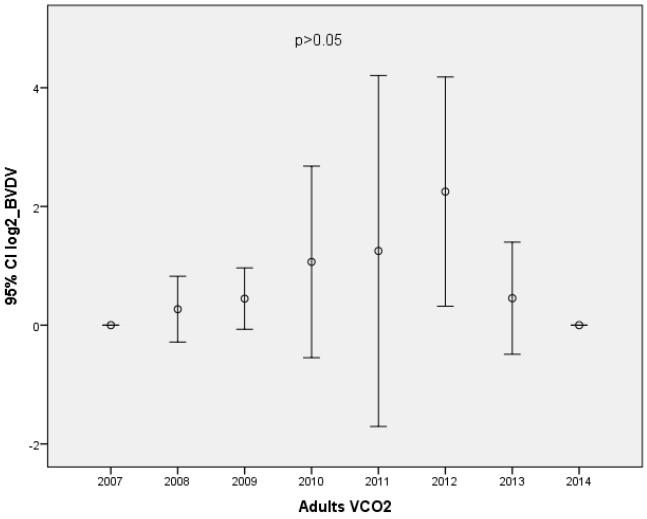




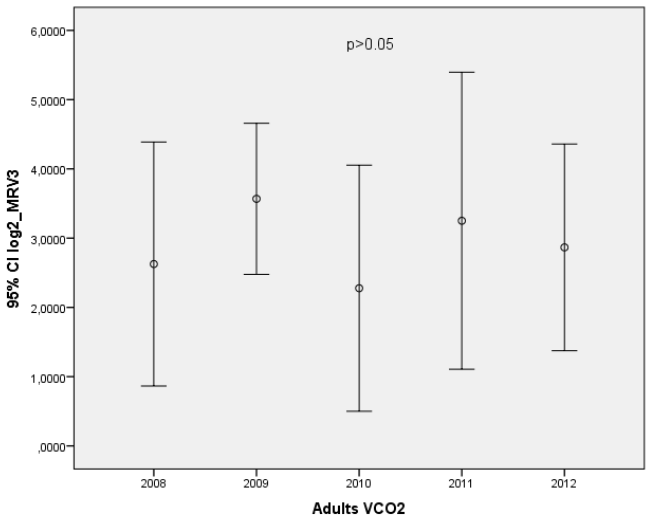
Graph 8. Error bar graph. Trend of yearlings antibody titres of MRV in VCO2.



Graph 9. Error bar graph. Trend of adults antibody titres of BRSV in VCO2.

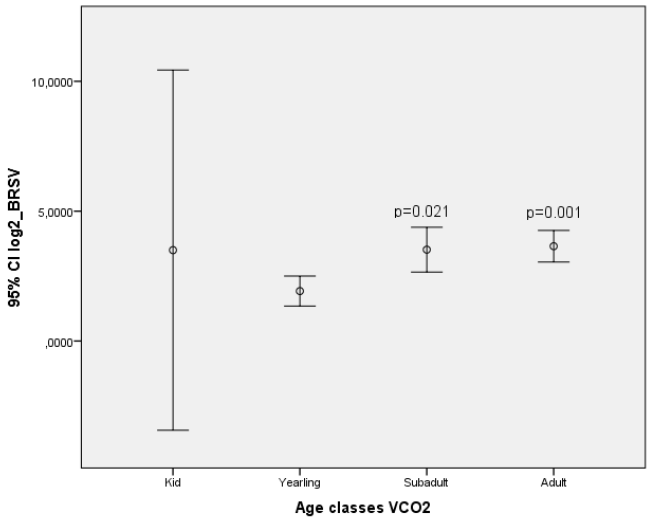


Graph 10. Error bar graph. Trend of adults antibody titres of BVDV in VCO2.



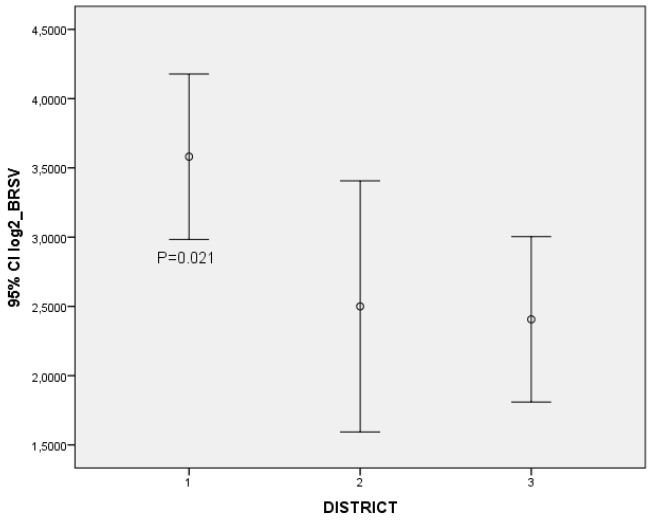
Graph 11. Error bar graph. Trend of adults antibody titres of MRV in VCO2.

Antibody titres of different age classes (kid, yearlings, subadults, adults) were compared with each other, giving evidence of statistical difference between yearlings and subdults (p=0.021) and between yearlings and adults (p=0.001) only in BRSV, as showed in graphs.

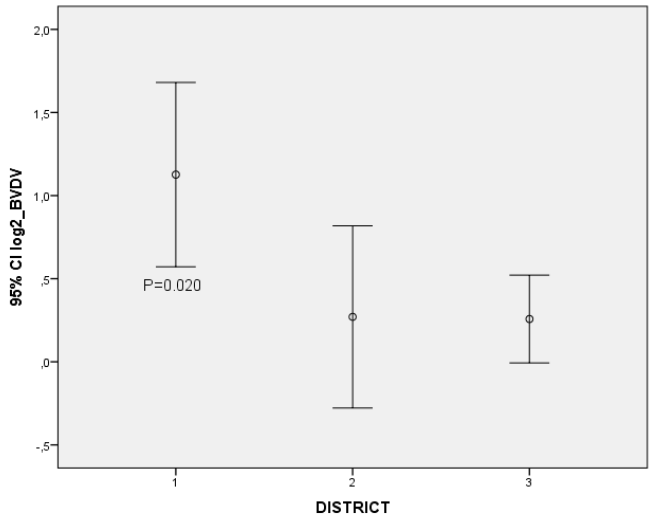


Graph 12. Error bar graph. Difference in BRSV antibody titres among different age classes in VCO2.

Then considering location of shooting in relation to three districts of VCO2 geographically well distinct, district 1 Antigorio (Crodo, Baceno, Premia, Formazza), district 2 Isorno (Montecrestese, Cravariola) and district 3 Vigezzo (Val Vigezzo), the statistical analysis shows a significant difference between district 1 and the others, both in BRSV and BVDV antibody titres (Graphs 13 and 14).



Graph 13. Error bar graph. Difference in BRSV antibody titres within districts in VCO2.



Graph 14. Error bar graph. Difference in BVDV antibody titres within districts in VCO2.

Table 5 and 6 show number of tested and seropositive chamois and seroprevalence during years of study in VC1.

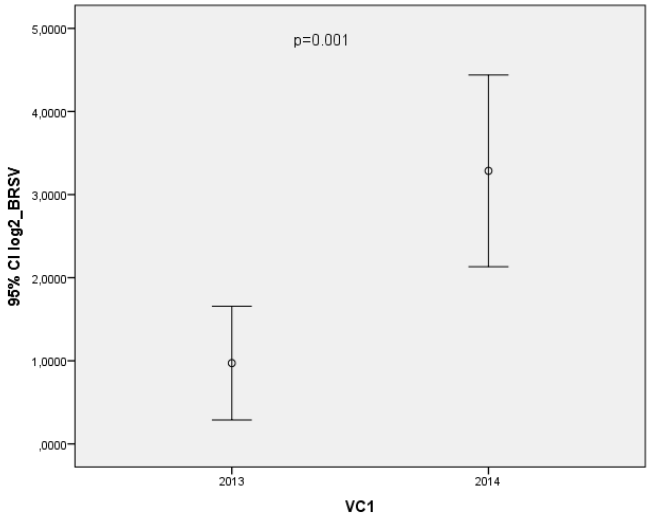
BRSV			
YEAR	N. TESTED	N. POSITIVE	% SEROPREVALENCE (C.I. 95%)
2013	36	7	19.44 (6.65-32.24)
2014	35	19	54.29 (37.95-70.62)

Table 5. Number of tested and positive chamois and % of seroprevalence for BRSV during hunting seasons in VC1.

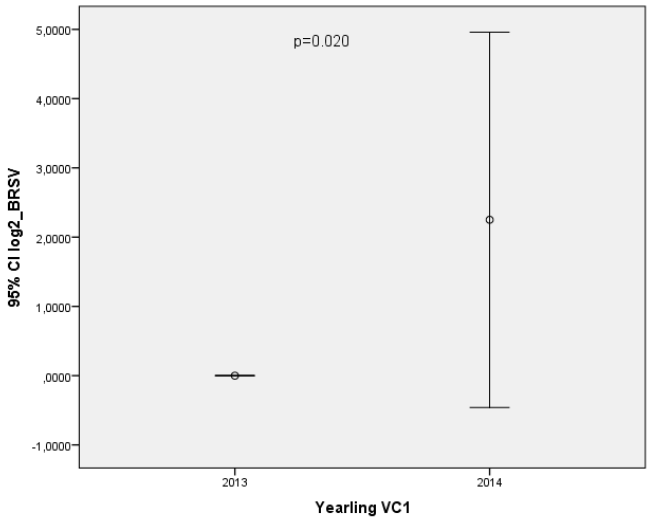
BVDV			
YEAR	N. TESTED	N. POSITIVE	% SEROPREVALENCE (C.I. 95%)
2013	40	1	2.50 (0.00-7.28)
2014	38	2	5.26 (0.00-12.28)

Table 6. Number of tested and positive chamois and % of seroprevalence for BVDV during hunting seasons in VC1.

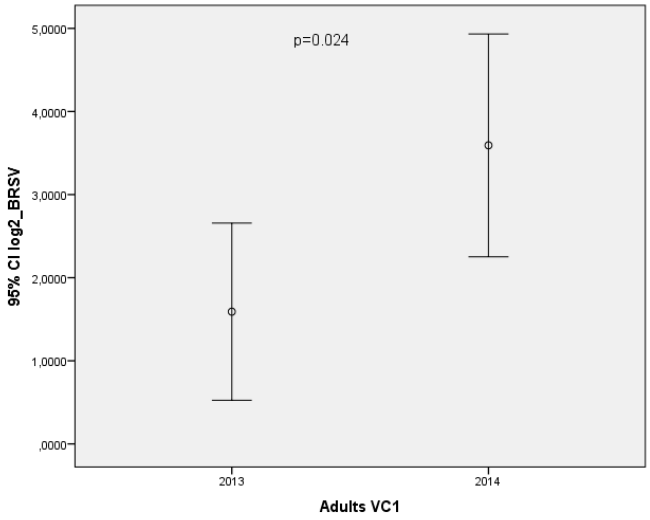
The analysis shows a significant increase of BRSV antibody titres in 2014 (p=0.001) (Graph 15), both in yearlings (p=0.020) and adults chamois (Graphs 16 and 17), also showing antibody titres higher in adults (p=0.033) than in yearlings (Graph 18).



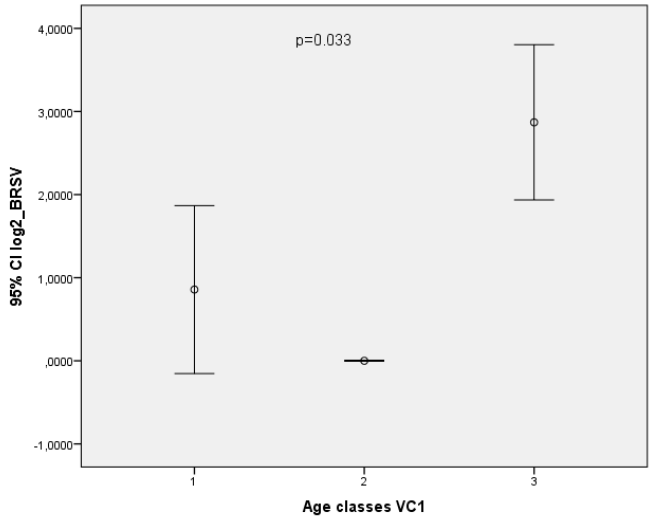
Graph 15. Error bar graph. Trend of antibody titres of BRSV in VC1.



Graph 16. Error bar graph. Trend of yearlings antibody titres of BRSV in VC1.



Graph 17. Error bar graph. Trend of adults antibody titres of BRSV in VC1.



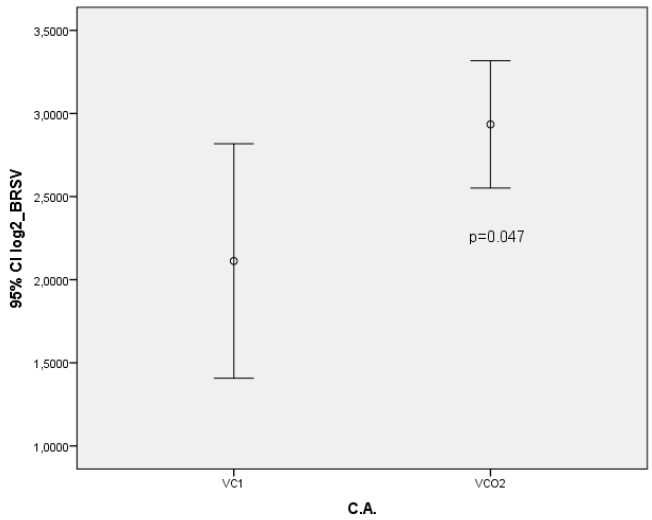
Graph 18. Error bar graph. Difference in BRSV antibody titres among different age classes in VC1.

Regarding BVDV, only three chamois result seropositive, the table below shows basic anamnestic characteristics of the subjects.

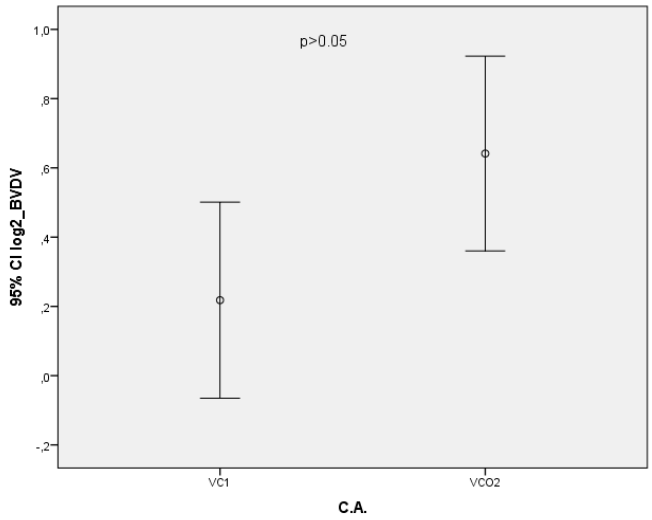
				BVDV	
YEAR	CHAMOIS	SEX	AGE	LOCATION	ANTIBOBY TITRE
2013	106596	F	1	Riva Valdobbia	11
2014	91835	M	1	Alagna	1024
2014	117200	F	1	Rimella	16

Table 7. Characteristics of BVDV seropositive chamois in VC1.

There is significant difference (p=0.047) between antibody titres resulted from the two areas only for BRSV, as showed in the graph (19).



Graph 19. Error bar graph. Difference in BRSV antibody titres between VCO2 and VC1.



While there's no statistical difference in antibody titres for BVDV, even if in VCO2 titres are higher than in VC1 (Graph 20).

Graph 20. Error bar graph. Difference in BVDV antibody titres between VCO2 and VC1.

VIRAL ISOLATION IN CELL CULTURE

During PhD study years 43 tissue lung samples were collected in VCO2, respectively 10 in 2012, 11 in 2013 and 22 in 2014. Twelve of those sampled in 2014 showed macroscopic lesions, eight of interstitial pneumonia and 4 of interstitial pneumonia and catarrhal bronchopneumonia. The sample of an adult male with both macroscopic lesions described showed ECP in cell culture characteristic of virus growth. MRV and pestiviruses can be excluded, but nowadays further investigations are in progress.

In VC1, sixteen tissue lung samples were collected during 2013 and 2014 hunting seasons. No macroscopic lesions were reported and any of the specimens showed ECP in cell culture.

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY

Histopathology and immunohistochemistry (IHC) were performed on lung tissue samples collected during 2013 and 2014 hunting seasons.

Histopathology examinations confirm the outbreak of interstitial pneumonia and catarrhal bronchopneumonia in VCO2 chamois.

IHC was performed in the laboratories of the Department of Anatomy and Comparative Pathology of Córdoba University using 15c5 monoclonal antibody against pestiviruses and results negative for all samples.

BACTERIOLOGICAL EXAMINATION

Bacteriological examination of lungs did not show any relevant pathogens for respiratory diseases. Bacteria of enteric origin have been seldom recovered, likely because of a cross-contamination caused by the shot and/or by the manipulation of carcass.

RT-PCR, SEQUENCING AND PHYLOGENETIC ANALYSYS

Virus identification was first confirmed by RT-PCR assay specific for the MRV L1 gene and all three samples resulted positive (Luzzago et al., 2011). Subsequently another RT-PCR specific for MRV S1 gene was performed to identify the type and the samples results MRV type 3.

Pair-wise nucleotide comparisons of the three chamois MRV strains showed a 100% and 99% identity of L1 and S1 segments respectively. The phylogenetic trees reported in figure 1 and 2 showed that chamois strains were classified as MRV type 3 and were closely related to Italian dog strain (Decaro et al., 2005) and Italian bats strains recently reported by (Lelli et al., 2013).

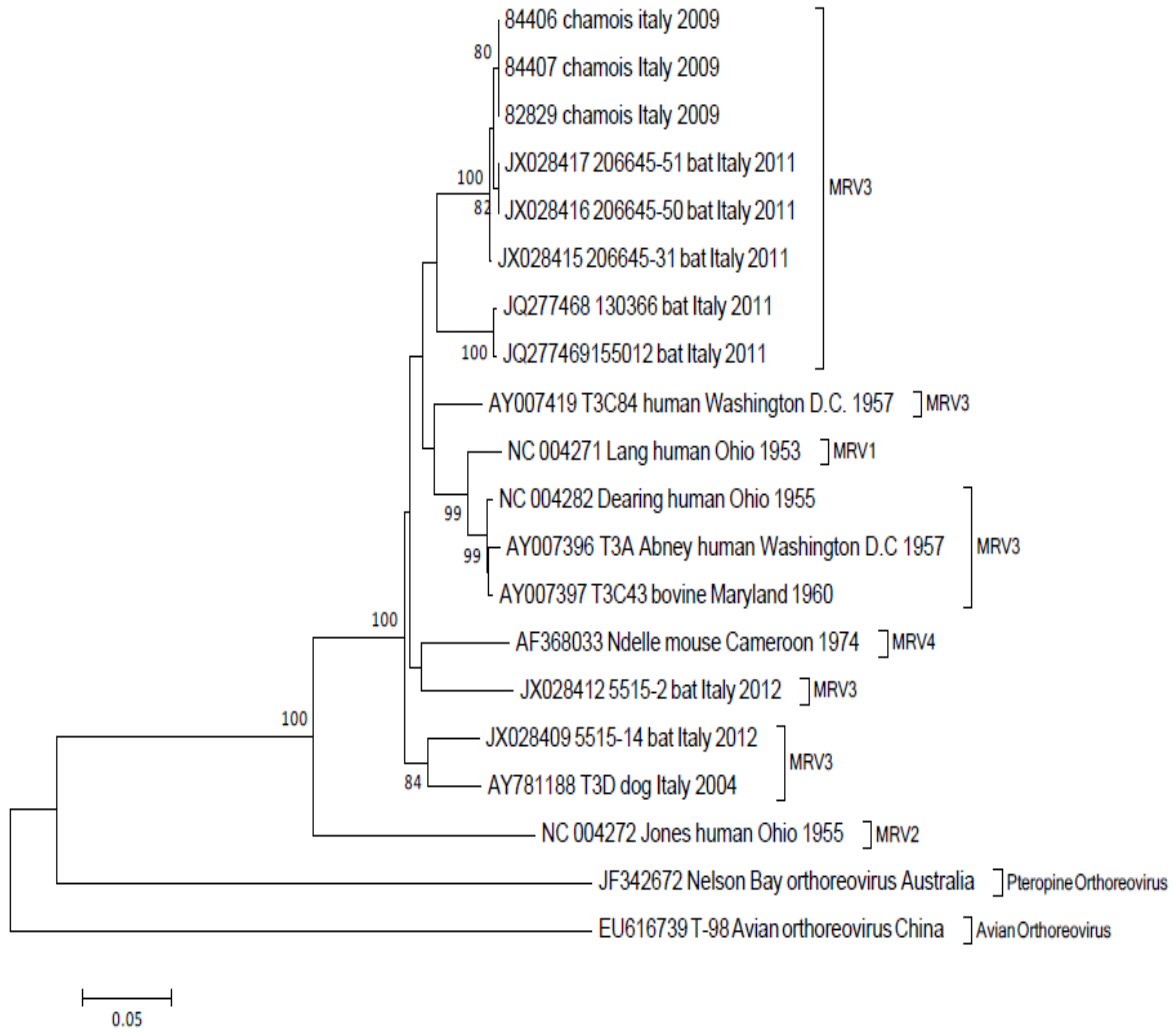


Figure 1. Phylogenetic tree of the partial L1 genome segments of MRV chamois sequences, reference strains and most related sequences from GenBank. Phylogenetic analyses were performed with MEGA5 using the NJ method. Bootstrap values > 80% are shown. Published sequences and references are identified by GenBank accession number (available at http://www.ncbi.nlm.nih.gov/pubmed/).

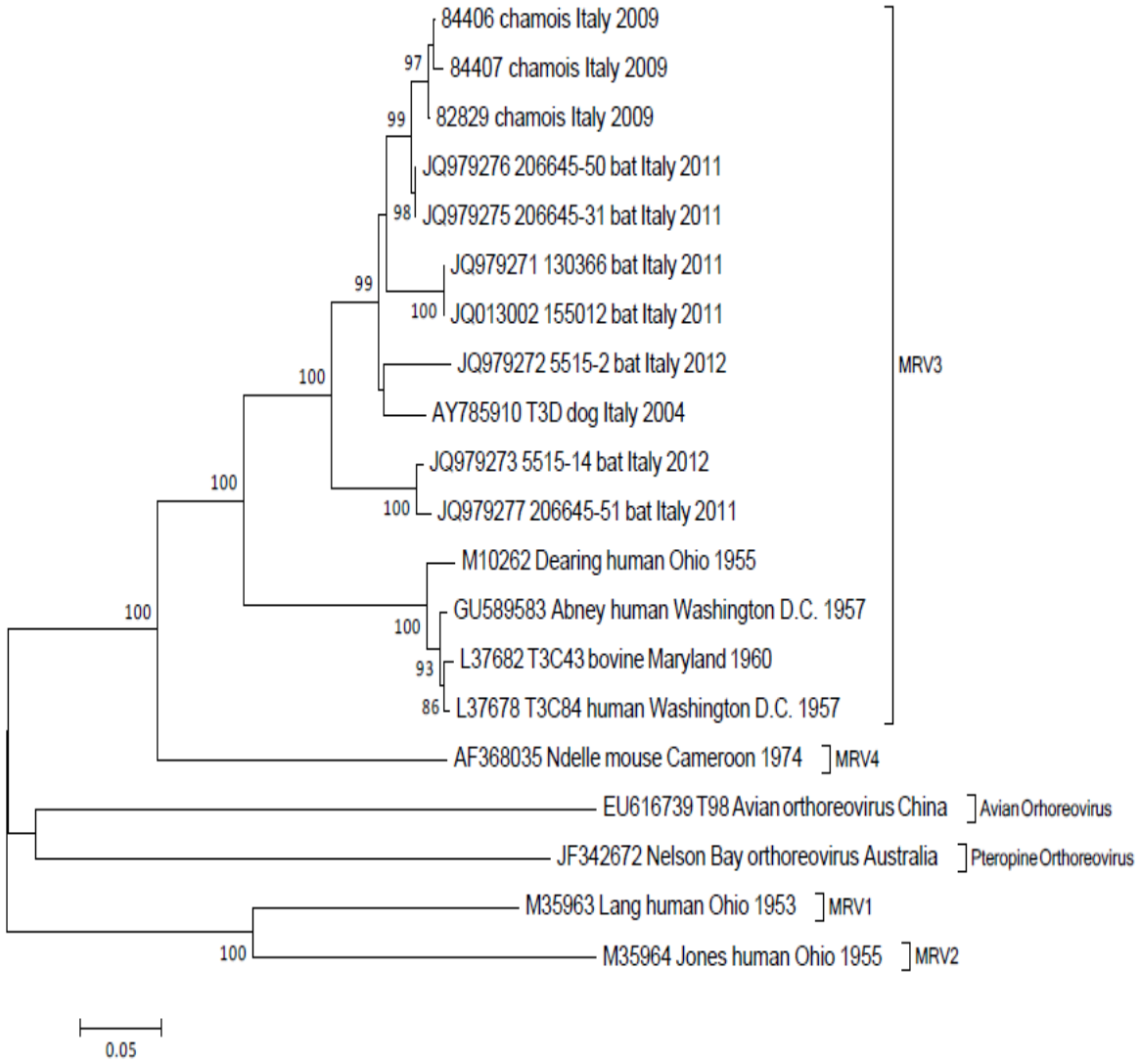


Figure 2. Phylogenetic tree of the partial S1 genome segments of MRV chamois sequences, reference strains and most related sequences from GenBank. Phylogenetic analyses were performed with MEGA5 using the NJ method. Bootstrap values > 80% are shown. Published sequences and references are identified by GenBank accession number (available at http://www.ncbi.nlm.nih.gov/pubmed/).

DISCUSSION

The blood sampling method performed by hunters in the field has been chosen in order to speed up the controls of carcasses at the Control Centres, but it is clear that the sample size is strictly related to hunters' collaboration and the serum quality not always optimal for further analysis. Despite this, we can consider that the sample size allowed the statistical analysis. Regarding lung tissue samples, the aim was screening in particular the young portion of the population, or subjects with macroscopic lesions of pneumonia (other than parasitic pneumonia), in fact, because of the ecology of chamois, yearlings are the subjects who may get in contact more easily with viruses and keep infection in the population.

In VCO2, the availability of samples collected in previous hunting seasons (2007 to 2011) allowed retrospective studies for MRV serology and a more robust statistical analysis of the results. In VC1, this was not possible because monitoring activities has been started in 2013.

In VCO2 seroprevalence of BRSV has a downtrend during years, from 76% [c.i.95% (59.37%-92.63%)] in 2007 to 29.41% [c.i.95% (7.89%-50.93%)], with a severe decrease in 2011 [21.43% c.i.95% (0.02%-42.83%)]. Antibody titres have the same trend in the whole population; at first yearlings' seem to decrease in 2011, year in which none result seropositive, but since 2012 seropositivities have been observed in adults BRSV titres decrease until 2011 too, but then remain at stable level. We can suppose that the first contact with BRSV occurred in years prior to those of the monitoring, it is reasonable that during last years the virus has stabilized at an endemic low level, as assumed for other wildlife populations also by Gaffuri et al., (2006). The continue seropositivity of

yearlings (except in 2011) suggests a continue re-infection, re-introduction of virus may derived from adult infected chamois, that moreover present antibody titres higher than yearlings, but also from domestic ruminants that share summer pastures with wildlife.

Regarding BVDV, both in VCO2 and VC1, the infection can be considered sporadic because of the low seroprevalence during years. The hypothesis is that these sporadic infections derived from domestic ruminants as a "spill-over" because of the interaction the two species have during summer grazing. In the current condition the maintenance of the circulation of pestivirus among wild populations is not possible probably because of the period in which wildlife and domestic ruminants have spatial and food interaction. In fact, for the maintenance of the infection in a population is fundamental the vertical transmission of the virus from a pregnant infected female to her foetus to give birth to a persistently infected (PI) subject spreading the virus. During interaction season, late spring/summer, female chamois are not pregnant because the mating season is in November and birth season is in early spring before the arrival of domestic ruminants in alpine pastures.

The assume of the spill-over from livestock is supported by the statistical difference between geographic district 1 and others, in fact in district 1 there is the mayor density of domestic ruminants during summer grazing.

In VC1, BRSV seroprevalence has a significant increase in 2014 [54.29% c.i.95% (37.95%-70.62%)]. In 2013 none yearling resulted seropositive, while in 2014 three yearlings were positive with high titres and in adults antibody titres increase significantly (p value=0.024). Further investigations during next years are necessary to verify the trend of the seroprevalence, because only two years

are not enough to understand the real trend of viruses, also considering the absence of clinical respiratory symptoms.

BVDV seropositive chamois in this area are three yearlings, one in 2013 and two in 2014, this means that the contact with domestic infected ruminants probably occur each year.

If BVDV infection can be considered sporadic in both study areas without difference, as regards BRSV the seroprevalence is overall significantly higher in VCO2 than in VC1, even if the trends seem to be opposites, in VCO2 the seroprevalence decreases while in VC1 increases, and in 2014 the seroprevalence is higher in VC1 than VCO2.

Regarding MRV, a retrospective serological screening has been done only in VCO2 since 2008 to 2012, because of the isolation of three MRV3 strains in three yearling lungs without any respiratory symptoms in 2009. The seroprevalence ranges from a minimum of 42.11% [c.i.95% (20.04%-64.17%)] in 2010 to a maximum of 75% [c.i.95% (50.59%-99.41%)], however remaining above 50% during other years. The stability of the seroprevalence on quite high values suggests an important circulation of the virus among chamois population during years. The isolation in cell culture of MRV3 from chamois lungs without lesions confirms the infection (Luzzago et al., 2011). Phylogenetic trees show the closely correlation of chamois MRV to strains isolated in Italian dog (Decaro et al., 2005) and Italian bats (Lelli et al., 2011). Considering the possible zoonotic infection and the isolation in human of a strain closely related to that of bat (Chua et al., 2007), we have to consider the possibility of an impact on Public Health. The sharing of Alpine environment by wildlife (for example chamois and bats) and humans, especially for some categories, such as hunters,

veterinary, gamekeepers, that interact directly with wildlife, expose them to a higher risk. In this contest, further investigations are needed also because this is the first isolation of MRV in a wild ruminant as chamois.

CONCLUSIONS

Analysing the actual condition of the two chamois populations studied, BRSV and BVDV seem to have no impact on demography, but their adaptation levels are different. In fact, BRSV seems to have a good adaptation level in wild ruminants convenient to maintain a low endemic circulation. Conversely, pestivirus infection can be considered sporadic as a spill-over from domestic ruminants with a low adaptation level of the virus in wildlife, so nowadays in our study areas the circulation of virus seems to be very different from that in Spain that caused a severe impact on demography (Marco et al., 2009).

The first isolation of MRV in chamois underlines the need for a continuous monitoring of wildlife with a “One World, One Health” view. Moreover, further investigations are necessary to understand the possible zoonotic implication in a heterogeneous territory of Alps and the necessity to understand the pathogenic potential of the virus, knowing that in domestic ruminants does not cause disease in absence of co-infection. In addition, the phylogenetic analysis extends the knowledge of MRV epidemiology, considering the limited amount of available sequences.

In general, the results need to integrate information on population dynamics (fitness, survival rate, reproductive success), carrying capacity (assessed on the total biomass), as well as ecological aspects (spatial interactions and competitions), which also are assessments that still remain descriptive level. In this framework the role of the Control Centre, which is often limited to report biometric measurement, should assume the function of "epidemiological observatory" comparing the health status of wild populations as a whole. Whereas, for obvious logistical reasons, it is not possible to be constantly present

at the sampling operations in field, the effective collaboration with the hunting world remained of crucial importance.

REFERENCES

- Alansari H, Potgieter LN. 1994. Molecular cloning and sequence analysis of the phosphoprotein, nucleocapsid protein, matrix protein and 22K (M2) protein of the ovine respiratory syncytial virus. *J. Gen. Virol.* 75:3597–3601.
- Alansari H, Duncan RB, Baker JC, Potgieter LN. 1999. Analysis of ruminant respiratory syncytial virus isolates by RNAse protection of the G glycoprotein transcripts. *Journal of Veterinary Diagnostic Investigation* 11: 215-20.
- Armaroli E, Carrain R, Guberti V. 2006. Sero-epidemiological survey on bovine respiratory syncytial virus in a wild roe deer (*Capreolus capreolus*) population in demographic decrease in the Northern Appennines, Italy (1995-2004). *VII Conference of the European Wildlife Diseases Association*. 27-30 Sept 2006, Aosta Valley, Italy.
- Attoui H, Biagini P, Stirling J, Martens PPC, Cantaloube J, Meyer A, de Micco P, de Lamballerie X. 2001. Sequence characterization of Ndelle virus genome segments 1, 5, 7, 8 and 10: evidence for reassignment to the genus *Orthoreovirus*, family *Reoviridae*. *Biochem. Biophys. Res. Commun*. 287:583-588.
- Aune KA, Anderson N, Worley D, Stackhouse L, Henderson J, Daniel J. 1998. A comparison of population and health histories among seven bighorn sheep populations. *Proc. Bien. Symp. North. Wild Sheep Goat Coun.*, vol. 11 (1998), pp. 46–69.
- Baker JC, Ames TR, Markham RJ. 1986. Seroepizootiologic study of bovine respiratory syncytial virus in a dairy herd. *Am. J. Vet. Res.* 47:240–245.
- Baker JC. 1987. Bovine viral diarrhea virus: a review. *J. Am. Med. Assoc.*, 190, pp. 1449-1458.
- Bassel-Duby R, Spriggs DR, Tyler KL, Fields BN. 1986. Identification of attenuating mutations on the reovirus type 3 S1 double-stranded RNA segment with a rapid sequencing technique. *J. Virol.* 60, 64-67.
- Becher P, Orlich M, Shannon AD, Homer G, Konig M, Thiel HJ. 1997. Phylogenetic analysis of pestiviruses from domestic and wild ruminants. *J Gen Virol.* 78, pp. 1357-1366.

- Becher P, Orlich M, Kosmidou A, Konig M, Baroth M & Thiel HJ. 1999. Genetic diversity of pestiviruses: identification of novel groups and implication for classification. *Virology* 262, 64–71.
- Becher P, Avalos Ramirez R, Orlich M, Cedillo Rosales S, Konig M, Schweizer M, Stalder H, Schirrmeier H, Thiel HJ. 2003. Genetic and antigenic characterization of novel pestivirus genotypes: Implications for classification. *Virology*. 2003;311:96–104.
- Belknap EB. 1993. Recognizing the clinical signs of BRSV infection. *Vet. Med.* 88:883–887.
- Bengins RG, Kock RA, Fisher J. 2002. Infectious animal diseases: The wildlife/livestock interface. *Rev Sci Tech* 21(1): 53-65.
- Besozzi M. 2012. Infezioni respiratorie virali nei camosci del Verbano Cusio Ossola. Tesi di Laurea a.a. 2011-2012.
- Besser TE, Cassirer EF, Potter KA, Vanderschalie J, Fischer A, Knowles DP, Herndon DR, Rurangirwa FR, Weiser GC, Srikumaran S. 2008. Association of Mycoplasma ovipneumoniae infection with population limiting respiratory disease in free-ranging Rocky Mountain bighorn sheep (Ovis canadensis canadensis). *J Clin Microbiol,* 46 (2008), pp. 423–430.
- Besser TE, A, Baker K, Cassirer EF, Anderson NJ, Ramsey JM, Mansfield K, Bruning DL, Wolff P, Smith JB, Jenks JA. 2012. Causes of pneumonia epizootics among bighorn sheep, Western United States, 2008-2010. *Emerg Infect Dis*; 18(3): 406-14.
- Boadella M, Carta T, Oleaga A, Pajares G, Muñoz M, Gortázar C. 2010. Serosurvey for selected pathogens in Iberian roe deer. *BMC Vet Res*. 16; 6:51.
- Boyce WM, Weisenberger ME, Penedo MCT, Johnson CK. 2011. Wildlife translocation: the conservation implications of pathogen exposure and genetic heterozygosity. *BMC Ecology* 2011, 11:5
- Bolin SR. 1995. The pathogenesis of mucosal disease. *Vet Clin North Am Food Anim Pract.* 11:489–500.
- Bregoli M, Constanzi C, Farina G, Guberti V, Pasolli C. 2006. Pestiviruses serological survey in wild ruminants in north-eastern Italy.

In: Proceedings of the 7th *Conference of the European Wildlife Disease Association*, Aosta valley, Italy, p. 41.

- Briand F, Dubost M, Pitt D, Ramband D. 1989. Le Alpi. Un sistema sotto pressione. Ed. Unione Internazionale per la Conservazione della Natura e delle Risorse Naturali (UICN) e Centro Internazionale per l'Ambiente Alpino (ICALPE), Le Bourget-du-Lac, Francia.
- Brownlie J, Clarke MC, Howard CJ. 1984. Experimental production of fatal mucosal disease in cattle. *Vet Rec*. 114:535–536.
- Brownlie J, Clarke MC, Howard CJ, Pocock DH. 1987. Pathogenesis and epidemiology of bovine virus diarrhoea infection of cattle. *Annales De Recherches Veterinaires* 18, 157–166.
- Brownlie J. 1990. Pathogenesis of mucosal disease and molecular aspects of bovine virus diarrhoea virus. *Vet Microbiol*. 23:371–382.
- Bryson DE. 1993. Necroscopy findings associated with BRSV pneumonia. *Vet. Med.* 88:894–899.
- Bunt AA, Milne RG, Sayaya T, Verbeek M, Vetten HJ, Walsh JA. 2005. Paramyxoviridae, in: Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (Eds.), *Virus taxonomy*, Eigth report of the International Committee on Taxonomy of Viruses, Elsevier, Academic Press, London, 2005, pp. 655–671.
- Burthe S, Telfer S, Begon M, Bennett M, Smith A, Lambin X. 2008. Cowpox virus infection in natural field vole Microtus agrestis populations: significant negative impacts on survival. *Journal of Animal Ecology*, 77, 110-119.
- Cabezón O, Rosell R, Sibila M, Lavín S, Marco I, Segalés J. 2010. Experimental infection of pigs with Border disease virus isolated from Pyrenean chamois (*Rupicapra pyrenaica*). *J Vet Diagn Investig* 2010, 22:360–365.
- Caldow GL, Edwards S, Nixon P, Peters AR. 1988. Associations between viral infection and respiratory disease in young beef bulls. *Vet. Rec.* 122:529–531.
- Callan RJ, Bunch TD, Workman GW, Mock RE. 1991. Development of pneumonia in desert bighorn sheep after exposure to a flock of exotic wild and domestic sheep. *J Am Vet Med Assoc*; 198: 1052-1056.

- Capurro AF, Gatto M, Tosi G. 1993. The demography and management of a chamois population in the Italian Alps. *6th Intern. Conf. on Nat. Resource, Modeling and Analysis:* 2-5.
- Carnevali L, Pedrotti L, Riga F, Toso S. 2009. Ungulates in Italy: status, distribution, abundance, management and hunting of Ungulates population in Italy. *Report ISPRA 2001-2005*, Vol. 117, pp. 115-129.
- Chapell J, Duncan R, Mertens P, Dermody T. 2005. Genus Orthoreovirus. In: Faquet CM, Mayo MA, Maniloff J, Desselberger U, Ball L (eds), Virus Taxonomy, Eight report of the Internatioal Committee on Taxonomy of Virus, pp. 455-465. *Elsevier Academic Press*, London.
- Chappuis G, Brun A, Kato F, Dauvergne M, Reynaud G, Duret C. 1986. Études Sérologiques et Immunologiques Réalisées à la Suite de l'isolement d'un Pestivirus dans un Foyer Ovin chez des Moutons de l'Aveyron. In: *Pestivirose des Ovinset des Bovins: Nouvelles Connaissances, Utilisation pour une Stratégie de Contrôle, Journées Nationales de la Société Française de Buiatrie et de son Groupe d'Étudesur la Pathologie des Ovins et des Caprins* (GEPOC). Eds J. Espinasse, M. Savey. Paris. pp 55–65.
- Chemini C, Gianelle D. 1999. Pascolo e conservazione della biodiversità. In "Presente e futuro dei pascoli alpini in Europa". Ed. Franco Angeli: 36-48.
- Chua KB, Crameri G, Hyatt A, Yu M, Tompang MR, Rosli J, McEachern J, Crameri S, Kumarasamy V, Eaton BT, Wang LF. 2007. A previously unknown reovirus of bat origin is associated with an acute respiratory disease in humans. *Proc. Natl Acad. Sci*. USA 104, 11424–11429.
- Chua KB, Voon K, Yu M, Keniscope C, Abdul Rasid K, Wang LF. 2011. Investigation of a Potential Zoonotic Transmission of Orthoreovirus Associated with Acute Influenza-Like Illness in an Adult Patient. *PLoS ONE* 6(10): e25434.
- Citterio CV, Luzzago C, Sala M, Sironi G, Gatti P, Gaffuri A, Lanfranchi P. 2003. Serological study of a population of alpine chamois (*Rupicapra r rupicapra*) affected by an outbreak of respiratory disease. *Vet. Rec*. 153(19):592-6.

- Collins PL, Chanock RM, Murphy BR. 2001. Respiratory syncytial virus. In: Kripe D.M., Howley P.M. (Eds.), Fields virology, 4th ed., Lippincott Williams and Wilkins, Philadelphia, pp. 1443–1485.
- Corlatti L, Lorenzini R, Lovari S. 2011. The conservation of the chamois *Rupicapra* spp. *Mammal Rev.*, vol. 41, n.2, 163-174.
- Cutler SJ, Fooks AR, Van der Poel WHM. 2010. Public health threat of new, reemerging, and neglected zoonoses in the industrialised world. *Emerging Infectious Diseases* Vol. 16, No. 1
- Day JM. 2009. The diversity of orthoreoviruses: molecular taxonomy and phylogenetic divides. *Infect. Genet. Evol.* 9:390-400.
- Daszak P, Cunningham AA, Hyatt AD. 2000. Emerging infectious diseases of wildlife—threats to biodiversity and human health. *Science*. 287(5452: 443-9); erratum in 287(5459): 1756.
- Decaro N, Campolo M, Desario C, Ricci D, Camero M, Loirusso E, Elia G, Lavazza A, Martella V, Buonavoglia C. 2005. Virological and molecular characterization of a mammalian orthoreovirus type 3 strain isolated from a dog in Italy. *Vet. Microbiol.* 109, 19-27.
- De Jong MCM, Van der Poel WHM, Kramps JA, Brand A, Van Oirschot JT. 1996. Quantitative investigation of population persistence and recurrent outbreaks of bovine respiratory syncytial virus on dairy farms. *Am. J. Vet. Res.* 57:628–633.
- Dermody TS, Nibert ML, Bassel-Duby R, Fields BN. 1990. Sequence diversity in S1 genes and S1 translation products of 11 serotype 3 reovirus strains. *J. Virol.* 64:4842-4850.
- Dobson A and Meagher M. 1996. The population dynamics of Brucellosis in Yellowstone National Park. *Ecology*, 77: 1026-1036.
- Doyle LG, Heuschele WP. 1983. Bovine viral diarrhea virus infection in captive exotic ruminants. *J Am Vet Med Assoc*, 183, pp. 1257-1259.
- Duffell SJ, Harkness JW. 1985. Bovine virus diarrhoea-mucosal disease infection in cattle. *Vet. Rec*. 117:240–245.
- Dunbar MR, Jessup DA, Evermann JF, Foreyt WJ. 1985. Seroprevalence of respiratory syncytial virus in free-ranging bighorn sheep. J *Am Vet Med Assoc*. 187(11):1173-4.

- Duncan R, Horne D, Cashdollar LW, Joklik, Lee PWK. 1990. Identification of conserved domains in the cell attachment proteins of the three serotypes of reovirus. *Virology* 174, 339-409.
- Duncan R. 1999. Extensive sequence divergence and phylogenetic relationships between the fusogenic and nonfusogenic orthoreovirus: a species proposal. *Virology* 260, 316-328.
- Elvander M. 1996. Severe respiratory disease in dairy cows caused by infection with bovine respiratory syncytial virus. *Vet. Rec.* 38:101–105.
- Evermann JF. 2006. Pestiviral infection of llamas and alpacas. *Small Ruminant Res*. 61, 201–206.
- Fernández-Sirera L, Cabezón O, Allepuz A, Rosell R, Riquelme C, Serrano E, Lavín S, Marco I. 2012. Two different epidemiological scenarios of border disease in the populations of Pyrenean chamois (Rupicapra p. pyrenaica) after the first disease outbreaks. PLoS One. 2012;7(12):e51031.
- Foreyt WJ, Jessup DA. 1982. Fatal pneumonia of bighorn sheep following association with domestic sheep. *J Wildl Dis*; 18: 163-168.
- Frolich K, Hofmann M. 1995. Isolation of bovine viral diarrhea virus-like pestiviruses from roe deer (Capreolus capreolus). *J. Wildl. Dis*. 31, 243–246.
- Frolich K, Thiede S, Kozikowski T, Jakob W. 2002. A review of mutual transmission of important infectious diseases between livestock and wildlife in Europe. *Ann NY Acas Sci*. 969: 4-13.
- Gaffuri A, Giacometti M, Tranquillo VM, Magnino S, Cordioli P, Lanfranchi P. 2006. Serosurvey of roe deer, chamois and domestic sheep in the central Italian Alps. Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna Bruno Ubertini, Via Rovelli 53, I-24100 Bergamo, Italy. *J Wildl Dis.* 42(3):685-90.
- Gamlen T, Richards KH, Mankouri J, Hudson L, McCauley J, Harris M, Macdonald A. 2010. Expression of the NS3 protease of cytopathogenic bovine viral diarrhea virus results in the induction of apoptosis but does not block activation of the beta interferon promoter. *Journal of General Virology* 91, 133–144.

- Giacometti M, Hartl GB, Volk F. 1997. Europe – regional summary for Caprinae. In: Shackleton DM (ed.) *Wild Sheep and Goats and their Relatives*, 143-147. International Union for Conservation of Nature, Gland, Switzerland.
- Giammaroli M, Saverina ALR, Steinbach F, Casciari C, De Mia GM. 2011. Genetic and antigenic typing of Border Disease Virus (BDV) isolates from Italy reveal the existence of a novel BDV group. *Vet mic*, 147: 231-236.
- Giovannini A, Cancellotti FM, Turilli C, Randi E. 1988. Serological investigations for some bacterial and viral pathogens in fallow deer (Dama dama) and wild boar (Sus scrofa). *Journal of Wildlife Diseases* 24, 127–132.
- Grooms DL. 2004. Reproductive consequences of infection with bovine viral diarrhea virus. *Vet. Clin. North Am. Food Anim. Pract*. 20:5–19.
- Guberti V, Stancampiano L, Ferrari N. 2014. Surveillance, monitoring and surveys of wildlife diseases: a public health and conservation approach. *Hystrix, the Italian journal of Mammalogy*. Vol. 25 (1): 3-8.
- Hall CB, Douglas RG Jr, Geiman JM. 1980. Possible transmission by fomites of respiratory syncytial virus. *J. Infect. Dis.* 141:98–102.
- Hamblin C, Hedger RS. 1979. The prevalence of antibodies to bovine viral diarrhea/mucosal disease virus in African wildlife. *Comp. Immunol. Microbiol. Infect. Dis.*, 2, pp. 295-303.
- Hamers C, Dehan P, Couvreur B, Letellier C, Kerkhofs P, Pastoret PP. 2001. Diversity among bovine pestiviruses. *Vet. J.* 161:112–122.
- Hatcher MJ, Dick JTA, Dunn MA. 2006. How parasites affect interactions between competitors and predators. *Ecology Letters*, 9, 1253-1271.
- Hodo C. 2010. Pnuemonia kills bighorn sheep. *SCWDS Briefs*, vol. 26, no. 1, pp. 5–6.
- Holt RD, Dobson AP. 2006. Extending the principles of community ecology to address the epidemiology of host-pathogen systems. *Disease Ecology Community Structure and Pathogen Dynamics.*(eds S.K. Collinge & C. Ray), pp 6-27, Oxford University Press, Oxford.

- Houe H. 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Vet. Microbiol*. 64, 89–107.
- Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeek H, Dobson AP. 2002. The ecology of wildlife diseases. Pp 187, *Oxford University Press.*
- Hurtado A, García-Pérez AL, Aduriz G, Juste RA. 2003. Genetic diversity of ruminant pestiviruses from Spain. *Virus Res.* 92(1): 67-73.
- Jackson GG, Muldoon RL. 1973. Viruses causing common respiratory infection in man. IV. Reoviruses and adenoviruses. *J. Infect. Dis*. 128: 811–833.
- Jiang J, Hermann L, Coombs KM. 2006. Genetic characterization of a new mammalian reovirus, type 2 Winnipeg (T2W). *Virus Genes* 33, 193–204.
- Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P. 2008. Global trends in emerging infectious diseases. *Nature* 451 (7181): 990:993.
- Kimman TG, Zimmer GM, Westenbrink F, Mars J, van Leeuwen E. 1988. Epidemiological study of bovine respiratory syncytial virus infections in calves: influence of maternal antibodies on the outcome of disease. *Vet. Rec.* 123:104–109.
- Krametter R, S, Loitsch A, Froetscher W, Benetka V, Moestl K, Baumgartner W. 2004. Pestivirus exposure in free-living and captive deer in Austria. *J Wildl Dis*. 2004 Oct;40(4):791-5.
- Leary PL, Erker JC, Chalmers ML, Cruz AT, Wetzel JD, Desai SM, Mushahwar IK, Dermody TS. 2002. Detection of mammalian reovirus RNA by using reverse transcription-PCR: sequence diversity within k3-encoding L1 gene. *Clin. Microbiol*, 40, 1368–1375.
- Lehmkuhl HD, Smith MH, Cutlip RC. 1980. Morphogenesis and ultrastructure of caprine respiratory syncytial virus. *Arch. Virol.* 65:269–276.
- Lent PC. 1974. Mother-infant relationships in ungulates. In: Geist V, Walther F (eds) The behaviour of ungulates and its relation to management. IUCN, Morges, pp 14–55.

- Letellier C, Kerkhofs P. 2003. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. J. *Virol. Methods* 114, 21–27.
- Lillehaug A, Vikøren T, Larsen IL, Akerstedt J, Tharaldsen J, Handeland K. 2003. Antibodies to ruminant alpha-herpesviruses and pestiviruses in Norwegian cervids. *J Wildl Dis*. 39(4): 779-86.
- Loison A, Jullien J-M, Menaut P. 1999b. Subpopulation structure and dispersal in two populations of chamois. *J Mammal* 80:620–632.
- Liu L, Xia H, Wahlberg N, Belàk S, Baule C. 2009. Phylogeny, classification and evolutionary insights into pestiviruses. *Virology*. 385(2):351-7.
- Luzzago C, Bronzo V, Salvetti S, Frigerio M, Ferrari N. 2010. Bovine respiratory syncytial virus seroprevalence and risk factors in endemic dairy cattle herds.*Vet Res Commun*. 34(1):19-24.
- Luzzago C, Lavazza A, Pisoni G, Viganò R, Besozzi M, Cordioli P, Sironi G, Lanfranchi P. 2011. Identificazione di Orthoreovirus in polmoni di camosci. *IV Workshop Nazionale di Virologia Veterinaria*, Brescia, 9-10 Giugno 2011, p.56.
- Mao L, Li W, Zhang W, Yang L, Jiang J. 2012. Genome sequence of a novel Hobi-like pestivirus in China. *J Virol.* 86(22):12444.
- Marco I, López-Olvera JR, Rosell R, Vidal E, Hurtado A, Juste R, Pumarola M, Lavín S. 2007. Severe outbreak of disease in the Southern chamois (*Rupicapra pyrenaica*) associated with border disease virus infection. *Veterinary Microbiology* 120, 33–41.
- Marco I, Rosell R, Cabezon O, Mentaberre G, Casas E, Velarde R, Lopez-Olvera JR, Hurtado A, Lavin S. 2008. Epidemiological study of border disease virus infection in Southern chamois (*Rupicapra pyrenaica*) after an outbreak of disease in the Pyrenees (NE Spain). *Vet. Microbiol*. 127, 29–38.
- Marco I, Rosell R, Cabezón O, Beneria M, Mentaberre G, Casas E, Hurtado A, López-Olvera JR, Lavín S. 2009. Serologic and virologic investigations into pestivirus infection in wild and domestic ruminants in the Pyrenees (NE Spain). *Research in Veterinary Science*, 87, 149–153.

- Marco I, Cabezón O, Rosell R, Fernández-Sirera L, Allepuz A, Lavín S. 2011. Retrospective study of pestivirus infection in Pyrenean chamois (*Rupicapra pyrenaica*) and other ungulates in the Pyrenees (NE Spain). *Vet Microbiol.* 2011 Apr 21;149(1-2):17-22.
- Marley MSD, Tabor JM, Givens MD, Kaproth M, Riddell KP, Galik PK, Zhang Y, Eason AB. 2009. Bovine viral diarrhea virus is inactivated when whole milk from persistently infected cows is heated to prepare semen extender. *Vet. Microbiol*.134:249–253.
- Mars MH, Bruschke CJ, van Oirschot JT. 1999. Airborne transmission of BHV1, BRSV, and BVDV among cattle is possible under experimental conditions. *Vet. Microbiol*. 66:197–207.
- Masini F, Lovari S. 1988. Systematics, phylogenetic relationships and dispersal of the chamois *Rupicapra* spp. *Quaternary Research* 30:339-349.
- Meyer G, Deplanche M, Schelcher F. 2008. Human and bovine respiratory syncytial virus vaccine research and development. *Comp Immunol Microbiol Infect Dis*.;31:191–225.
- Meyers G, Thiel HJ. 1996. Molecular characterization of pestiviruses. *Adv. Virus Res*. 47, 53–118.
- Meyling A, Houe H, Jensen AM. 1990. Epidemiology of bovine virus diarrhoea virus. *Revue Scientifique et Technique* (International Office of Epizootics) 9, 75–93.
- Miller MW. 2001. Pasteurellosis. In: Infectious diseases of wild mammals, Williams ES and Barker IK, editors. Iowa State University Press, Ames, Iowa, pp. 330–339.
- Miller DS, Weiser GC, Aune K, Roeder B, Atkinson M, Anderson NJ, Keating KA, Chapman PL, Kimberling C, Rhyan J, Clarke PR. 2011. Shared bacteria and viral respiratory agents in bighorn sheep (Ovis canadensis), domestic sheep (Ovis aries) and goats (Capra hircus) in Montana. *Vet Med Int.* 2011; 2011: 162520.
- Miller DS, Hoberg E, Weiser G, Aune K, Atkinson M, Kimberling C. 2012. A review of hypothesized determinants associated with bighorn sheep (Ovis canadensis) die-offs. *Vet Med Int.* 2012; 2012: 796527.

- Morin MJ, Warner A, Fields BN. 1994. A pathway for entry of reoviruses into the host through M cells of the respiratory tract. *J. Exp. Med*. 180:1523–1527.
- Morse SS, Mazet JA, Woolhouse M, Parrish CR, Carroll D, Karesh WB, Zambrana-Torrelio C, Lipkin WI, Daszak P. 2012. Prediction and prevention of the next pandemic zoonosis. *Lancet* 380(9857):1956-1965.
- Mustoni A, Pedrotti L, Zanon E, Tosi G. 2002. Ungulati delle Alpi. 1° ed. 265-309 pp.
- Nettleton PF, Entrican G. 1995. Ruminant pestiviruses. *British Veterinary Journal*. 151(6):615–642.
- Nettleton PF, Gilray JA, Russo P, Dlissi E. 1998 Border disease of sheep and goats. *Veterinary Research* 29, 327–340.
- Nibert ML, Dermody TS, Fields BN. 1990. Structure of the reovirus cell-attachment protein; a model for the domain organization of sigma 1. *J. Virol*. 64, 2976–2989.
- Nielsen SS, Roensholt L, Bitsch V. 2000. Bovine virus diarrhea virus in free-living deer from Denmark. *Journal of Wildlife Diseases* 36, 584–587.
- Nettleton PF. 1990. Pestivirus infections in ruminants other than cattle. *Rev. Sci. Tech. Off. Int. Epizoot.*, 9, pp. 131-150.
- Nettleton, P.F., 2000. Border disease. In: Diseases of Sheep, Blackwell Science, Oxford, UK, pp. 95–102.
- Nibert, M. L., and L. A. Schiff, 2001. Reoviruses and their replication. In: Knipe, D. M., and P. M. Howley (eds), Fields virology 4th edn, pp. 1679–1728. Lippincott-Raven, Philadelphia, PA.
- Nocture M, Cabaret J, Hugonnet-Chapelle L. 1998. Protostrongylid nematode infection of chamois (Rupicapra rupicapra) at the Bauges massif (French Alpes). *Vet Parasitol*; 77(2-3): 153-61.
- Oguzoglu TC, Tan MT, Toplu N, Demir AB, Bilge-Dagalp S, Karaoglu T, Ozkul A, Alkan F, Burgu I, Haas L, Greiser-Wilke I. 2009. Border disease virus (BDV) infections of small ruminants in Turkey: A new BDV subgroup? *Vet Microbiol*, 135:374–379.

- Olde Riekerink RG, Dominici A, Barkema HW, De Smit AJ. 2005. Seroprevalence of pestivirus in four species of alpine wild ungulates in the High Valley of Susa, Italy. *Vet Microbiol.* 108(3-4):297-303.
- Panayotova-Pencheva MS, Alexandrov MT. 2010. Some pathological features of lungs from domestic and wild ruminants with single and mixed protostrongylid infections. *Vet Med Int*; 2010: 741062.
- Passler T, Walz PH, Ditchkoff SS, Brock KV, DeYoung RW, Foley AM, Givens MD. 2009. Cohabitation of pregnant white-tailed deer and cattle persistently infected with Bovine viral diarrhea virus results in persistently infected fawns. *Vet Miocrobiol.* 134:362-367.
- Pedrotti L, Dupré E, Preatoni D, Toso S. 2001. Banca dati ungulati. Status, distribuzione, consistenza e gestione delle popolazioni di Ungulati in Italia. *Biologia e Conservazione della Fauna*, 109:110 pp.
- Pellerin C, Van Den Hurk J, Lecomte J, Tijssen P. 1994. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalieties. *Virology*, 203, pp. 260-268.
- Petney TN, Andrews RH. 1998. Multiparasite communities in animals and humans: frequency, structure and pathogenic significance. *International Journal of Parasitology.* 28, 377-393.
- Pioz M, Loison A, Gibert P, Dubray D, Menaut P, Le Tallec B, Artois M, Gilot-Fromont E. 2007. Transmission of a pestivirus infection in a population of Pyrenean chamois. *Vet. Microbiol*. 119, 19–30.
- Posautz A, Loncaric I, Kübber-Heiss A, Knoll A, Walzer C. 2014. Acute die-off of chamois (Rupicapra rupicapra) in the Eastern Austrian Alps due to bacterial bronchopneumonia with Pasteurellaceae. *J Wildl Dis*. 50(3):616-20.
- Rehbinder C, Belák S, Nordkvist M. 1992. A serological, retrospective study in reindeer on five different viruses. *Rangifer* 12, 191–195.
- Richomme C, Gauthier D, Fromont E. 2006. Contact rates and exposure to inter-species disease transmission in mountain ungulates. *Epidemiol. Infect*. 134, 21–30.
- Rivera H, Madewell BR, Ameghino E. 1987. Serologic survey of viral antibodies in the Peruvian alpaca (*Lama pacos*). *Am. J. Vet. Res.* 48:189–191.

- Rossi S, Artois M, Pontier D, Crucière C, Hars J, Barrat J, Pacholet X, Fromont E. 2005. Long-term monitoring of classical swine fever in wild boar (*Sus Scrofa* sp.) using serological data. *Vet Res* 36:27-42.
- Ruckstuhl K, Ingold P. 1994. On the suckling behaviour of Alpine chamois *Rupicapra rupicapra rupicapra*. *Zeitschr Säugetierkd* 59:230–235.
- Ruckstuhl KE, Ingold P. 1999. Aspects of mother-kid behaviour in Alpine chamois, *Rupicapra rupicapra rupicapra*. *Zeitschr Säugetierkd*. 64:76–84.
- Rudolph KM, Hunter DL, Rimler RB, Cassirer EF, Foreyt WJ, Delong WJ, Weiser GC, Ward AC. 2007. Microorganism associated with pneumonic epizootic in Rocky Mountain bighorn sheep (Ovis canadensis canadensis). *J Zoo Wildl Med*, 38: 548–558.
- Rughetti, M. & Festa-Bianchet, M. 2010. Compensatory growth limits opportunities for artificial selection in Alpine chamois. *Journal of Wildlife Management*, 74, 1024–1029.
- Sabin AB. 1959. Reoviruses: a new group of respiratory and enteric viruses formerly classified as ECHO type 10 is described. *Science* 130, 1387–1389.
- Sausker EA, Dyer NW. 2002. Seroprevalence of OHV-2, BVDV, BHV-1, and BRSV in ranch-raised bison (*Bison bison*). *J. Vet. Diagn. Invest.* 14:68–70.
- Schiff LA, and BN Fields. 1990. Reoviruses and their replication. In Virology. 2nd ed., vol. 2. B.N. Fields and D.M. Knipe, editors. Raven Press, Ltd., New York. 1275-1306.
- Schmitt D, Wittowski G. 1999. Studies of the spread of BVD-virus infections in hoofed game in Bavaria. *Tierarztliche Umschau* 54; 284–288.
- Sharp MW, Rawson BC. 1986. The cost of border disease infection in a commercial flock. *Vet Rec*, 119: 128–130.
- Smith MH, Lehmkhul HD, Phillips SMI. 1979. Isolation and characterization of respiratory syncytial virus from goats. *Am. Assoc. Vet. Lab. Diagn.* 22:259– 269.

- Stott EJ, Thomas LH, Collins AP, Crouch S, Jebbett J, Smith GS, Luther PD, Caswell R. 1980. A survey of virus infections of the respiratory tract of cattle and their association with disease. *J. Hyg. (Lond.)* 85:257–270.
- Stott EJ, Taylor G. 1985. Respiratory syncytial virus. Brief review, *Arch. Virol.* 84:1–52.
- Strong R, La Rocca SA, Ibata G, Sandvik T. 2010. Antigenic and genetic characterisation of border disease viruses isolated from UK cattle. *Vet Microbiol*, 141:208–215.
- Thabti F, Letellier C, Hammami S, Pépin M, Ribière M, Mesplède A, Kerkhofs P, Russo P. 2005. Detection of a novel border disease virus subgroup in Tunisian sheep. *Arch Virol.* 150(2): 215-29.
- Tarry DW, Bernal L, Edwards S. 1991. Transmission of bovine virus diarrhea virus by blood feeding flies. Vet. Rec. 128, 82–84.
- Telfer S, Bennett M, Bown K, Cavanagh R, Crespin L, Hazel S, Jones T, Begon M. 2002. The effects of cowpox virus on survival in natural rodent populations: increases and decreases. *Journal of Animal Ecology*, 71, 558-568.
- Thomas LH, Stott EJ, Jones PW, Jebbett NJ, Collins AP. 1980. The possible role of respiratory syncytial virus and *Pasteurella* spp. in calf respiratory disease. *Vet. Rec.* 107:304–307.
- Thomas LH, Stott EJ, Collins AP, Jebbett J. 1984. Experimental pneumonia in gnotobiotic calves produced by respiratory syncytial virus. *Br. J. Exp. Pathol.* 65:19–28.
- Thiel HJ, Plagemann PGW, Moenning V. 1996. Pestiviruses. In: BN Fields, DM Knipe, PM Howley (Eds.), Fields Virology, 3rd ed., Vol. 1, pp. 1059-1073.
- Thurmond MC. 2005. Virus transmission. Pages 91–104 in Bovine Viral Diarrhea Virus Diagnosis, Management, and Control. S. M. Goyal and J. F. Ridpath, ed. Blackwell Publishing, Ames, IA.
- Tosi G and Pedrotti L. 2003. Rupicapra rupicapra. In Boitani L, Lovari S, Vigna Taglianti A (Eds.) *Mammiferi carnivore e Artiodattili*. Serie Fauna d'Italia, ed. Calderini Bologna.

- Trudel M, Nadon F, Simard C, Belanger F, Alain R, Seguin C, Lussier G. 1989. Comparison of caprine, human and bovine strains of respiratory syncytial virus. *Arch. Virol.* 107:141–149.
- Tyler, K. L., and B. N. Fields. 1990. Reoviruses, p. 1307–1328. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed., vol. 2. Raven Press, Ltd., New York.
- Tyler, K. L., 2001: Mammalian reoviruses. In: Knipe, D. M., and Howley, P. M. (eds), Fields Virology 4th edn, pp. 1729–1745. Lippincott Williams & Wilkins, Philadelphia.
- Tyler, K. L., E. S. Barton, M. L. Ibach, C. Robinson, J. A. Campbell, S. M. O'Donnel, T. Valyi-Nagy, P. Clarke, D. Wetzel, and T. S. Dermody, 2004: Isolation and Molecular Characterization of a Novel Type 3 Reovirus from a Child with Meningitis. *J. Infect. Dis*. 189, 1664–1675.
- Valarcher JF, Schelcher F, Bourhy H. 2000. Evolution of bovine respiratory syncytial virus. *J. Virol.* 74:10714–10728.
- Valarcher JF, Taylor G. 2007. Bovine respiratory syncytial virus infection. *Vet. Res.*, 38, pp. 153–180.
- Vallat B. 2008. Improving wildlife surveillance for its protection while protecting us from the diseases it transmits. Editorials from the Director General, OIE World Organization for Animal Health.
- Van Campen H, Ridpath J, Williams E, Cavender J, Edwards J, Smith S, Sawyer H. 2001. Isolation of bovine viral diarrhea virus from a free-ranging mule deer in Wyoming. *J Wildl Dis*. 37(2): 306-11.
- VanderLey B, Ridpath JF, Sweiger S. 2011. Comparison of detection of bovine virus diarrhea virus antigen in various types of tissue and fluid samples collected from persistently infected cattle. *J. Vet. Diagn. Invest.* 23:84–86.
- Van der Poel WHM, Brand A, Kramps A, Van Oirschot JT. 1994. Respiratory syncytial virus infections in human beings and in cattle. *J. Infect. Dis.* 29:215–228.
- Van der Poel WHM, Langedijk JPM, Kramps JA, Middel WGJ, Brand A, Van Oirschot JT. 1995. Bovine respiratory syncytial virus antibodies in non-bovine species. *Arch. Virol*. 140:1549–1555.

- Verhoeff J, Van der Ban M, van Nieuwstadt AP. 1984. Bovine respiratory syncytial virus infections in young dairy cattle: clinical and haematological findings. *Vet. Rec.* 114:9–12.
- Viganò R. 2009. Integrazione dei parametri parassitologici, metabolici e sierologici nella gestione degli ungulati selvatici alpini. Phd project a.y. 2008-2009.
- Vilcek S, Nettleton PF. 2006. Pestivirus in wild animals. *Vet Miocrobiol.* 116:1-12.
- Vilcek S, Leskova V, Meyer D, Postel A, Becher P. 2014. Molecular characterization of border disease virus strain Aveyron. *Vet Miocrobiol*. 171: 87-92.
- Viuff B, Tjornehoj K, Larsen LE, Rontved CM, Uttenthal A, Ronsholt L, Alexandersen S. 2002. Replication and clearance of respiratory syncytial virus. Apoptosis is an important pathway of virus clearance after experimental infection with bovine respiratory syncytial virus. *Am. J. Pathol.* 161:2195–2207.
- Weiner, H. L., and B. N. Fields, 1977: Neutralization of reovirus: the gene responsible for the neutralization antigen. *J. Exp. Med*. 146; 1305–1310.
- Weiser GC, De Long WJ, Paz JL, Shafii B, Price WJ, Ward ACS. 2003. Characterization of Pasteurella multocida associated with pneumonia in bighorn sheep. *J Wildl Dis*. 39: 536–544.
- White, D.O., and F.J. Fenner. 1986. Medical Virology. 3rd ed. Academic Press, Inc., Orlando. 119-145.
- Wiethoelter AK, Beltrán-Alcrudo D, Kock R, Siobhan MM. 2015. Global trends in infectious diseases at the wildlife-livestock interface. *Proc Natl Acad Sci USA.* 112: 9662-7.
- Wolfe ND, Dunavan CP, Diamond J. 2007. Origins of major human infectious diseases. *Nature* 447(7142): 279-283.
- Wolfe LL, Diamond B, Spraker TR, Sirochman MA, Walsh DP, Machin CM, Bade DJ, Miller MW. 2010. A bighorn sheep die-off in Southern Colorado involving a Pasteurellaceae strain that may have originated from syntopic cattle. *J Wildl Dis* 46: 1262-8.

- Woolhouse MEJ. 2002. Population biology of emerging and re-emerging pathogens. *Trends Micorbiol.* 10: s3-s7.
- Zanzi L. 2004. Le Alpi nella storia d'Europa. Edizione CDA Vivalda, Torino.
- Zunino F. 2003. L'idea wilderness o della via italiana alla preservazione delle terre selvagge. Documenti Wilderness Anno XVIII, n 2, aprile-giugno 2003.

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