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Infectious agents involved in infectious keratoconjunctivitis in semi-domesticated reindeer (*Rangifer tarandus tarandus*) in Fennoscandia.

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SUMMARY

Infectious keratoconjunctivitis (IKC) is one of the most common ocular diseases in ruminants worldwide. Animals affected by this disease can show a wide variety of clinical symptoms, including keratitis, uveitis, corneal ulcer, conjunctivitis and in severe cases, blindness. *Moraxella* spp., *Chlamydia* spp. and *Mycoplasma conjunctivae* have been described as primary causative agents in ruminant species such as cattle (*Bos taurus*), sheep (*Ovis aries*) or alpine ibex (*Capra ibex*), but previous studies indicated that the reindeer alphaherpesvirus (cervid herpesvirus 2; CvHV2) could be associated with IKC as a primary agent in reindeer (*Rangifer tarandus* spp.). To address this further, 344 semi-domesticated reindeer (*R. t. tarandus*), with (n = 127) or without (n = 126) clinical symptoms of IKC, or with no details on clinical symptoms provided (n = 91), were sampled in Norway, Sweden and Finland between 2010 and 2014. Serum was tested for antibodies against CvHV2 (enzyme-linked immunosorbent assay; ELISA) and swab samples obtained from conjunctiva were subjected to bacteriological cultivation and also tested (polymerase chain reaction; PCR) for the presence of *Chlammydiaceae*, *M. conjunctivae* and CvHV2 specific deoxyribonucleic acid (DNA). This master project summarizes the data obtained from the sampled reindeer populations and evaluates the assumed link between the presence of CvHV2 and clinical symptoms of IKC. The significant association detected in the present study between clinical symptoms of IKC and the presence of CvHV2 in affected eyes, which is not present for any of the other microorganisms studied, leads to the hypothesis that CvHV2 is the primary agent of the IKC in semi-domesticated reindeer in Fennoscandia.

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INTRODUCTION

1. BACKGROUND

Infectious keratoconjunctivitis (IKC) is a severe transmissible ocular disease, which affects domestic and wild ruminants worldwide. It is considered the most important eye disease in cattle (*Bos taurus*) (Postma et al., 2008). In cattle, the bacterium *Moraxella bovis* is considered as the main causative agent of the disease, but also other bacteria, viruses and environmental factors can play a role in the disease (Angelos, 2010; Zbrun et al., 2011). IKC also occurs in small ruminants. In Norway, *Mycoplasma conjunctivae* and possibly the bacterium *Branhamella (Moraxella) ovis* are considered as the main causative agents in sheep (*Ovis aries*) (Åkerstedt and Hofshagen, 2004). The disease has also been described in several wildlife species (Gortázar et al., 1998; Meagher et al., 1992), e.g. chamois (*Rupicapra rupicapra*) (Giacometti et al., 2002; Marco et al., 2009), alpine ibex (*Capra ibex*) (Giacometti et al., 2002) and moose (*Alces alces*) (Dubay et al., 2000a).

As in other ruminants, IKC in reindeer is considered a multi-factorial disease, associated to infectious agents, but also environmental factors such as high animal density, stress, UV light exposure and dust (Dubay et al., 2000b; Nayar and Saunders, 1975; Rehbinder and Nilsson, 1995). Various microorganisms such as *Moraxella bovoculi*, *Moraxella ovis*, *Escherichia coli*, *Staphylococcus* sp. and cervid herpesvirus 2 (CvHV2) have been isolated from animals with clinical symptoms of IKC (Aschfalk et al., 2003; Oksanen et al., 1996; Tryland et al., 2009), and may thus play a role in the development of the disease. IKC is most often detected in connection with the collection, transport and feeding of reindeer within a fence, which also coincides with the period when animals are observed more carefully and closely. In contrast, in periods when reindeer are free ranging, the prevalence of the disease is unknown, since animals are less often closely observed. This disease has been described in reindeer since more than 100 years ago (Bergman, 1912) and particularly affects calves and young animals (Tryland et al., 2009). It occurs sporadically and affects individual animals, but may also appear as regular outbreaks, affecting tens of animals in a herd and being a great issue for both reindeer herders and animal welfare (Tryland et al., 2009).

2. REINDEER (*Rangifer tarandus*)

Rangifer tarandus spp., known as reindeer in Eurasia and caribou in North America, is the most numerous cervid species in the arctic region, but the number of animals is declining globally (Vors and Boyce, 2009).

There are seven recognized subspecies of reindeer, all being distributed in the arctic and subarctic region (Fig. 1) (Røed, 2005). Most of these subspecies are located in North America, the Canadian barren-ground caribou (*R.t. groenlandicus*), woodland caribou (*R.t. caribou*), Peary caribou (*R.t. pearyi*) and Alaska tundra caribou or Porcupine caribou (*R.t. grantii*). Only two subspecies are present in continental Europe, the Eurasian tundra reindeer (*R.t. tarandus*) and the wild forest reindeer (*R.t. fennicus*). Besides these subspecies, the Svalbard reindeer (*R.t. platyrhynchus*) may be found on the Norwegian Svalbard archipelago. In earlier times, the Arctic reindeer (*R.t. eogroenlandicus*) lived on the eastern coast of Greenland, but this species has been considered extinct since the beginning of the 20th century (Banfield, 1961; Gravlund et al., 1998).

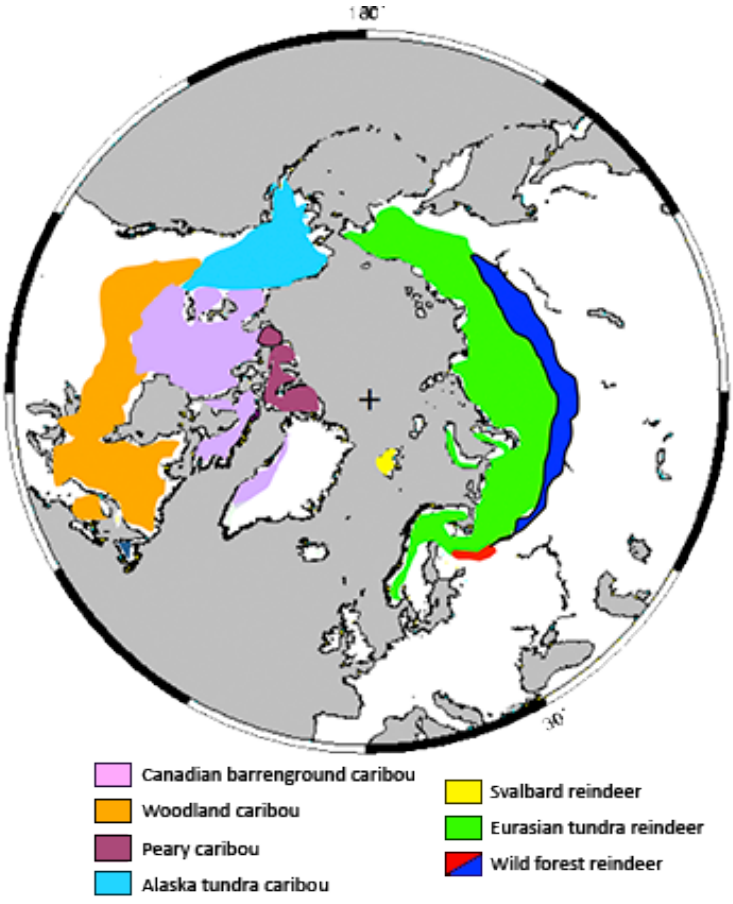

Fig. 1. Distribution of the 7 subspecies of reindeer (*Rangifer tarandus*) through the arctic region. Adapted from the original map of Røed 2005.

Semi-domesticated reindeer in Norway, Sweden and Finland belong to the Eurasian tundra reindeer subspecies. Around 1000 years ago, during the Viking age, reindeer use in those areas changed from exclusively hunting of wild animals to an extensive production system, and within the last 150 - 200 years the "modern" concept of Sámi pastoralism emerged (Bjorklund, 2013).

One of the most important characteristics of the reindeer species is the growth of the antlers. Reindeer hinds can grow antlers, an exclusive feature among deer species. Reindeer antlers are furthermore the largest relative to body size among cervids and the fastest growing mammalian bone structure (Banfield, 1974; Whitaker and Hamilton, 1998).

Generally, female reindeer are considerably smaller than males. The height of adult male reindeer at the withers (the ridge between the shoulder blades) is about 110 cm and the weight varies between 90 - 180 kilograms. Adult female reindeer height at the withers is about 90 cm and the weight is usually 60 - 100 kilograms. Reindeer coloring varies from black, via shades of brown, to almost completely white (Banfield, 1961; Whitaker and Hamilton, 1998).

Reindeer reproduction has many similarities to the reproduction processes of other cervids, being seasonal and influenced by decreasing light conditions in autumn (Ropstad, 2000). Semi-domesticated reindeer reach sexual maturity at the age of one and a half years old, although particularly large animals can get pregnant in their first mating season. The rutting season is from the middle of September until the middle of October and the gestation period can vary from 203 to 240 days (Rowell and Shipka, 2009). Females give birth to one calf, between May and June, with some very rare cases of twin calves (Banfield, 1974; Whitaker and Hamilton, 1998).

Besides the semi-domesticated reindeer, there are small populations of wild reindeer in Norway and Finland. Norway has wild Eurasian tundra reindeer, which belong to the same subspecies as the semi-domesticated reindeer, with little genetic differentiation between the domestic and wild reindeer herds in Fennoscandia (Røed et al., 2008). There is a population of approximately 25.000 wild reindeer in Norway, located in 23 management units in the mountainous areas in the southern part of the country. Wild reindeer from some of those units can interact with the semi-domesticated reindeer populations (Reimers, 2007; Ulvevadet and Klokov, 2004). There is a seasonal hunting of this wild reindeer, with 4.000 - 5.000 animals

being harvested every year (Ulvevadet and Klokov, 2004). Finland has some small populations of wild forest wild reindeer, making a total of approximately 2.500 animals, of which individual animals are killed only in order to minimize interaction with semi-domesticated reindeer (Anonymous, 2007b). It differs from the semi-domesticated reindeer in several body measurements and ratios, e.g. are the legs significantly longer (Nieminen and Helle, 1980).

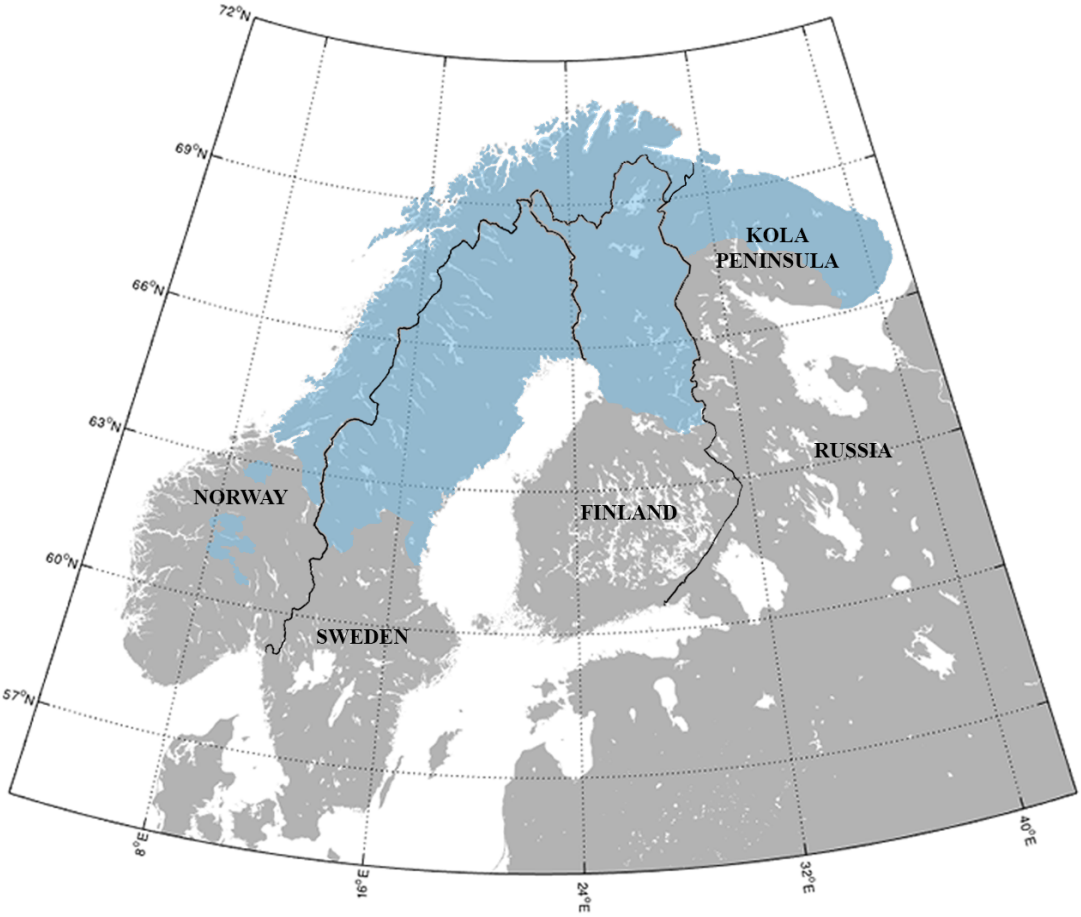

Fig. 2. Map of Fennoscandia (Norway, Sweden, Finland) and the Kola Peninsula (Russia) and overview of the area dedicated to reindeer husbandry highlighted in blue.

3. REINDEER HUSBANDRY

Reindeer and its husbandry has been a fundamental part of the arctic societies since hundreds of years ago, involving more than 20 different indigenous peoples (Anonymous, 2006; Huntington and Fox, 2005). In Fennoscandia, up to 40 % of the land is used as reindeer pasture (Fig. 2) (Pape and Löffler, 2012). The Sami are one of the indigenous groups that have traditionally made reindeer herding one of the bases of their livelihood (Reinert, 2006).

They are the native inhabitants of an area known as Sápmi, which is located in the northern part of Norway, Finland, Sweden and a part of the Kola Peninsula in Russia. According to the Sami Parliaments in Norway, Finland and Sweden, there are between 40.000 and 80.000 Sami living in Fennoscandia and about 10 % of them are somehow involved in reindeer husbandry (Anonymous, 2008).

Generally, reindeer grazes in habitats rich in green plants during the snow-free season while lichen-rich habitats are grazed during winter (Moen and Danell, 2003), although there are different patterns of reindeer movements and husbandry systems depending of the country.

Norway is divided in 90 pasture districts that can be used by reindeer herders as grazing areas (Ulvevadet and Klokov, 2004). Most of the animals feed along the coast during summer and in the mountainous inlands in winter (Moen and Danell, 2003). According to Norwegian Agriculture Agency approximately 40 % of the land in Norway was dedicated to grazing pastures for 253.092 reindeer in 2012 (Anonymous, 2013), involving about 2.700 Sami that have reindeer husbandry as their main or part-time occupation (Pentha, 2014; Ulvevadet and Klokov, 2004). The Norwegian Reindeer Herding Act from 2007, which regulates reindeer herding in Norway, states that only people licensed with a official reindeer earmark can perform reindeer husbandry in the Sami reindeer herding area. To qualify for the right to earmark reindeer, a person has to belong to the Sami community and has the reindeer husbandry as his or her main activity, or their parents or grandparents have had it as their primary occupation. This right can also be acquired by adoption or marriage. All reindeer owners must be part of a siida, which are regional organizations of reindeer herders (Anonymous, 2007a; Bjorklund, 2013). An exception to this are the non-Sami reindeer herds, which require a special permission from the administration to operate and are located in Nord-Gudbrandsdal and Valdres. Total meat production from the reindeer husbandry in 2012 was 1.516 tons, with 67.037 animals slaughtered (Anonymous, 2013).

In Sweden, the right to herd reindeer is exclusive for members of the Sami community (Anonymous, 1971). The herding system is similar to the one used in Norway. The reindeer herding areas are divided in districts, some of them close to and including the mountain areas for summer pastures, and the winter grazing grounds located at lower altitudes (Moen and Danell, 2003). There are approximately 160.000 square kilometers or 34 % of the Swedish land dedicated to reindeer herding, divided in 51 areas of reindeer husbandry (Ulvevadet and

Klokov, 2004). The number of reindeer in Sweden is approximately 252.550 and about 1.500 tons of meat are produced yearly, involving 4.500 Sami people (Anonymous, 2005, 2015a).

In Finland, all citizens can own reindeer and practice reindeer herding, including citizens from other countries of the European Union (EU) who are permanent residents in the Finnish reindeer herding area (Fig. 2) (Anonymous, 1990; Ulvevadet and Klokov, 2004). A more intensive reindeer husbandry system is used in Finland, as compared to Norway and Sweden, with less movement of animals between different grazing areas. Reindeer owners tend to keep the animals corralled in winter, with supplementary feeding and more protection against predators (Moen and Danell, 2003; Ulvevadet and Klokov, 2004). According to the Finnish Association of Reindeer Herding Cooperatives (Paliskuntain Yhdistys), the reindeer herding area occupies 122.936 square kilometers or 36 % of Finland's total area (Anonymous, 2015b), and the activity is regulated by the Ministry of Agriculture and Forestry through the Reindeer Herding Law. This law establishes that the number of grazing reindeer cannot exceed the sustainable production capacity of reindeer winter pastures (Anonymous, 1990). This number is currently 203.700 reindeer, with around 7.000 reindeer owners and close to 2.000 tons of reindeer meat reaching the market every year (Anonymous, 2015c).

Mainly in Norway and Sweden, some reindeer owners practice cross-border migration between the different countries, with their reindeer feeding in one country during summertime and in another during wintertime (Ulvevadet and Klokov, 2004). Additionally animals may move across the borders between Norway, Sweden and Finland without being herded, and reindeer moving across the border between Norway and Russia have also been observed (Pers. comm. border patrol agent Mathisen 25.02.15).

Reindeer herding is considered a traditional herding system, but nowadays snow scooters, quads or helicopters have replaced the traditional skis and sledges. The use of these new technologies has facilitated the management and care of the animals. On the downside the easier management has led to a reduced need for human resources and time, which may have contributed to an increase in the number of reindeer in some areas of Fennoscandia.

Traditionally, one of the main challenges for reindeer herding was considered to be the damage caused by large carnivores, however, recent studies demonstrated that the claims for predator damages may have been overestimated for many years, and pointed to

overpopulation as the most important cause of winter mortality (Tveraa et al., 2014). Climate change is another important challenge as it affects reindeer husbandry both directly and indirectly. For example, with the temperature increase during summers there is an increment in the populations of warble flies and mosquitoes (Åsbakk et al., 2014; Rydén et al., 2009; Schäfer and Lundström, 2009), which is related to the risk of disease transmission and parasite load in reindeer. Additionally the reindeer spend more time avoiding these insects at the expense of food intake. Moreover, warmer winters combined with increased precipitation in the form of rain, and an increased frequency of freeze-thaw cycles, may produce an ice crust effect over the pastures, locking them and intensifying the risk of starvation of the reindeer during winter (Moen, 2008; Pape and Löffler, 2012).

There have been considerable changes in the reindeer grazing areas over the past few decades. Several types of human infrastructures, such as roads, power lines, windmills or dams, have been developed in Fennoscandia. In addition, human activities in reindeer herding areas, including mountain sports and tourism, have also increased. All these changes may have an effect on different levels, from altering the behavior of reindeer, which tend to avoid any human contact, to changing the composition of the pasture (Anttonen et al., 2011).

The combination of overgrazing due to the large number of animals, the rise in human presence and climate change, has contributed to the degradation of pastures and therefore, to the decline of available winter food. As a consequence, the reindeer herders find themselves forced to provide supplementary food to the animals to ensure that as many as possible survive winter. In the case of corralled animals, they are entirely dependent on food provided by humans, increasing the working load of reindeer herders and threatening the traditional herding system.

4. INFECTIOUS KERATOCONJUNCTIVITIS

In many ruminant species, IKC is considered a multifactorial disease. The primary cause of this disease in reindeer remains unconfirmed, but different infectious agents and environmental factors, such as stress, dust or UV light have been proposed to contribute to the development of the disease (Rehbinder and Nilsson, 1995; Tryland et al., 2009).

Different symptoms can appear in reindeer affected by IKC. Light sensitivity, increased lacrimation and whitish or bluish eyes due to corneal oedema are usually the early symptoms of the disease (Fig. 3). As the disease progresses, the symptoms become more severe, including conjunctivitis, periorbital oedema, purulent exudate, sometimes associated with blood, corneal ulceration, necrosis and in severe cases, blindness. During the development of IKC, a variety of clinical diagnosis may appear, including conjunctivitis, keratitis, uveitis and ulcus cornea (Fig. 4) (Tryland et al., 2009).

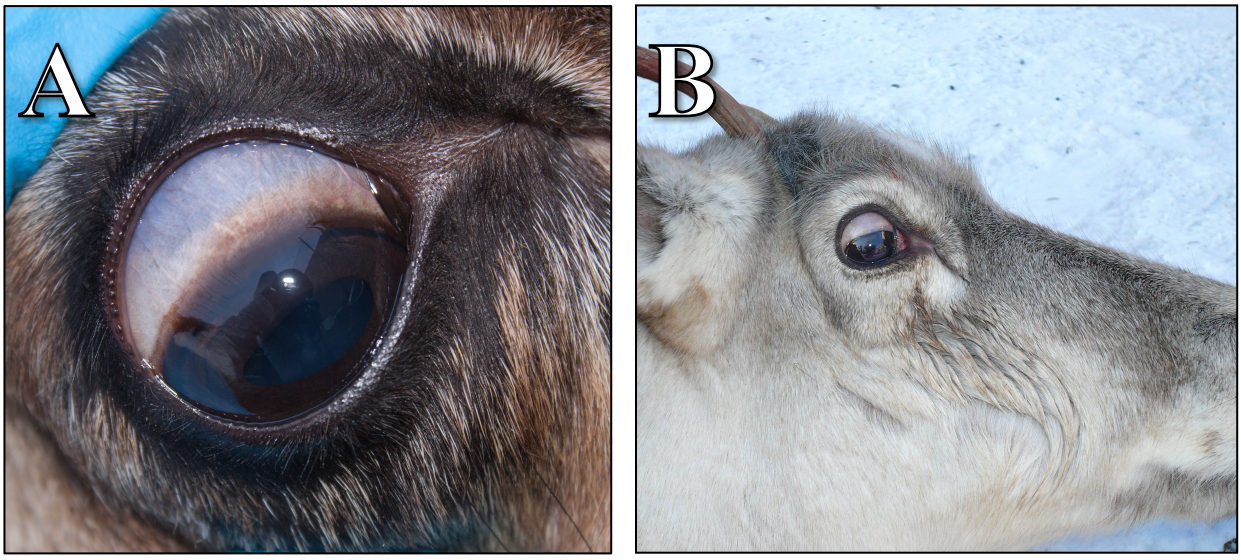

Fig. 3. Reindeer eyes. **A.** Apparently healthy eye (score 0). **B**. Initial stage of eye disease, with an increased lacrimation, which is visible as a browning of the fur under the eye. This symptom can be caused by IKC or other eye problems, depending of the cause and how it will develop. Credit: Morten Tryland (B)

Even if the causative agent(s) of IKC has not yet been identified, some preventive measures and symptomatic treatments are used during outbreaks of this disease. Due to the transmissible nature of IKC, the first measure can be to isolate the affected animals from the animals without clinical symptoms of IKC in order to control the spreading of the disease. The treatment, described by Tryland et al. (2009) during an outbreak of IKC in semi-domesticated reindeer in Norway, consisted in cleaning of the eye and surrounding area with paper, and flushing with sterile physiological salt water. An antibiotic ointment (Fucithalmic Vet 1%; VetXX, Uldum, Denmark) was also provided in order to control possible bacterial infections; at least 1 - 2 times per day as long as the animals had symptoms. Due to the special characteristics of reindeer husbandry, capturing and treating a large number of animals is challenging, laborious and costly. Thus, in most cases, herders choose to slaughter animals at an early stage of the disease. Alternatively, clinically affected animals are euthanized (not for human consumption), or treated.

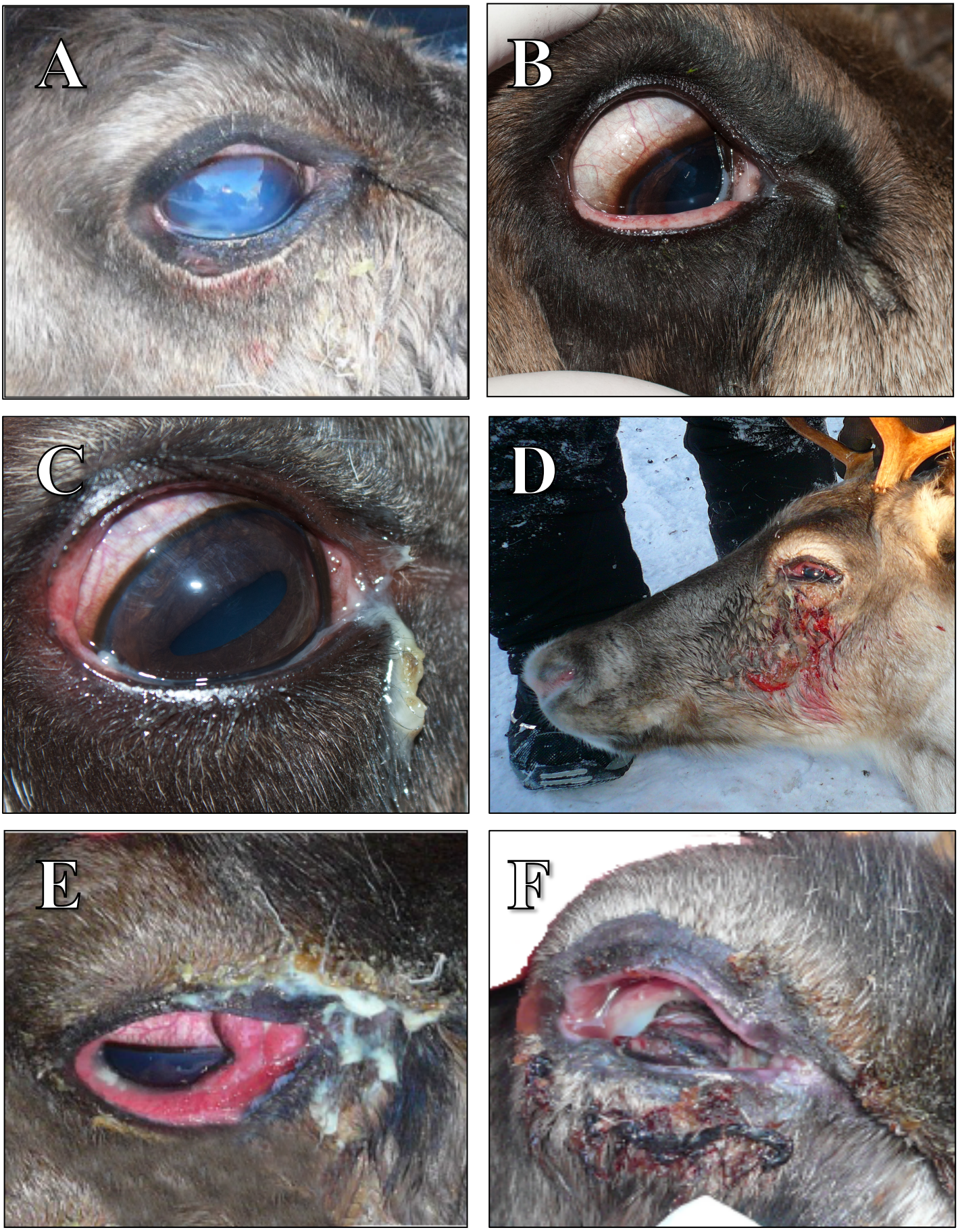

Fig.4. Evolution of the clinical symptoms of infectious keratoconjunctivitis in reindeer. **A-B.** Milder symptons of IKC: corneal oedema, with the animal showing a characteristic bluish/whitish eye (A) and mild conjunctivitis (B). **C-E**. Conjunctivitis, periorbital oedema and purulent exudate, in some cases associated with blood, can appear in later stages. **F**. Necrosis followed by disorganization and collapse of the ocular structures in the most severe cases. Credit: Morten Tryland (A, D, E, F)

Several outbreaks have been reported in the Scandinavian countries in recent years, e.g. in Finland in the winter 1992-1993 (Oksanen, 1993), in Troms County, Norway, in 1999, 2009 and 2012 (Aschfalk et al., 2003; Smits et al., 2013; Tryland et al., 2009), and in Sweden in 2011, 2013 and 2014 (unpublished data). All these outbreaks were detected in connection with the gathering and handling of the reindeer, for tagging or selection of animals for slaughter. In previous studies, it was concluded that reindeer show an acute stress response to handling and transport (Wiklund et al., 2001), which can weaken the cellular and humoral immune responses of the animals (Laudenslager et al., 1988) and therefore, increase the risk of disease. The cellular immune response is driven by T helper 1 cytokines (Th1), while T helper 2 cytokines (Th2) are associated to the humoral immune response. Salak-Johnson and McGlone (2007) hypothesize that stress can modify the Th1/Th2 balance, affecting disease susceptibility.

5. AGENTS POSSIBLY INVOLVED IN IKC IN REINDEER

5.1 General bacteriology

Different species of bacteria can be cultured from the eyes of apparently healthy ruminants. Barber et al. (1986) showed that bacteria could be cultured from the eyes of up to 87 % of cows and according to Egwu el al. (1989) in 40 % of sheep. *Corynebacterium* sp., *Streptococcus* sp., *Staphylococcus* sp., Non-hemolytic *Moraxella* sp., *Acinetobacter* sp., *Bacillus* sp. or *E. coli* have been identified by various authors in the eyes of domesticated animal species with no clinical symptoms of IKC (Åkerstedt and Hofshagen, 2004; Barber et al., 1986; Egwu et al., 1989). Therefore, several species of bacteria might also be present in the eyelids and conjunctiva of healthy reindeer. In order to differentiate between the microorganisms that can be considered as normal microbial flora and pathogens, we have to initially distinguish between the terms colonization and infection. Colonization means that the concentration of a given microorganism is enough to allow its detection, but it is not causing any harm to the host. On the contrary, infection implies the existence of signs of disease. Colonization can progress to infection under specific circumstances, e.g. if a concurrent infection with another microorganism take place, or in cases where the immune competence of the host is reduced. A third term that has to be well understood is contamination, i.e. the organism is not present in the eye, but has been introduced into the specimen during sampling, or during any of the subsequent steps until the final investigation.

M. bovis is considered to be the primary causal organism associated with IKC in cattle, also known as infectious bovine keratoconjunctivitis (IBK) (O'Connor et al., 2012). There are two different virulence factors that are determinant for the capacity of *M. bovis* to cause IBK; the presence of type IV pili (TFP) on the bacterial surface, which allows the adherence of the bacteria to the corneal surface, the first step in the pathogenesis of IBK (Postma et al., 2008), and the secretion of a bacterial protein called cytotoxin A, which produce a cytolytic effect in cells of the immune system and corneal epithelial cells (Angelos et al., 2001; Hoien-Dalen et al., 1990; Kagonyera et al., 1989).

A species closely related to *M. bovis*, namely *Moraxella bovoculi,* has also been isolated from corneal ulcers and associated with IBK in cattle (Angelos et al., 2007b; Galvão and Angelos, 2010) and a retrospective analysis study conducted by Loy and Brodersen (2014) identified this species as the only *Moraxella* sp. in the majority of the cases investigated. Several samples recovered from cattle eyes were misidentified as *M. ovis* before the isolation of *M. bovoculi*, and have been reclassified to *M. bovoculi* (O'Connor et al., 2012).

M. bovoculi also contains cytotoxin A encoding genes (Angelos et al., 2007a) and a single pili-encoding gene (Calcutt et al., 2014). The virulence of different strains of this bacterium can be associated to the existence of these virulence factors (Angelos et al., 2007a). However, an experimental study by Gould et al. (2013) suggested that *M. bovoculi* was not a causal agent for IBK, since it could be recovered from apparently healthy eyes, and there was no strong evidence of association between exposure to *M. bovoculi* and the development of IBK and neither evidences of the capacity of *M. bovoculi* to cause corneal ulcer, attributing an opportunistic character to this bacteria.

TFP are also present in several other Gram-negative bacteria such as *Neisseria* spp. or *Pseudomonas* spp., promoting the attachment to host epithelial cells and regulating virulence factors (Persat et al., 2015; Prieto et al., 2013). A second characteristic of pathogenic Gram-negative bacteria is the formation of biofilms (Reisner et al., 2003). *E. coli*, *Klebsiella* spp., *Citrobacter* spp. and *Enterobacter* spp. are among the microorganisms that present these characteristics, however, they are not considered primary pathogens but rather opportunistic bacteria (Gelatt et al., 2013; Ong et al., 2010). *Pseudomonas* spp. has been associated with purulent infections of the conjunctiva and eyelids in humans, and other Gram-negative bacteria such as *Enterobacter* spp., *E. coli*, *Citrobacter* spp., *Klebsiella pneumonia* or

Serratia spp. have been associated with keratitis and conjunctivitis as potential opportunistic pathogens in humans (Ben-Tovim et al., 1974) and sheep (Åkerstedt and Hofshagen, 2004).

Acinetobacter spp. is a non-motile, non-fermenting Gram-negative coccobacillus. These are characteristics that are shared with the species in the *Moraxella* genus (Vaneechoutte et al., 2015; Wilcox, 1970). This bacterium is considered as an environmental organism which can cause opportunistic infections in animals. *Acinetobacter* spp. may be part of the commonly found conjunctival flora of production animals, but isolation of this bacterium in cases of equine keratoconjunctivitis have been reported (Gelatt et al., 2013). Although severe infections with *Acinetobacter baumanii* have been documented, colonisations are much more frequent than infections (Vaneechoutte et al., 2015).

Members of the genus *Aeromonas* are usually found in aquatic environments, and have been isolated from stagnant and flowing fresh water, tap water, diluted seawater, sewage, soil and foodstuffs (Sire et al., 1990). They are opportunistic pathogens of fish and reptiles, and more rarely mammals (Quinn et al., 2011). These organisms usually cause septicaemia, but eye infections with *Aeromonas hydrophila* have been reported in laboratory-maintained lizards (Cooper et al., 1980), purulent conjunctivitis have been observed in sea turtles (Isler et al., 2014) and periorbital infections, keratitis or endophthalmitis have been registered in humans (Ben-Tovim et al., 1974; Motukupally et al., 2014; Sohn et al., 2007).

Also some Gram-positive bacteria have been identified in cases of eye infection. *Corynebacterium* spp., *Staphylococcus* spp., *Bacillus* spp., *Micrococcus* spp. and *Streptococcus* spp. were identified in sheep with IKC, but no relation between their presence and clinical symptoms could be established (Åkerstedt and Hofshagen, 2004). Staphylococcal species and a large number of streptococcal species are well known as potentially pathogenic agents. While the other Gram-positive species like *Corynebactrium* spp., *Bacillus* spp. and *Micrococcus* spp. have an unclear role in the disease pathogenesis, as they in some cases are present as opportunistic microorganisms, or as a co-infections with other pathogenic agents (Gelatt et al., 2013). The bacteria *Listeria monocytogenes* has also been reported as an eye pathogen in large animals, including sheep (Åkerstedt and Hofshagen, 2004; Kummeneje and Mikkelsen, 1975), cattle, horse (*Equus ferus caballus*) (Evans et al., 2004) and fallow deer (*Dama dama*)(Welchman et al., 1997). Contaminated silage seems to be the primary source of infection in farm animals, as recently shown in horse in Norway (Revold et al., 2015).

5.2 Family *Chlamydiaceae*

The bacterial family *Chlamydiaceae* belongs to the phylum *Chlamydiae*, order *Chlamydiales*. Members of this family are considered to be anaerobic Gram-negative obligate intracellular bacteria (Quinn et al., 2011). Some of the species are involved in the development of IKC in sheep and goats (*Capra aegagrus hircus*) (Constable et al., 2007). The classification of the family *Chlamydiaceae* was revised based on ribosomal RNA, and the microorganisms known to cause conjunctivitis in small ruminants were reclassified as *Chlamydophyla abortus* and *Chlamydophyla pecorum* (Everett et al., 1999a). A possible role in the development of IKC in sheep has been suggested for two additional species of the same genus, *Chlamydophyla suis* and *Chlamydophyla psittaci* (Osman et al., 2013; Polkinghorne et al., 2009; Rodolakis and Laroucau, 2015). Chlamydial keratoconjunctivitis has been also reported in wild ruminants, such as bighorn sheep (*Ovis canadensis*) (Meagher et al., 1992).

5.3 *Mycoplasma conjunctivae*

Mycoplasma species lack a cell wall around their cell membrane and are therefore resistant to many common antibiotics that target cell wall synthesis (Quinn et al., 2011). One of the species of this genus, *M. conjunctivae* is considered the primary agent of ovine and caprine keratoconjunctivitis (Egwu et al., 1989; Trotter et al., 1977). The disease is usually mild, but can last up to two months in the most severely affected animals (Barile et al., 1972; DaMassa et al., 1992). This microorganism can also infect wild *Caprinae*. In these cases, animals may be clinically affected with severe symptoms of the disease, but they may also become carriers, hosting the bacterium without any clinical symptoms for up to two years, as observed in chamois, alpine ibex and bighorn sheep (Jansen et al., 2006; Mavrot et al., 2012; Ryser-Degiorgis et al., 2009). It has also been demonstrated that *M. conjunctivae* can be transmitted between domestic sheep and wild *Caprinae* (Belloy et al., 2003a).

5.4 Cervid herpesvirus 2

CvHV2 is a member of the genus *Varicellovirus*, and belongs to the order *Herpesvirales*, family *Herpesviridae*, and subfamily *Alphaherpesvirinae*. CvHV2 is one of the virus species related to the prototype of the ruminant alphaherpesviruses, bovine herpesvirus 1 (BoHV1), along with bovine herpesvirus 5 (BoHV5), bubaline herpesvirus 1 (BuHV1), caprine

herpesvirus 1 (CpHV1), cervid herpesvirus 1 (CvHV1) and elk herpesvirus 1 (ElHV1) (Thiry et al., 2006). The CvHV2 strain Salla 82 was isolated in 1982 from a semi-domesticated reindeer in Finland (Ek-Kommonen et al., 1986). Particular characteristics shared by these viruses are a short replication cycle (approximately 18 h) as compared to members of the subfamily *Betaherpesvirinae,* and the capacity to induce life long and latent infections (Thiry et al., 2006).

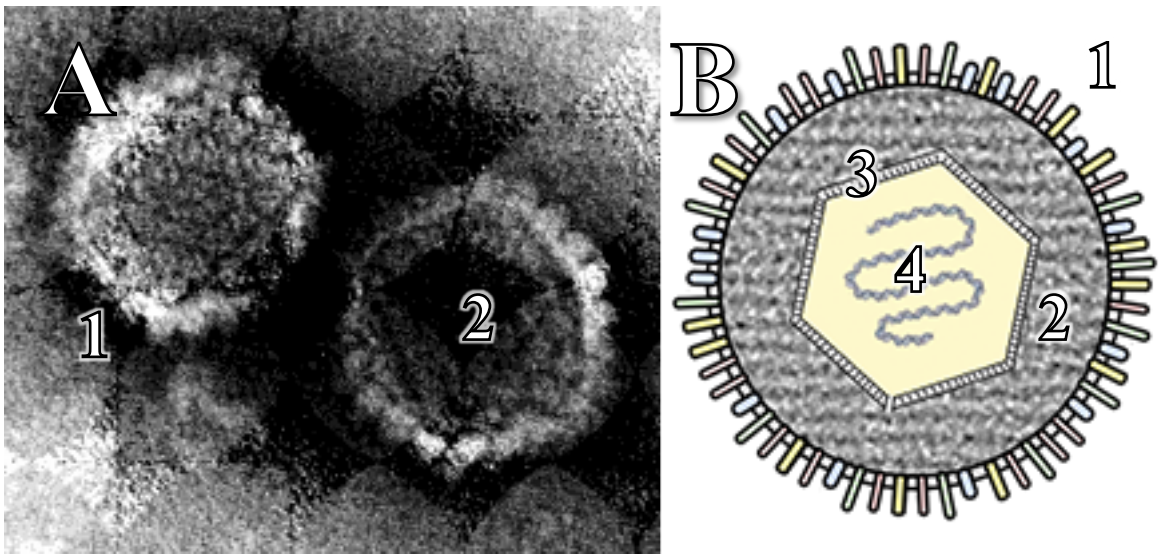

Fig. 5. **A.** Image of two herpesvirus-like particles isolated from reindeer, where the envelope (1) and the icosahedral nucleocapsid (2) are visible. Image modified from Rockborn et al. (1990). **B**. Diagram of CvHV2, with the envelope glycoproteins (1), tegument (2), icosahedral capsid (3) and the DNA molecule (4).

Morphologically, the herpesviruses have a double-stranded linear deoxyribonucleic acid (DNA) molecule, with an estimated size of 145.1 ± 2 kbp (Vanderplasschen et al., 1993), which is protected by a surrounding capsid. This icosahedral nucleocapsid is surrounded by tegument proteins and an external lipid bilayer membrane (the envelope), in which 10 different envelope glycoproteins are placed (Fig. 5) (Thiry et al., 2006).

The herpesviruses genomic organization consists of one long unique unit (U_L) and one short unit (U_S), both flanked by two inverted repeat sequences, the internal repeat sequence (IR), with genes encoding six of the glycoproteins; and the terminal repeat sequence (TR), with genes for the remaining four envelope glycoproteins (Fig. 6) (Schwyzer and Ackermann, 1996). These glycoproteins play a very important role in the interactions between the virus and the host cells and are involved in several steps of the lytic cycle, latent cycle and reactivation of latent virus. They are also important targets for the host' immune system cells (Engels and Ackermann, 1996). Glycoprotein B (gB) gene sequence (UL27) is the most

conserved among alphaherpesviruses, the percentage of nucleotide sequence identity between BoHV1 gB and CvHV2 gB being 84.0 % (Ros and Belak, 2002). This glycoprotein is involved in the process of attachment to, and entrance into, the host cells by the virus (Griffin, 1991). A conserved part of the gC gene (UL44) has also been identified (Thiry et al., 2006). gB and gC are two of the most abundant envelope glycoproteins (Thiry et al., 2006).

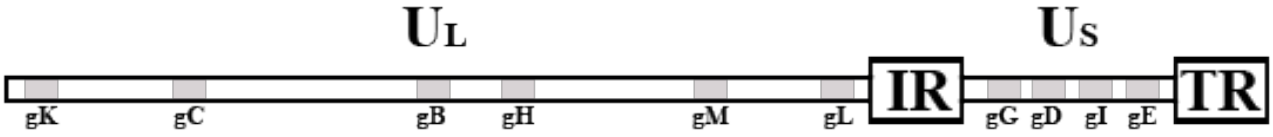

Fig. 6. Genomic organization of the cervid herpesvirus 2 and distribution of the viral envelope glycoproteins. The long unique unit (U_L) contais the gene sequences for 6 of these envelope glycoproteins, including the most conserved among ruminant alphaherpesviruses, the glycoprotein B. The short unique unit (U_S) contains the other 4 gene sequences for envelope glycoproteins. Both, U_L and U_S, are flanked by the internal (IR) and the terminal repeat (TR) sequences. Based on the diagram designed by Thiry et al. (2006).

CvHV2 can be transmitted via direct contact between animals, contact with ocular, nasal or genital secretions, or through aerosols (Engels and Ackermann, 1996). From previous studies, we know that CvHV2 is transmitted from mother to foetus during pregnancy; viral DNA has been detected in foetuses in the slaughterhouse, and the virus has been detected in cells of the uterine wall of experimentally infected female reindeer (das Neves et al., 2009a; das Neves et al., 2009b). If an association between CvHV2 and abortion could be established, one could speculate that infection might be a factor in the loss of calves *in utero* in reindeer, due to the high prevalence in adult animals. It has furthermore been shown that CvHV2 was associated with bronchopneumonia. The virus was detected in goblet cells in the bronchial respiratory epithelium, which are cells responsible for the mucus production of the mucosal membrane of the respiratory tract (das Neves et al., 2009b). As other members of the subfamily *Alphaherpesvirinae*, CvHV2 can cause viremia, spreading to other tissues via blood vessels and possibly lymphatic vessels (das Neves et al., 2009a; das Neves et al., 2009b). Experimentally infected reindeer excreted viral particles up to 24 days post-inoculation (das Neves et al., 2009b).

Alphaherpesviruses active replication cycle follows a cascade-like expression with three phases: immediate early (IE), early (E) and late (L) phases. Proteins expressed during the IE phase activate the E gene expression; it is during this phase that viral DNA replication occurs.

The L gene expression is also activated by an IE protein, the bICP0, and thereafter the cycle finishes with the assembly and release of the virion (Jones and Chowdhury, 2007).

As other herpesvirus, CvHV2 can enter a latency stage. This stage of the CvHV2 cycle starts with neuroinvasion; the virus invades the central nervous system ganglia. In animals affected by IKC, the trigeminal ganglion is the regional ganglion. Therefore, CvHV2 can migrate through the trigeminal nerve and establishes latency in this local neuronal ganglion (das Neves et al., 2009a; Engels and Ackermann, 1996). Sacral ganglia are also a place of choice for the establishment of latency for other ruminant alphaherpesviruses (Pastoret et al., 1982), and the recovery of CvHV2 DNA from reindeer genital swabs after a reactivation protocol could indicate that sacral ganglia are also a place of choice for latency in CvHV2 (Ek-Kommonen et al., 1986). After clearance of infectious viral particles as such, it is still possible to detect viral DNA in the latency sites. According to Jones et al. (2006), the latency related (LR) gene promotes the establishment and maintenance of latency and increase neuronal survival of the virus. The LR gene also inhibits bICP0 expression, and therefore inhibits productive infection (Geiser et al., 2002). Stressful situations or corticosteroids, inducing immunosupresion, can lead to the reactivation of the virus, which then enters a new lytic cycle, with replication and spreading of the viral particles (das Neves et al., 2009a; Rockborn et al., 1990).

CvHV2 is enzootic in reindeer populations in Norway, Sweden and Finland and the virus has also been detected by polymerase chain reaction (PCR) in reindeer and caribou in Alaska and Canada (das Neves et al., 2010). Although CvHV2 has been identified as the primary agent of IKC in an outbreak in Norway, it has not been established whether this viral infection may have clinical significance in relation to IKC in general (Tryland et al., 2009).

AIM OF THE STUDY

The main objective of this study was to evaluate the role of different microorganisms in the pathogenesis of infectious keratoconjunctivitis (IKC) in semi-domesticated reindeer.

A secondary objective of this project was to identify possible causative agent(s) of the disease, a necessary first step in order to be able to identify the primary agent of IKC in reindeer.

Research questions

1. Which bacterial agents are present in the eyes of semi-domesticated reindeer with and without clinical symptoms of IKC?

2. Is the presence of any of these bacteria associated to IKC in semi-domesticated reindeer?

3. Is the presence of CvHV2 in eyes of reindeer or antibodies against CvHV2 in blood associated with IKC in semi-domesticated reindeer?

MATERIAL AND METHODS

1. SAMPLING OF REINDEER

The reindeer husbandry system makes the sampling challenging, as compared to many other production animals. The semi-domesticated reindeer roam freely in the Scandinavian mountains during most of the year and the opportunities to sample are reduced to the period during which animals are collected and handled for several purposes, such as marking, counting, antiparasitic treatment or selection of slaughter animals. Hence, the sampling events are few and the conditions are usually not optimal, including bad weather, dusty or muddy working environments, and consequently, risk of contamination of the samples.

Due to the aim of this study, reindeer with clinical symptoms of IKC were prioritized during sampling. Whenever possible, apart from the symptomatic animals, we have also sampled animals from the same herd without such symptoms, for comparison. In some settings we have sampled only apparently healthy animals, since no animals showed clinical symptoms in the available herd. Thus, the samples were not randomly collected, and are thus not necessarily representative for the herds they were gathered from. It is important to stress this since the results presented here, such as the prevalence of reindeer with antibodies against CvHV2, do not necessarily reflect the real epidemiological situation of IKC in reindeer within the actual herds or in the reindeer populations in the Scandinavian countries.

Three hundred and thirty-five semi-domesticated reindeer have been sampled between 2010 and 2014 in Norway (Sennalandet and Ifjordfjellet in Finnmark county, Lødingen, Sørreisa and Tønsvika in Toms county), Sweden (Karesuando, Kikkejaure, Kiruna, Lainiovuoma Sameby, Malå Sameby and Rans Sameby) and Finland (Kallioluoma, Käsivarsi and Muotkatunturi). In addition, samples from nine wild Norwegian reindeer have also been included in this study (Dovre/Rondane) (Fig. 7). One hundred and twenty-seven of these animals had symptoms related to IKC, 126 did not show such symptoms, and for 91 animals, information on clinical symptoms was not available.

Severity of the ocular disease was classified from 0 to 3, simplifying the 5 grades classification given by Tryland et al. (2009). Asymptomatic animals were scored with 0, animals with increased lacrimation and/or mild conjunctivitis were scored with 1 (mild),

Fig.7. Distribution of the sample sites in Fennoscandia, with the identification of the reindeer district name and number in Norway, and Sami villages and cooperatives in Sweden and Finland respectively. Blue squares represent sampling of live animals, red circles represent sampling of slaughter animals and yellow diamond represents that both, live and slaughter animals have been sampled in that location.

animals with corneal oedema, periorbital oedema, conjunctivitis, keratitis or pus were scored with 2 (moderate) and animals with pus and blood, corneal ulcus or collapse and fibrosis of the eye were scored with 3 (severe).

Sampling of live animals was conducted when animals were corralled for tagging of the calves or for selection of animals for slaughter. In this setting, the animals were physically immobilized inside the fence and fixed during sampling. At the slaughterhouses, animals were stunned with a captive bolt gun and suspended by the hind legs and bled by cutting the jugular vein. After the bleeding, heads were cut off and subsequently sampled.

Blood samples were collected from the jugular vein in BD Vacutainer® Blood collection tubes (silicon-coated serum tubes and spray-coated K2EDTA tubes; BD, Plymouth, UK), either from live animals, using a venoject needle (Terumo, Leuven, Belgium), or from stunned and bled animals, collecting blood directly in open tubes. All tubes were set aside for a few hours at room temperature and then centrifuged for 10 minutes at 3.000 g to separate serum or plasma from blood cells. Serum or plasma was collected and stored at -20 ºC until further analysis.

Swab samples for bacteriology were obtained from the conjunctiva and placed in Amies transport medium with charcoal (Transwab® Amies Charcoal Transport; MWE, Wiltshire, England), transported without freezing to the laboratory and cultured within 2 - 9 days after sampling, depending on transport time to the laboratory.

Swab samples for virology (Applimed SA, Châtel-St-Denis, Switzerland) were obtained from the conjunctiva and placed in sterile cryotubes with 1 ml of Eagle's Minimum Essential Medium (EMEM) with antibiotics in final concentrations of 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 50 µg/ml of gentamicin and 2.5 µg/ml amphotericin B. Swabs were then stored at -80 ºC until further analysis.

2. CULTIVATION OF BACTERIA

Conjunctival swabs were plated onto three agar plates, two agar plates with 5 % ovine blood (blood agar) and one MacConkey agar plate. Blood agar is a differential medium that can distinguish non-haemolytic and haemolytic bacteria (Murray and Baron, 2015), whereas MacConkey is a selective medium that inhibits the growth of Gram-positive bacteria and indicates if the bacteria are lactose fermentative (Murray and Baron, 2015). One blood agar and one MacConkey agar were incubated aerobically at 37 ºC, while the remaining blood agar plate was incubated anaerobically at 37 ºC. All plates were incubated for 48 h and examined visually for bacterial growth after 22 - 24 h and 46 - 48 h.

The bacterial growth was registered as rich, moderate or poor. Dominant colonies or colonies suspected as pathogenic, were subcultured for further identification. These colonies of interest were stained with the Gram's method, in order to make a first presumptive identification of the bacteria (Garrity and Bergey, 2001).

Subsequently, API® strips (bioMérieux, Marcy l'Etoile, France) were used for bacterial identification. These strips include up to 20 ampoules with diverse biochemical test, which will detect enzymatic activity, assimilation and fermentation of sugars by the tested microorganism. Following the general overview provided by the Gram staining, bacteria growing in pure cultures have to be diluted in 2 to 5 ml of distillate water and introduced in the corresponding tubes, as indicated by the manufacturer. Then, the API® strip is incubated and depending on the test, the medium changes colour or opacity, indicating a positive result for that biochemical test. Results were introduced into the APIweb™ software (bioMérieux, Marcy l'Etoile, France), which compare them with the API® databases, giving an interpretation of the strip result.

If identification at species level was not possible by standard methods, bacterial colonies were sent to the Veterinary Institute in Oslo, Norway, for further identification by 16S ribosomal RNA (16S rRNA) gene sequencing. The 16 rRNA gene sequencing is a phylogenetic analysis based on ribosomal RNA, which is present in all self-replicating organisms and has not evolved much along time (Woese and Fox, 1977). The use of universal primers that are able to amplify DNA from a wide variety of bacteria ensure, in most of the cases, the identification of the tested microorganism (Weisburg et al., 1991).

3. DETECTION OF ANTIBODIES

Serum samples were tested for the presence of antibodies against CvHV2 with a commercial BoHV1 blocking enzyme-linked immunosorbent assay (ELISA) kit (IBRG/10, LSI, Lissieu, France) previously validated for the testing of reindeer serum samples for CvHV2 specific antibodies (das Neves et al., 2009c). The test is based on the strongly immunogenic gB as the antigen. Positive and negative controls for cattle provided in the ELISA kit were included on each plate.

This ELISA kit is designed as a blocking ELISA. The serum or plasma sample to be tested is added to an antigen-coated 96-well microtiter plate. The outer wells of the ELISA plates are more vulnerable to error since they are more exposed to evaporation or changes of temperature than the inner wells, and can behave differently during the test. To reduce this problem, known as edge effect, all serum samples were tested in duplicate. If specific antibodies are present in the serum or plasma sample, they will bind to the antigens, forming

antigen-antibody complexes. Subsequently, the plate is washed in order to remove unbound antibodies, and a horseradish peroxidase conjugated monoclonal antibody against the envelope gB of BoHV1 is added. These conjugated antibodies will bind with the remaining antigens that are not already covered by antibodies from the test sample. The final step is the addition of a coloured substrate, which will react with the peroxidase of the conjugate, meaning that the more antibodies that are present in the serum or plasma sample, the fewer antigens in the microtiter plate will be available for the conjugate, and thus less peroxidase to react with the substrate. Since the reaction between the peroxidase and the substrate induces a change of colour in the well, the results can be read quantitatively with a spectrophotometer in an ELISA plate reader, using a wavelength of 450, and a reference wavelength of 620 nm. Since antibodies in the sample and the conjugated antibodies compete for the antigens, a weakly coloured test well indicates a positive sample, i.e. abundance of specific antibodies in the sample, which will bind at the expense of the conjugated antibodies, whereas a strongly coloured test well indicates that only the added conjugate has bound, i.e. no relevant antibodies in the sample (Hornbeck et al., 2001). Samples with a competition percentage above 50 % were considered as seropositive, between 45 and 50 % were considered as doubtful and below 45 % were considered as seronegative, as recommended for cattle and verified for reindeer (das Neves et al., 2009c).

4. POLYMERASE CHAIN REACTION ANALYSIS

PCR is a rapid and versatile *in vitro* method for amplifying defined target DNA sequences present within a source of DNA (Strachan and Read, 2011). The PCR consists in decreasing and increasing temperature cycles, in which the DNA replication can be performed. In each cycle we can see three principal steps: denaturation, annealing and extension. Denaturation is run at high temperature (95°C) and is used to convert the double-stranded DNA into single strands. During the annealing, specific primers for the targeted microorganism anneal with the known gene sequences of this target. Finally we have the extension, with an optimal temperature for the activity of the DNA polymerase.

DNA was extracted from all swab samples with a QIAamp DNA MiniKit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Purity and concentration of the DNA was measured with a NanoDrop^{TM} 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific). Nucleic acids can absorb UV light at a wavelength of 260 nm (A260), while the

aromatic amino acids, which are present in the proteins, absorb it at 280 nm (A280). In order to determine the purity of the DNA, the ratio between the absorbances at A260 and A280 is calculated. DNA with an A260/A280 ratio of 1.7 - 2.0 and a final concentration > 5 ng/µl was considered as good quality DNA.

4.1. *Chlamydiaceae* detection

A subset of the samples (n = 135) was analyzed at the Swedish Veterinary Institute (Uppsala, Sweden), where a TaqMan real-time PCR specific for members of the family *Chlamydiaceae* was performed. This real-time PCR, based on the method described by Everett and coworkers (1999b); targets the 23S rRNA operon.

The primer set included primers TQF (5'-GAAAAGAACCCTTGTTAAGGGAG-3') and TQR (5'-CTTAACTCCCTGGCTCATCATG-3'), which are specific for all known *Chlamydiaceae*, and a fluorescence-labeled probe (5'-FAM-CAAAAGGCACGCCGTC AAC-BHQ1-3'). If the sequence targeted by the primers is present, the probe anneals with the sequence and the DNA polymerase cleaves off the probe during the extension phase of the qRT-PCR. When this occurs the fluorescent dye is liberated, emitting fluorescence that can be detected by the PCR system. The primer set amplifies a fragment of 132 bp from the DNA template (Everett et al., 1999b). Following an incubation period of 3 min at 94 °C, samples were subjected to 45 cycles of 3 s at 95 °C and 30 s at 60 °C. The use of an Applied Biosystems® 7500 Fast Real Time PCR System (ThermoFisher Scientific) and the PerfeCTa qPCR Toughmix (Quanta Biosciences) made the reduction of times in the PCR cycles with regard to those shown in the original protocol possible. Samples with threshold cycle (C_t) values up to 38 were considered positive while the rest of the samples were considered negative for the presence of *Chlamydiaceae*.

4.2. *Mycoplasma conjunctivae* detection

In order to cultivate *Mycoplasma* spp., specific mycoplasma media are needed due to the requirement of concrete nutrients, such as sterol for cytoplasmic membrane stability (Bannerman and Nicolet, 1971; Friis, 1975). Since the media used in our bacteriological study were more of a general kind and since the cultivation period was set at a maximum of 48 hours, a specific PCR assay based on unique sequences of the *LppS* gene was used for

detection of *Mycoplasma* spp. (Belloy et al., 2003b). This molecular technique allows the direct detection of *M. conjunctivae* in swabs sampled from eyes of domestic and wild ruminants (Belloy et al., 2003b; Giacometti et al., 1999; Vilei et al., 2007).

The primer set specific to the conserved 5′ terminal part of gene *LppS* of *M. conjunctivae* (Belloy et al., 2003b), consisted of the primers LPPS-TM-L (5′- CAGCTGGTGTAGCACTT TTTGC-3′) and LPPS-TM-R (5′- TTAACACCTATGCTCTCGTCTTTGA-3′) (Vilei et al., 2007). Jump Start Red Taq Ready Mix (Sigma) was included as a source of DNA polymerase, dNTPs and $MgCl_2$. DNA purified from *M. conjunctivae* strain HRC/581 (ATCC 25834; NCTC 10147), originally isolated from a sheep with "pink eye" and kindly provided by Dr. Branko Kokotovic (Technichal University of Denmark) was used as positive control, while diethylpryocarbonate (DEPC) water was used as negative control (Kobisch and Friis, 1996).

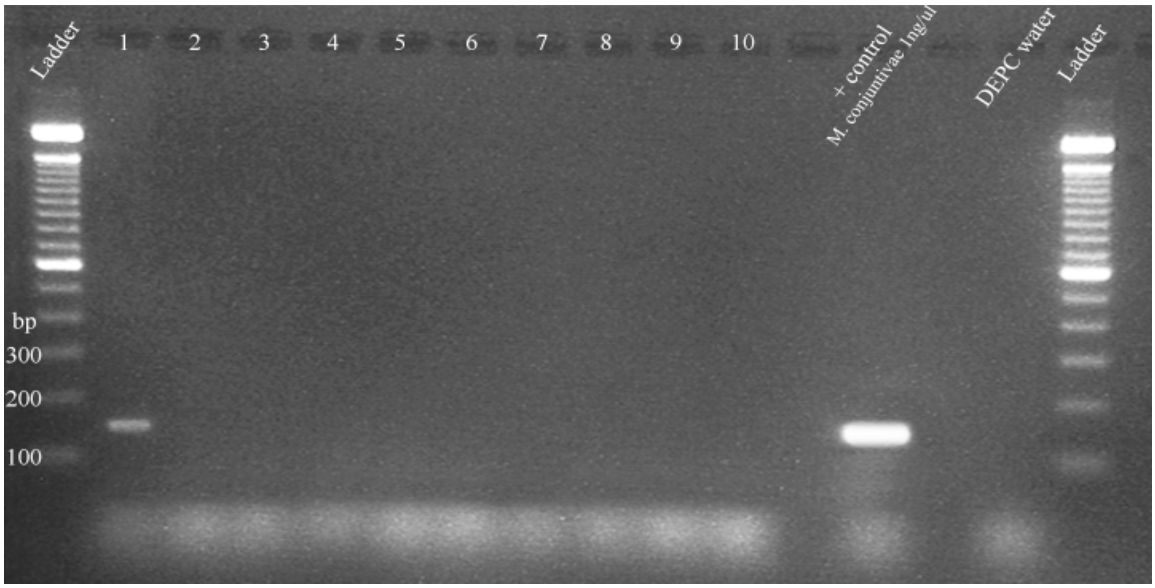

Fig. 8. *LppS* of *M. conjunctivae* PCR amplification from DNA from *M. conjunctivae* strain HRC/581 at a concentration of 1 ng/µl (+ control) and DNA extracted from semidomesticated reindeer eye swabs. 1kbp+ ladder is shown in both sides of the gel. An amplicon of approximately 139 bp was detected in sample 1, while samples 2 - 10 were negative.

The chosen primers amplified a fragment of 139 bp from the DNA template. After 5 min incubation at 95 °C, samples went through 35 cycles of 30 sec at 95 °C, 1 min at 60 °C and 30 sec at 72 °C. To separate the produced amplicons, agarose gel electrophoresis (1 %) was performed for all PCR products and stained with ethidium bromide (Fig. 8). Amplicons of the expected size (139 bp) were purified and sequenced to confirm the presence of *M. conjunctivae.*

4.3. Cervid herpesvirus 2 detection

A nested pan-alphaherpesvirus PCR targeting the UL27 gene coding for gB of CvHV2 was performed as described by Ros and Bèlak for rangiferine herpesvirus 1 (RanHV1) (1999).

The primer pair for the first PCR consisted of the forward primer CR30 (5'-TCGAARGC CGAGTACCTGCG-3') and the reverse primer CR31 (5'-CCAGTCCCAGGCRACCG TCAC-3'). The inner primer set, consisted of the forward primer CR32 (5'-TGGTGGCC TTYGACCGCGAC-3') and the reverse primer CR33 (5'-GCTCCGGCGAGTAGCTGG TGTG-3') (Ros and Belák, 1999). Jump Start Red Taq Ready Mix was used as source of DNA polymerase. Purified CvHV2 diluted 1:100 (strain Salla 82, Finland; Ek-Kommonen et al., 1986) was used as positive control and DEPC water was used as negative control.

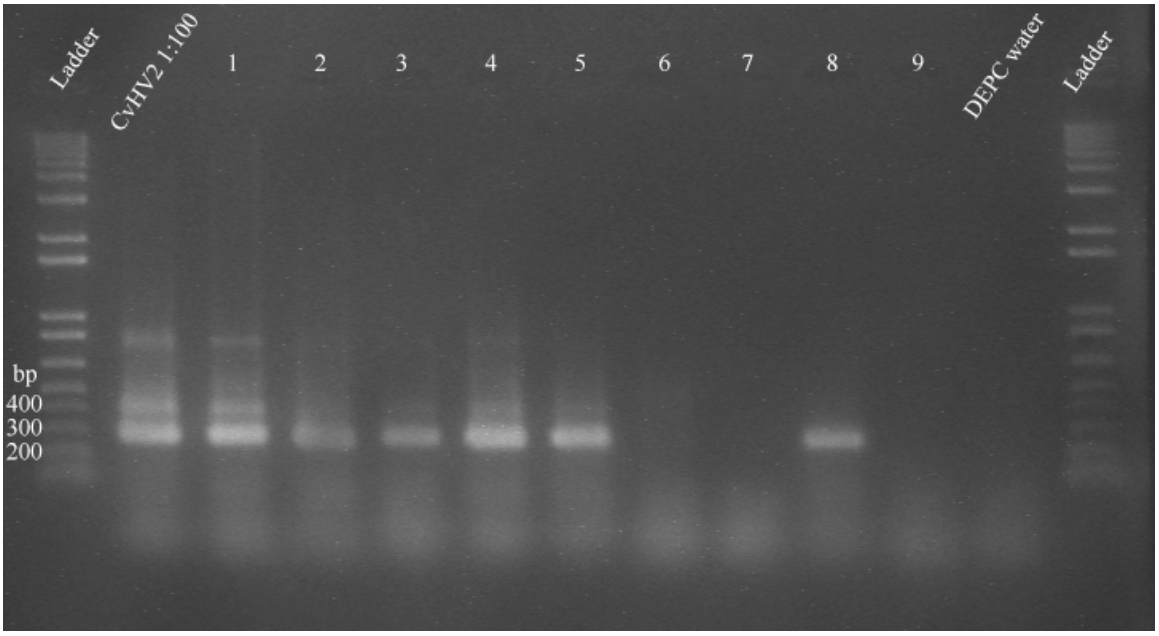

Fig. 9. Glycoprotein B PCR amplification from purified CvHV2 diluted 1:100 and DNA extracted from semidomesticated reindeer eye swabs. 1kbp+ ladder is shown in both sides of the gel. Samples 1 - 5 and 8 show the presence of an amplicon of approximately 294 bp in the tested PCR product, while samples 6, 7 and 9 are negative.

The first primer set amplifies a fragment of 443 bp from the DNA template during 40 cycles (denaturation: 95 ºC for 1 min, annealing: 60 ºC for 1 min, and extension: 72 ºC for 1 min). The second primer set amplifies a fragment of 294 bp from the first PCR product during 35 cycles (denaturation: 95 ºC for 1 min, annealing: 62 ºC for 1 min, and extension: 72 ºC for 1 min). PCR products were separated by 1 % agarose gel electrophoresis and stained with ethidium bromide for visualization on an Gel Doc™ 2000 UV transilluminator (Bio-Rad®,

Hercules, CA, USA) (Fig. 9). Amplicons similar to the expected size (294 bp) were purified and sequenced to compare with the sequences deposited in GenBank (NCBI, USA), a public nucleotide sequences database, in order to confirm the CvHV2 presence.

4.4. Post-processing of the PCR products

Amplified fragments were purified for sequencing using ExoSAP-IT enzymes (USB Corp, Cleveland, USA). Multiple bands were visible in some gels run with PCR products with CvHV2 DNA. In order to only sequence the region of interest, the band corresponding to approximately 294 bp was cut from the gel and cleaned with the MinElute gel extraction kit (Qiagen, Hilden, Germany) following the instructions of the producer.

After purification, a cycle sequencing was conducted in reverse and forward directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Norway). Amplicons from both directions were submitted for purification/precipitation and sequencing at the DNA sequencing core facility laboratory at the University Hospital of North Norway (UNN; Applied Biosystems 3130xl Genetic Analyzers, Warrington, UK). Consensus amplicon sequences were assembled with the Chromas pro software (version 1.7.7, Technelysium Pty Ltd, South Brisbane, QLD, Australia) and blasted in GenBank (NCBI, USA) to compare them with available matching sequences.

5. STATISTICAL ANALYSIS

All statistical analysis was performed using SYSTAT 13 for Windows.

The statistical analysis in this project has been performed using categorical data. In order to investigate whether distributions of this categorical variables differs, the use of a test such as Person's chi-squared (X^2) statistical test is generally recommended. X^2 test can be use to evaluate the grade of relationship between two categorical variables when a normal distribution cannot be assumed, since it is a nonparametric test. This test is suitable for unpaired data from large samples and was defined by Karl Pearson in 1900 (Pearson, 1900). It is necessary to define a null hypothesis (H_0), an alternative hypothesis (H_1), and a significance level (α).

In this test we defined the null hypothesis as equality in the distributions of the different variable groups. If we reject H_0, we accept H_1, and consider that the variables are not equally distributed. With the X^2 test we obtain a p-value. This p-value helps us to quantify the statistical significance of evidence. An easy interpretation of this definition is that the p-value is the probability of observing our sample values if the null hypothesis were true. We defined a significance level of 5 %, so if the p-value is lower than 0.05, the observed data would be inconsistent with the assumption that H_0 is true and our null hypothesis must be rejected.

This test can be trusted as long as we have large sample sizes ($n > 20$). In the case of lower sample size, it is necessary to perform an appropriate exact test such as Fisher's exact test, which is used for the analysis of 2 x 2 contingency tables when sample sizes are small. This test is used to examine the significance of association between the two given classifications. As before, if the p-value is lower than 0.05, we are forced to reject H_0 and consider that the variables are not associated.

RESULTS

Three hundred and forty-four animals were sampled from 15 different regions in Norway, Sweden and Finland (Fig. 4). The material comprised 127 reindeer with clinical symptoms of IKC, 126 non-symptomatic animals and 91 reindeer for which the current status with regards to clinical symptoms was unknown. This information in summarized in table 1 (total number of animals), table 2 (calves) and table 3 (adults), showing the number of animals sampled in different parts of Fennoscandia and the data associated to them.

Table 1 Total number of animals sampled (2010 - 2014) in Norway (white), Sweden (shaded) and Finland (darker shaded), and severity of the clinical signs, classified as 0 for asymptomatic animals, 1 for animals with mild symptoms, 2 for animals moderate symptoms and 3 for animals with severe symptoms (see text for more detailed descriptions).

Location	Animals sampled	0	1	2	3	Not registered
Ifjordfjellet (13)	28	-	2	-	-	26
Sennalandet (22)	23	-	2	-	-	21
Tromsdalen (17)	39	33	6	-	-	-
Sørreisa (20)	35	9	10	6	10	-
Lødingen (34)	40	20	-	-	-	20
Dovre/Rondane *	9	9	-	-	-	-
Karesuando	34	16	7	4	7	-
Lainiouoma Sameby	33	-	21	11	1	-
Kiruna	20	8	12	-	-	-
Kikkejaure	32	2	1	3	2	24
Malå Sameby	5	-	5	-	-	-
Rans Sameby	15	1	14	-	-	-
Muotkatunturi cooperation	10	10	-	-	-	-
Käsivarsi cooperation	11	9	1	-	1	-
Kallioluoma cooperation	10	9	-	1	-	-
	344	126	81	25	21	91

* Samples from hunted wild Norwegian reindeer.

Table 2 Number of calves sampled (<1 year) in Norway, Sweden and Finland, and severity of the clinical signs, classified as 0 for asymptomatic animals, 1 for animals with mild symptoms, 2 for animals moderate symptoms and 3 for animals with severe symptoms (see text for more detailed descriptions).

Location	Animals sampled	0	1	2	3	Not registered
Ifjordfjellet (13)	9	-	1	-	-	8
Sennalandet (22)	6	-	-	-	-	6
Tromsdalen (17)	30	30	-	-	-	-
Sørreisa (20)	26	5	8	4	9	-
Dovre/Rondane *	2	2	-	-	-	-
Karesuando	34	16	7	4	7	-
Lainiouoma Sameby	27	-	20	6	1	-
Kiruna	20	8	12	-	-	-
Malå Sameby	2	-	2	-	-	-
Rans Sameby	10	1	9	-	-	-
Muotkatunturi cooperation	10	10	-	-	-	-
Käsivarsi cooperation	11	9	1	-	1	-
Kallioluoma cooperation	10	9	-	1	-	-
	197	90	60	15	18	14

* Samples from hunted wild Norwegian reindeer.

Table 3. Number of adults sampled (>1 year) in Norway and Sweden*, and severity of the clinical signs, classified as 0 for asymptomatic animals, 1 for animals with mild symptoms, 2 for animals moderate symptoms and 3 for animals with severe symptoms (see text for more detailed descriptions).

Location	Animals sampled	0	1	2	3	Not registered
Ifjordfjellet (13)	12	-	1	-	-	11
Sennalandet (22)	12	-	-	-	-	12
Tromsdalen (17)	9	3	6	-	-	-
Sørreisa (20)	9	4	2	2	1	-
Lødingen (34)	20	20	-	-	-	-
Dovre/Rondane **	7	7	-	-	-	-
Lainiouoma Sameby	6	-	1	5	-	-
Malå Sameby	3	-	3	-	-	-
Rans Sameby	4	-	4	-	-	-
	82	34	17	7	1	23

* No adult reindeer were sampled in Finland.
** Samples from hunted wild Norwegian reindeer.

1. CULTIVATION OF BACTERIA

Two hundred and eleven animals were sampled for bacteriology. Samples obtained from 51 of the animals with clinical symptoms of IKC and 93 of the animal without clinical symptoms of IKC were cultivated (54.3 and 73.8 % respectively). Bacteria of different species were isolated from the conjunctiva of 158 reindeer, whereas no bacteria were detected in the samples from the remaining 53 animals. All bacteriological findings are presented in Table 4.

Moraxella sp. was isolated from eleven IKC-affected animals (21.6 %) and from five healthy animals (3.4 %) during two different outbreaks of IKC in Sørreisa (Norway) and Karesuando (Sweden). Seven of those isolates were identified at species level as *Moraxella bovoculi*. However, no significant differences between the presence of *Moraxella* sp. in reindeer with IKC and reindeer without IKC were present in the outbreaks ($p = 0.148$).

Possible pathogenic bacteria such as *A. hydrophila*, *E. coli*, *Pseudomonas* sp. or *Staphylococcus* sp. were also isolated from the eyes of reindeer with clinical symptoms of IKC, but statistical association between the presence of any of these bacteria and clinical symptoms of IKC could not be established (Fig. 10).

A. baumanii was isolated from nine healthy animals (9.7 %); in addition *Acinetobacter iwoffi* and an unidentified *Acinetobacter* species were isolated in three other asymptomatic reindeer (3.2 %). *A. iwoffi* was also isolated from the eyes of two animals with mild symptoms of keratoconjunctivitis (3.9 %). For these bacteria, there was no significant association between their presence and the clinical symptoms of IKC ($p = 0.801$).

A great variety of other species of bacteria were isolated from the eyes of non-symptomatic semi-domestic reindeer, as shown in Figure 11. More than one type of microorganisms dominated in the agar plates with samples from four asymptomatic animals (*Enterobacter cloacae* and *Pseudomonas putrida* / *Cryseobacterium indologenes* and *Staphylococcus scuri* / *K. pneumoniae* and *Yersinia kristensenii* / *E. brevis* and *Weeksella virosa*). In 54 cases the results were not conclusive due to poor bacterial growth or the absence of a dominant species in the plates.

Table 4 Dominating species of bacteria in agar plates cultivated from conjunctival swabs of semi-domesticated reindeer in Norway, Sweden and Finland (2010 – 2014).

Location	Total	No IKC Symptoms	IKC Symptoms	Not registered
Acinetobacter sp.	12	1	-	11
A. baumanii	9	6	1	2
A. iwofii	3	2	1	-
Aeromonas hydrophila	11	2	4	5
Brevudimonas diminuta	1	1	-	-
Citribacter sp.	1	-	-	1
Cryseobacterium indologenes	2	2	-	-
Empedobacter brevis	1	1	-	-
Enhydrobacter sp.	3	3	-	-
Enterobacter sp.	-	-	-	-
Enterobacter aerogenes	1	1	-	-
Enterobacter cloacae	2	2	-	-
Enterobacter salazakii	1	1	-	-
Escerichia coli	10	1	-	9
Klebsiella sp.	1	-	-	1
Klebsiella pneumoniae	2	2	-	-
Macrococcus sp.	1	-	-	1
Micrococcus lylae	8	-	-	8
Mobilicoccus sp.	1	1	-	-
Moraxella sp.	9	3	6	-
M. bovoculi	7	2	5	-
Myroides sp.	3	-	-	3
Pantoea sp.	3	1	-	2
Pseudomonas sp.	1	-	1	-
P. fluorescens	1	1	-	-
P. putrida	1	1	-	-
P. strutzeri	1	-	-	1
Psychrobacter sp.	1	-	1	-
Raistonia picketti	1	1	-	-
Rathayibacter rathayi	1	1	-	-
Shewanella putrefaciens group	1	-	-	1
Staphylococcus sp.	3	2	-	1
S. aureus	1	-	1	-
S. scuri	1	1	-	-
Serratia liquefaciens	1	1	-	-
Weeksella virosa	1	1	-	-
Yersinia sp.	1	-	-	1
Y. kristensenii	1	1	-	-
Non-identified bacteria	54	23	15	16
No bacterial growth	53	35	16	2

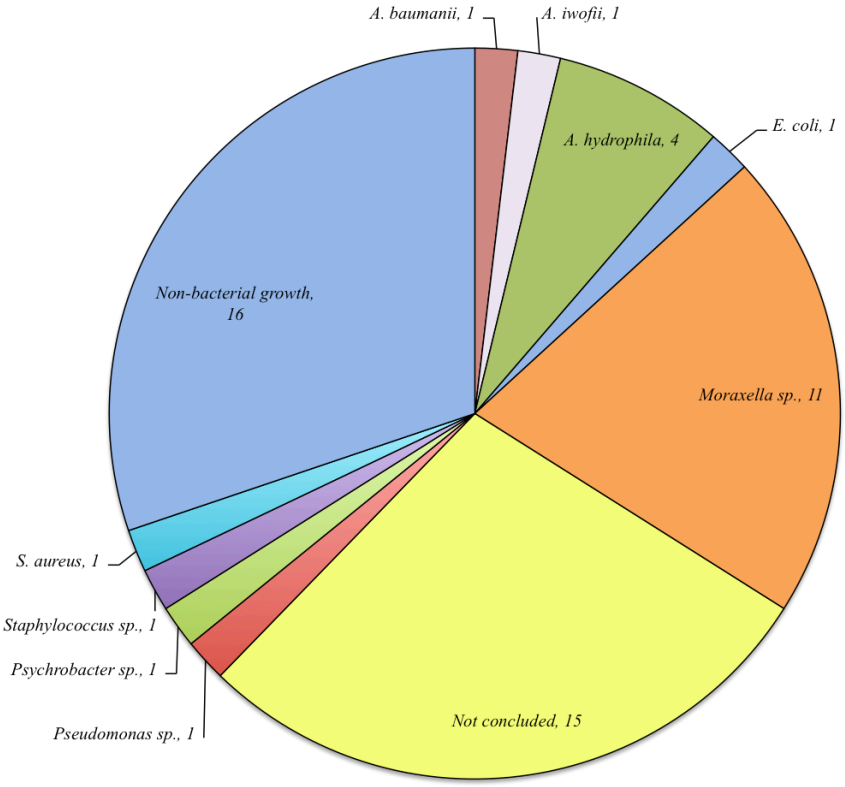

Fig. 10. Bacterial species that dominanted the culture plates inoculated with swab samples from semidomesticated reindeer (*Rangifer tarandus tarandus*) with clinical symptoms of infectious keratoconjunctivitis (IKC), including the number of swabs animals from which the bacteria were isolated.

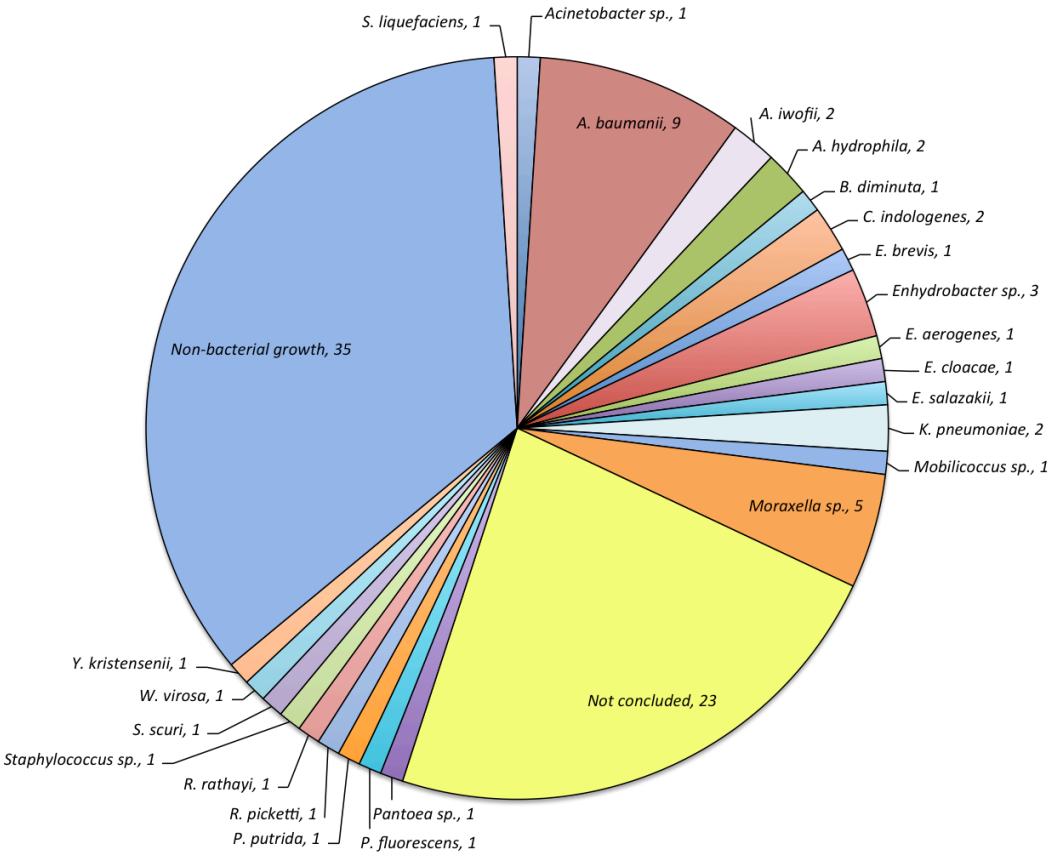

Fig. 11. Bacterial species that dominanted the culture plates inoculated with swab samples from semidomesticated reindeer (*Rangifer tarandus tarandus*) without clinical symptoms of infectious keratoconjunctivitis (IKC), including the number of animals from which the bacteria were isolated.

2. DETECTION OF ANTIBODIES

As it is shown in Fig. 12, the seroprevalence, i.e. the number of animals with antibodies against CvHV2 over the total number of animals tested, differed between calves and adults. For calves, the number of seropositive animals increased relatively to the number of cases when the clinical symptoms of IKC increased, but remained at a relatively low percentage (15.7 - 57.1 %) (Fig. 12 - Left).

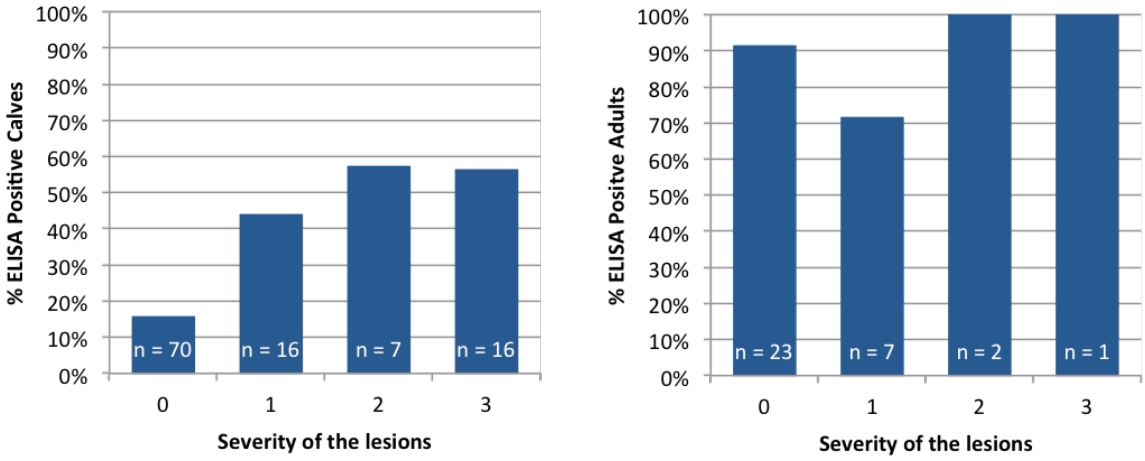

Fig. 12. The percentage of seropositive reindeer calves and adults in the ELISA test for antibodies against CvHV2 (n = number of seropositive animals).

For adults, the ratio between animals with and without antibodies against CvHV2 remained high (71.4 - 100 %) regardless of the clinical score (Fig. 12 - Right). The seroprevalence was much higher in adults (87.9 %) than in calves (28.4 %).

Figures 13 and 14 show the differences in the presence of antibodies between calves and adults, with more seronegative samples in calves than in adults, independently of the presence or not of clinical symptoms of IKC and the severity of those symptoms.

There was a statistical significant association between the presence of clinical symptoms of IKC and the presence of antibodies against CvHV2 in calves ($p = 0.001$). Moreover, this association was also present between the presence of antibodies and the increase of the score of clinical symptoms ($p = 0.002$). This association between the worsening of the clinical symptoms and the presence of antibodies against CvHV2 did not exist for adults (Fisher's exact test, $p = 0.567$), or for the whole population, i.e. adults and calves together ($p = 0.090$).

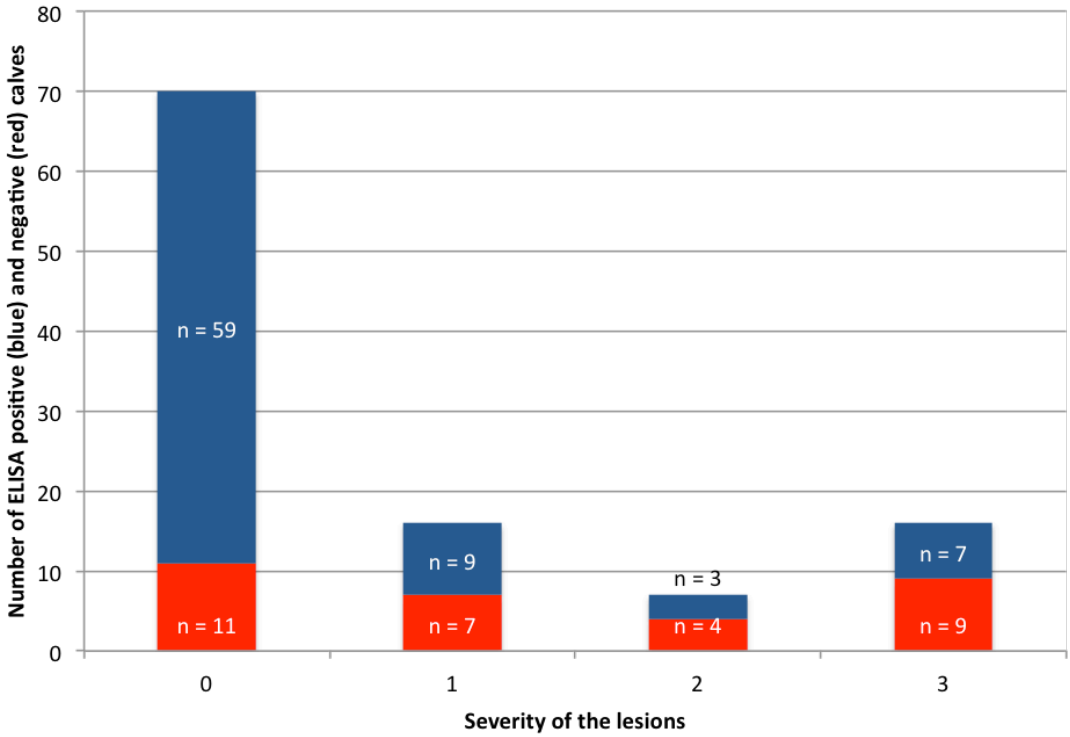

Fig. 13. The figure displays the results of the ELISA test for antibodies against CvHV2 in reindeer calves without clinical symptoms of IKC (0) and with increasing severity of clinical symptoms (1-3) (n = number of animals). ELISA positive reindeer calves are represented in blue and ELISA negative reindeer calves are represented in red.

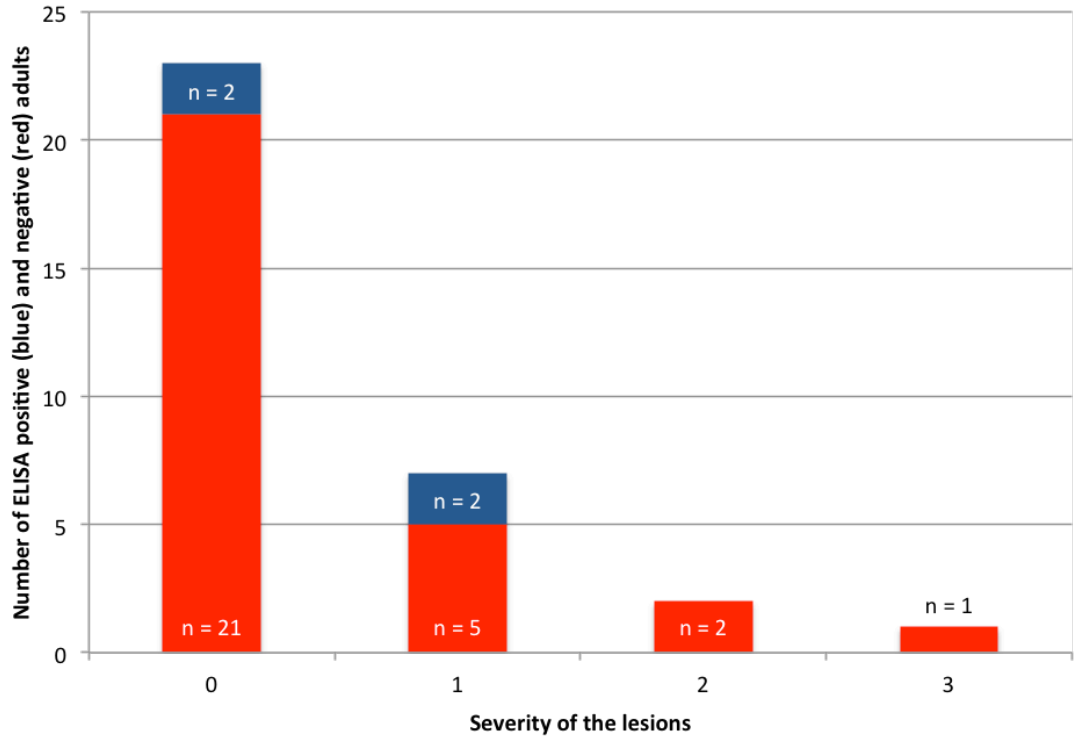

Fig. 14. The figure displays the results of the ELISA test for antibodies against CvHV2 in adult reindeer without clinical symptoms of IKC (0) and with increasing severity of clinical symptoms (1-3) (n = number of animals). ELISA positive adult reindeer are represented in blue and ELISA negative adult reindeer are represented in red.

3. POLYMERASE CHAIN REACTION ANALYSIS

3.1. *Chlamydiacea* detection

One hundred and thirty-five samples were analysed for the presence of *Chlamydiacea* bacteria. DNA from these bacteria was isolated from sixteen different animals (11.8 %) and was associated with clinical symptoms of IKC in half of those cases (15.1 %).

No statistical association could be established ($p = 0.873$) between the presence of clinical symptoms of IKC and the detection of bacteria from the family *Chlamydiacea*.

Eight of the positive animals were detected in Karesuando (Sweden) during an outbreak of IKC in 2011. Among these eight animals, there were no significant differences between the presence of *Chlamydiacea* in healthy and sick (IKC) animals ($p = 0.550$) and the proportion of positive samples was higher among animals with no clinical symptoms of IKC. More specifically, 50.0 % of the apparently healthy reindeer sampled during this outbreak were positive for *Chlamydiacea*, while only 35.0 % of the animals with clinical symptoms of IKC presented DNA from these bacteria in the eyes.

3.2. *Mycoplasma conjunctivae* detection

A subset of one hundred and ninety-seven samples were analysed for the presence of *M. conjunctivae*. The prevalence of *M. conjunctiva* among those samples was 1.0 %, with only two positive samples for the presence of this bacterium. The presence of *M. conjunctivae* was associated with clinical symptoms of IKC in one case (1.5 %).

The two positive animals were detected in Tønsvika (Troms County, Norway) in 2010. No link between the presence of clinical symptoms and *M. conjunctivae* could be established ($p = 0.992$).

3.3. Cervid herpesvirus 2 detection

The PCR results for the presence of CvHV2 in eye swab samples showed an increase in the number of positive cases compared with the negatives when the IKC clinical score increases for both calves and adults (Fig. 15).

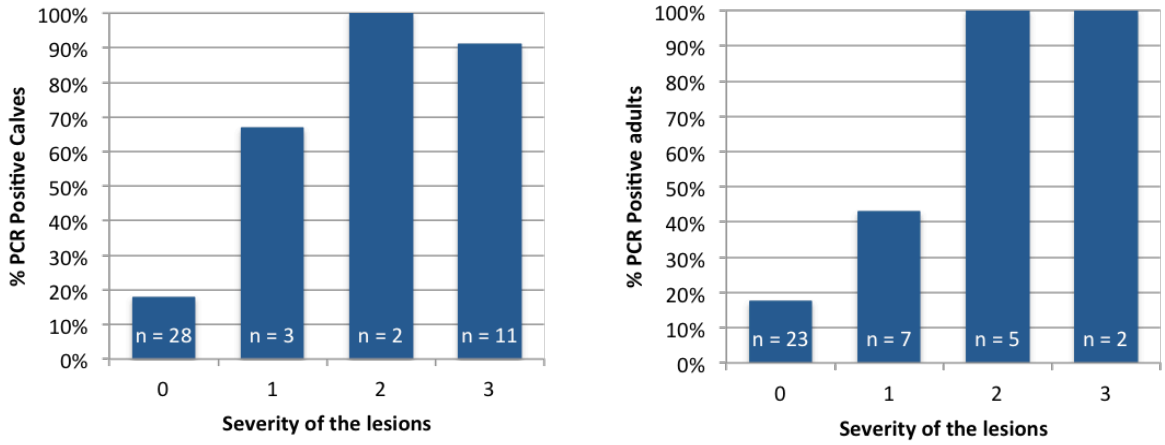

Fig. 15. The percentage of CvHV2-PCR positive reindeer calves and adults (n = number of positive animals) without clinical symptoms of IKC (0) and with increasing severity of clinical symptoms (1-3).

Less than 20.0 % of the asymptomatic animals presented CvHV2 DNA in their eye swabs. The percentage of animals with CvHV2 DNA in their eyes increased with the severity of the clinical symptoms of IKC. The only exception was the group of calves with the most severe symptoms. The percentage decreases from 100.0 % in the group of calves with moderate symptomatology (clinical score 2), to 90.9 % among the most affected animals (clinical score 3).

Figures 16 and 17 display a different graphical representation of these results, where it is aditionally shown the different amount of samples analysed from asymptomatic animals and animals included in any of the three categories of IKC clinical symptoms.

There was an association between the presence of symptoms (IKC) and the presence of viral DNA in the eyes of the animals. This association was valid both for the total number of animals independently of the age ($p < 0.001$) and for the calves ($p < 0.001$) and the adults ($p = 0.015$) as separate groups.

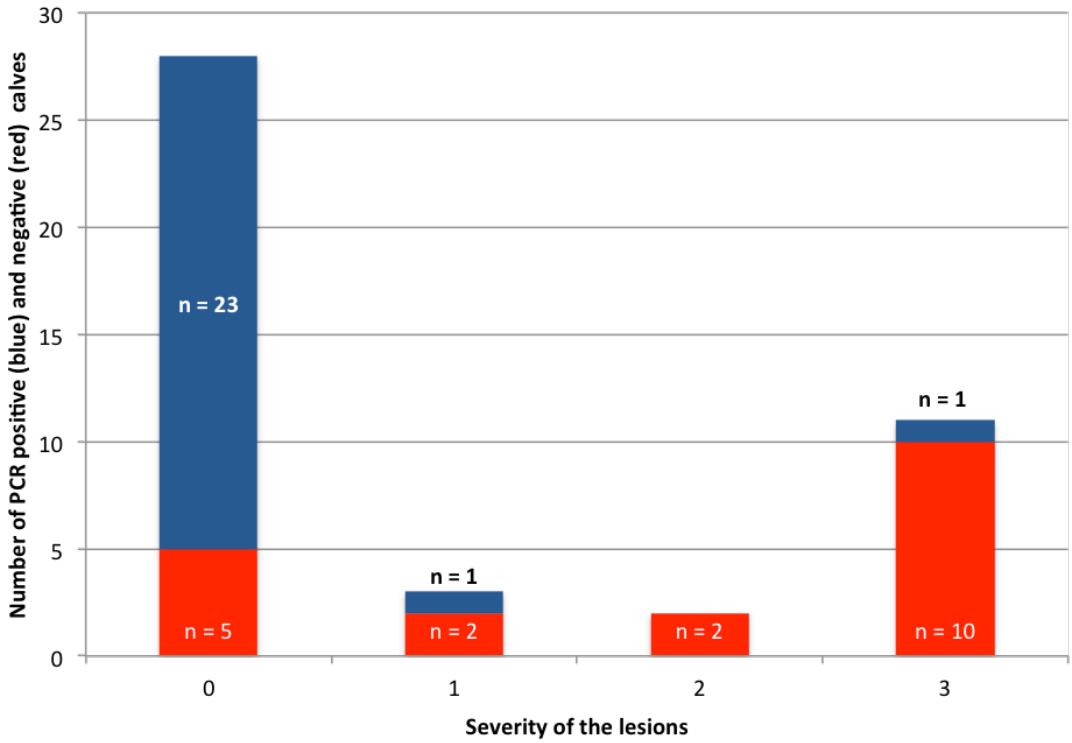

Fig. 16. The figure displays the results of the nested PCR for CvHV2 in reindeer calves eye swabs without clinical symptoms of IKC (0) and with increasing severity of clinical symptoms (1-3) (n = number of animals). CvHV2-PCR positive reindeer calves are represented in blue and CvHV2-PCR negative reindeer calves are represented in red.

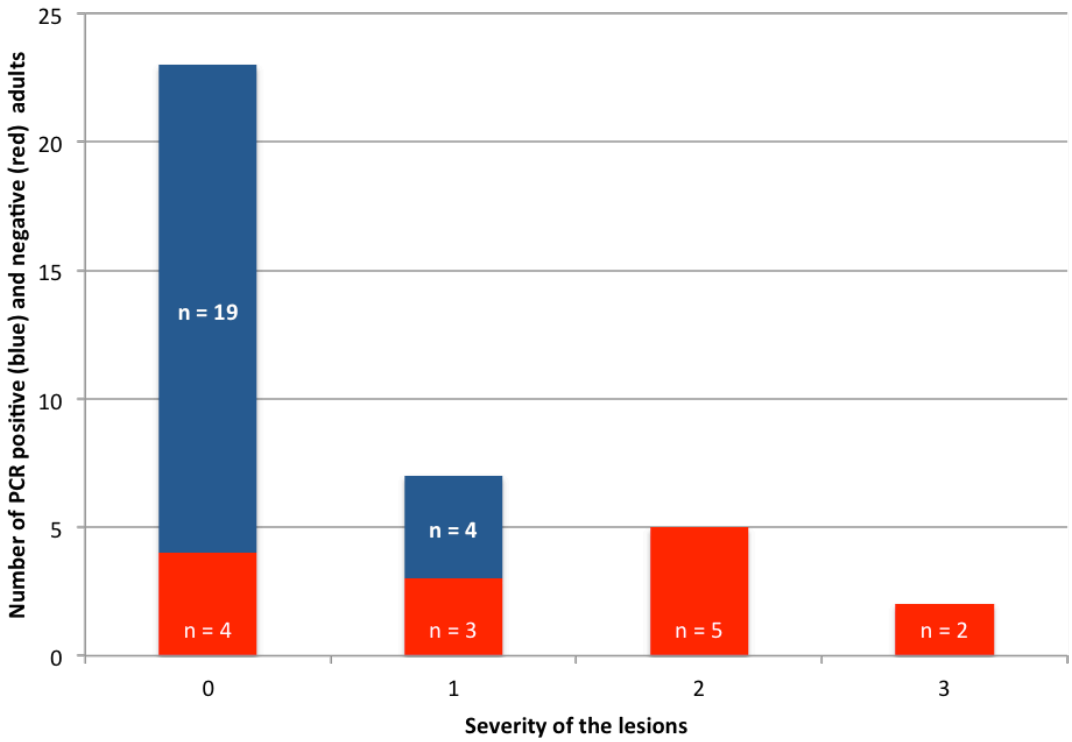

Fig. 17. The figure displays the results of the nested PCR for CvHV2 in adult reindeer eye swabs without clinical symptoms of IKC (0) and with increasing severity of clinical symptoms (1-3) (n = number of animals). CvHV2-PCR positive adult reindeer are represented in blue and CvHV2-PCR negative adult reindeer are represented in red.

DISCUSSION

Three hundred and thirty-five semi-domesticated and nine wild Eurasian tundra reindeer were sampled in Norway, Sweden and Finland between 2010 and 2014, prioritizing animals with clinical symptoms of IKC.

In this set of sampled reindeer, which not necessarily represent the whole reindeer population of Fennoscandia, IKC cannot be considered an age dependent disease, since there are no significant differences between the number of affected calves and adults ($p = 0.259$), although Tryland et al. (2009) reported that young animals are more affected than adults in natural outbreaks of IKC. A possible reason for this discrepancy is the selective sampling performed for this study, prioritizing sampling of animals with clinical symptoms of IKC, including several disease outbreaks.

1. CULTIVATION OF BACTERIA

Bacteria belonging to the genus *Moraxella* were isolated from the eyes of eleven animals with clinical symptoms of IKC. Seven of the isolates were analysed at species level and *M. bovoculi* was the identified bacteria. *M. bovoculi* has been associated as the causative agent of IBK in cattle (Angelos et al., 2007b; Galvão and Angelos, 2010).

A statistical association did not exist between the presence of *Moraxella* spp. and the clinical symptoms of IKC in reindeer ($p = 0.461$), but there still, might be a possible biological significance of this bacterium in the development of IKC, since almost 94 % of those bacteria were isolated during the IKC outbreaks in Sørreisa (Norway) and Karesuando (Sweden). If these two populations were studied independently of the rest, the proportion of animals with clinical symptoms of IKC in association with the presence of *Moraxella* sp. in the infected eye was 37.5 % during the outbreak in Sørreisa and 50 % in Karesuando, supporting the hypothesis of a biological significance of *Moraxella* spp. in the development of IKC in reindeer, at least for these two disease outbreaks. Since IKC is considered a multifactorial disease, the absence of this bacterium in any of the other IKC cases could be due to other pathogens being the causative agent.

A great variety of bacteria were isolated from the eyes of healthy animals. These bacteria can be divided in two different groups. 1) Bacterial species that can be considered as environmental or saprophytic bacteria. In this category we can include *Acinetobacter* spp. (*A. baumanii* and *A. iwofii*), *B. diminuta*, *C. indologenes*, *E. brevis*, *Enterobacter* spp. (*E. aerogenes*, *E. cloacae* and *E. salazakii*), *Mobilicoccus* spp., *Pantoea* spp., *R. picketti*, *R. rathayi*, *Staphylococcus* spp. and *W. virosa* (Crawford and Hurst, 2007; Willey et al., 2013). Even if under normal circumstances all these bacteria are non-pathogenic, some of them, such as *Acinetobacter* spp. or *Enterobacter* spp., may show pathogenic characteristics under specific conditions (Quinn et al., 2011; Willey et al., 2013). 2) Potentially pathogenic bacteria such as *Pseudomonas* spp., *Klebsiella* spp., *Staphylococcus* spp. and *Yersinia* spp. (Garrity and Bergey, 2001; Quinn et al., 2011). However, since these species of bacteria were isolated from animals without any clinical symptoms of IKC, we cannot assume that they play an important role in the development of IKC in reindeer, probably requiring some previous damage of the mucosal membrane in order to be able to settle and start a secondary infection, as it occurs with the cytopathic effect (CPE) produce by CvHV2 during its lytic cycle. A depression of the cell mediated immunity due to virus-induced lymphocytoslyis after infection with BoHV1 has been described (Griebel et al., 1990), and it is likely to think that CvHV2 can produce a similar effect on the reindeer's immune system. This immune system depression could also favour secondary bacterial infections.

No significant association could be identified between the presence of any of the other isolated bacteria and the presence or not of clinical symptoms of IKC, which may suggest that none of these bacteria have a significant importance for the development of IKC in reindeer, and may also hint that most of the isolated bacteria may come from contamination of the eyes prior to, or during the sampling, since none of the sampling procedures were performed under sterile conditions, in which the working environment and gear are free of all biological material, and therefore contamination of the eyes or the samples with environmental bacteria may occur. This theory is also supported by the growth of a mix of bacterial species in the agar plates cultivated from 15 animals with and 23 without clinical symptoms of IKC, in which it was impossible to isolate any bacteria in pure culture, and thus remaining as not identified species.

This possible bacterial contamination of the reindeer eyes during gathering, corralling or handling of the animals may, anyway, contribute to the conversion of mild stages of the

disease into more severe affections, as suggested by Egwu and coworkers for *Staphylococcus aureus* and IKC in sheep (1989), and the possible role of different species of bacteria as opportunistic pathogens, such as e.g. *E. coli*, *L. monocytogenes*, *Staphylococcus* spp. and *Streptococcus* spp., should not be discarded.

The possible role of bacterial infection as a trigger for the reactivation of CvHV2 can likewise not be discarded. Bovine Herpesvirus 4 (BoHV4) can enter a lytic replication cycle from latency after endometrial infection with *E. coli*, causing uterine disease in cattle (Donofrio et al., 2008). However, BoHV4 belongs to the subfamily *Gammaherpesvirinae*, and whether or not this finding can be applied to CvHV2 or any other ruminant alphaherpesviruses remains unclear and should be studied in further detail.

Thirty-three samples were excluded from the bacteriological study due to suboptimal temperature conditions during the transport to the laboratory. These samples froze and thawed, and the transport medium liquefied. In 30 of these samples, *A. hydrophila* was isolated, but this finding cannot be considered relevant for this study, since this bacterium is known to be present in several liquid environments and can grow more easily than other bacterial species in aquatic mediums (Crawford and Hurst, 2007). There are many sources of contamination in our sample sites in corrals and slaughterhouses (Gill, 2007; Vaarala and Korkeala, 1994; Vaarala and Korkeala, 1999) and *A. hydrophila* can be considered a contaminant in this subset of samples.

2. DETECTION OF ANTIBODIES

Regarding the presence of antibodies against CvHV2, the highet seroprevalence in adults as compared to calves was expected, since alphaherpesviruses produce lifelong infections (Smits et al., 2013). Once the animal is infected, it will carry the virus for its whole life. CvHV2 antibodies can appear as soon as 7 - 10 days after respiratory or vaginal infection (das Neves et al., 2009b). We do not know the longevity of antibodies against CvHV2 in reindeer, but in cattle, it is possible to detect antibodies against BoHV1 up to three years after the animals have been infected (Hage et al., 1998). In summary, older animals have had more chances to be exposed to the virus than younger animals and hence they are more likely to have developed antibodies against CvHV2, and the prevalence of seropositives accumulate

throughout the age classes. CvHV2 can also be reactivated from latency in previously infected animals, with a subsequent immune response and production of new antibodies.

This is also in line with the statistical analysis, that indicated an association between the presence of antibodies and an increasing severity of the clinical symptoms of IKC. While this association was present in calves, which seemed to be suffering from more acute forms of the disease as compared to older animals, this association was not present in adults, which can be latently infected and may have been exposed in the past; without and active infection or presence of clinical symptoms at the time of sampling.

Another interesting finding was the increase of seropositive animals, together with an increase of the severity of the clinical symptoms of IKC. This may suggest that previously unexposed animals seroconvert during the development of the disease, producing antibodies against CvHV2 in later stages, which will contribute to the remission of the disease and the possible recovery of the animals.

3. POLYMERASE CHAIN REACTION ANALYSIS

3.1 *Chlamydiacea* detection

Chlamydiacea DNA was present in 11.8 % of the subset of samples analysed for the presence of these bacteria. The lack of association between their presence in the reindeer eyes and the presence of clinical symptoms of IKC may suggest that these bacteria are not relevant in the establishment of IKC in reindeer. However, as for most of the other identified bacteria in this study, the contribution of members of the family *Chlamydiacea* in later stages of IKC as opportunistic pathogen cannot be discarded, since *C. abortus* and *C. pecorum* are involved in the development of IKC in domesticated and wild small ruminants such as sheep, goat or bighorn sheep. A possible role of two others species, *C. suis* and *C. psittaci*, in the development of IKC in sheep has also been suggested (Meagher et al., 1992; Osman et al., 2013; Polkinghorne et al., 2009; Rodolakis and Laroucau, 2015).

3.2 *Mycoplasma conjunctivae* detection

The lack of association between the presence of clinical symptoms of IKC and *M. conjunctivae* in the present study was even more remarkable. The bacterium was present in only 1 % of the tested animals. *M. conjunctivae* has been previously detected in sheep and reindeer affected by IKC in Norway (Åkerstedt and Hofshagen, 2004; Kummeneje, 1976), and it is known to be the primary agent of IKC in sheep (Egwu et al., 1989), goat (Trotter et al., 1977) and wild *Caprinae*, such as chamois, alpine ibex or bighorn sheep (Jansen et al., 2006; Mavrot et al., 2012; Ryser-Degiorgis et al., 2009). However, in the present study, no relevance of this microorganism in the establishment or development of the IKC in reindeer was identified.

3.3 Cervid herpesvirus 2 detection

The detection of CvHV2 DNA in eye swabs by PCR, and the fact that the increase of positive samples was directly related to the severity of the clinical symptoms of IKC can be interpreted as a possible role of CvHV2 as a primary agent in the development of IKC in reindeer.

CvHV2 DNA was amplified by PCR from the eyes of apparently healthy reindeer. A reason for this could be that, during the sampling period, the animals were in the incubation period of the disease, the initial stage of an active infection without any clinical symptoms. Another possibility is that the virus is active but the reindeer is suffering a subclinical infection, without any clinical symptoms of IKC, but with replication of the virus in its eyes. Contrariwise, it was not possible to isolate CvHV2 DNA from some animals with symptoms of IKC. PCR sensitivity might not have been sufficient to detect the presence of the virus. In a natural outbreak, it was not possible to detect the presence of CvHV2 by PCR from the animals with more severe symptoms (Tryland et al., 2009), and it was hypothesized that the peak of viral excretion was produced much earlier than the most severe symptoms of IKC in reindeer. It is possible that in the final stages of IKC, the virus is entering in the latency stage, shutting down the active replication cycle and having little or no replication of viral particles.

The statistical analysis of our data supports these observations. There is a strong association between the presence of CvHV2 DNA in the eye of the animals and the presence of clinical

symptoms ($p < 0.001$). These results indicate that during an acute infection, with active replication of the virus in the mucosa of the eyes, it is possible to find clinical symptoms characteristic of IKC.

4. FUTURE PERSPECTIVES

Even if for many years Koch's guidelines have been used for determining the causative agent(s) of a disease, but for multi-factorial and viral diseases, those postulates can be considered difficult to accomplish. New postulates, based on updated molecular techniques, have been proposed and should be taken into account (Evans, 1976; Fredericks and Relman, 1996). Some of the criteria suggested by Fredericks and Relman (1996) for the establishment of microbial disease were that the detection of the specific DNA of a pathogen should be possible in most cases of the infectious disease, with a decrease of the number of copies or even no detection of DNA of the microorganism in animals recovered from the disease or without the disease. On the other hand, the number of copies should increase along with the severity of the clinical symptoms. In order to address these issues further, an experimental infection should be performed, new molecular identification techniques, such as a qRT-PCR for CvHV2, should be established for the quantification of viral particles in the samples, and the role of different pathogens as possible causative agents for IKC should be evaluated.

The development of alternatives to animal experiments, such as theoretical models or *in vitro* experiments, is helping to decrease the total amount of the animals involved, but so far, it is still no possible to completely leave aside the animal models in science. Determining the primary agent of the disease in the natural environment is difficult due to uncontrolled factors. On these occasions, the amount of samples available, time to work with the animals and in some cases the access to information about the herd, individual animals or previous history, are limited, and the primary objective is usually to stop the progression of the disease rather than to study its development. Contrariwise, in an experimental setting, times of inoculation and sampling, clinical history and animal condition can be controlled. Sterile conditions cannot be reached in a field experimental setting, but IKC is a complex disease and the sterility provided by a laboratory setting, such as cell culture infection, would not allow us to fully understand how IKC develops. And also not which cell types are infected, the amount of viral particles being shed or what is the specific immune response of the reindeer against CvHV2 at different clinical stages of the disease.

Nevertheless, before opting for an experimental infection, pros and cons have to be taken into consideration. On one side of the scale lies the possibility to improve the knowledge about the pathogenesis of IKC in reindeer, to determine the causative agent and possibly to find a suitable treatment for the disease. While on the other side lies the possible suffering of the animals and also a possible insignificant outcome from the experiment, which would imply a null benefit with a high cost for the animals used in the experiment. Considering the possible outcomes, the cost-benefit ratio will leave us with a presumable long-term benefit for the species and the society, which justifies the individual costs for the experimental animals.

Therefore, further studies should be also carried out in order to understand the infection biology of these microorganisms when IKC develops under natural (live or slaughtered animals) and experimental conditions.

If it can be shown that CvHV2 is the main agent in IKC in reindeer, antiviral treatments used in other animal species, such as idoxuridine and ganciclovir against feline herpesvirus type 1 (FHV1) in cats (Maggs and Clarke, 2004), or cidofovir against CpHV1 in goats (Tempesta et al., 2007), could be tested in order to find a suitable treatment for reindeer. Different vaccines have also been developed against *Moraxella* sp. in cattle (Angelos, 2010; Angelos et al., 2012; Angelos et al., 2014) and against other virus of the genus *Varicellovirus* (Khattar et al., 2010; Marinaro et al., 2012; Romera et al., 2014).

If the primary agent(s) are identified during the experimental infection between CvHV2 and *M. bovoculi*, which are the most promising candidates in this study, the treatment options and our capacity for the prevention of this disease will increase, alongside with animal welfare and reindeer production. On one hand, the cost of possible preventive measures or treatments can be seen as an impediment in the fight to control this disease, and the use of additional human resources to apply these measures can also be problematic, since it can be considered as a waste of time and money from the reindeer herders point of view. On the other hand, nowadays most of the reindeer herders use antiparasitic treatments on a regular basis and a lot of time and effort has been invested in symptomatic treatments during IKC outbreaks, with the hope of recovering the animals. So if it is possible to develop effective vaccines or affordable and specific treatments against the causative agent of IKC, they will certainly be welcomed by a majority of the herders.

CONCLUSIONS

IKC can be defined as a syndrome for a severe transmissible eye infection and affects many different ruminants, but the causative agent(s) can be different depending on the species.

Among the microorganism identified in this study, CvHV2 is the most plausible candidate as the causative agent of IKC in semi-domesticated reindeer.

The isolation of *M. bovoculi* in two different outbreaks of IKC makes this bacterium the second best candidate as possible primary agent of this disease in reindeer.

We can also assume that some of the other bacteria identified in the eyes of apparently healthy and sick (IKC) reindeer, such as *Enterobacter* spp., *E. coli*, *K. pneumonia* *Pseudomonas* sp. or *Staphylococcus* spp. may play a role in the later stages of IKC as opportunistic pathogens, taking advantage of the mucosal lesions produced by other pathogens and the weakened immune system of the sick animals and thereby worsening the clinical picture.

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APPENDIX 1: LSIVet^{TM} Bovine IBR gB Serum ELISA kit protocol

BoHV1 blocking ELISA kit (IBRG/10, LSI, Lissieu, France)

1. Dilute 100 µl of the serum samples, and positive and negative controls with 400 µl of the Reagent B (Dilution Buffer).
2. Add 100 µl of the dilutions into the wells of a 96 well tray in duplicates. Negative control should be placed in wells A1-B1, and positive control should be placed in wells A2-B2.
3. Mix by agitating the plate and cover with a plate adhesive.
4. Incubate at 37 ºC for 2 h.
5. Wash 5 times with a dilution 1:10 of the Reagent A (Wash Solution) in the automatic washing machine.
6. Add 100 µl of the Reagent C (Substrate Solution) and mix by agitating the plate.
7. Incubate for 10 min at room temperature in darkness.
8. Add 100 µl of Reagent D (Stop Solution) and mix by agitating the plate.
9. Read the plate at 450-620 nm.

APPENDIX 2: DNA extraction protocol

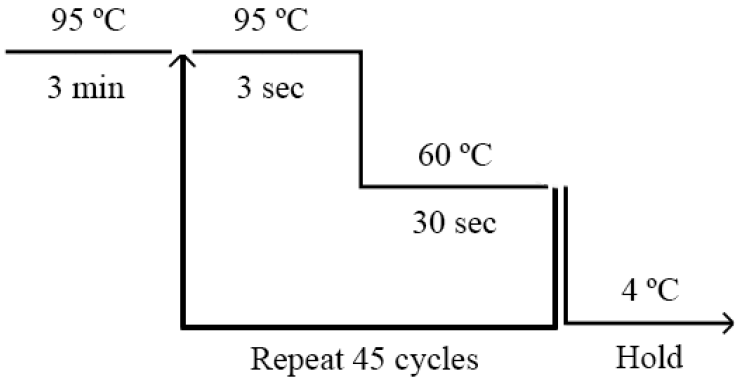
QIAamp DNA MiniKit (Qiagen, Hilden, Germany)

1. Add 200 µl of the sample (swab medium) into a clean Eppendorf tube.
2. Add 20 µl of Proteinase K and mix by vortexing for 15 sec.
3. Add 200 µl of AL Buffer (Lysis Buffer) and mix by vortexing for 15 sec.
4. Incubate at 56 ºC for 2 h.
5. Incubate at 70 ºC for 10 min.
6. Add 200 µl of ethanol and mix by inverting the tube. Centrifuge to remove drops from the lid.
7. Transfer the mix into a QIAamp Mini Spin column and centrifuge at 8.000 rpm for 1 min.
8. Change collection tube.
9. Add 500 µl of AW1 (Wash Buffer) and centrifuge at 8.000 rpm for 1 min.
10. Repeat 8.
11. Add 500 µl of AW2 (Wash Buffer) and centrifuge at 8.000 rpm for 1 min.
12. Repeat 8. And centrifuge at 13.000 rpm for 1 min.
13. Place the QIAamp Mini Spin column into a clean 1.5 ml microcentrifuge tube. To elute DNA, add 30 µl of Buffer AE (Elution Buffer) or water to the center of the membrane, let the column stand for 1 min, and then centrifuge at 8.000 rpm for 1 min.
14. Measure the DNA concentration.

APPENDIX 3: *Chlamydiadiacea* qRT-PCR protocol

Primer	Name	Sequence (5'- 3')
Forward Primer	TQF	GAAAAGAACCCTTGTTAAGGGAG
Probe	Probe	FAM-CAAAAGGCACGCCGTCAAC-BHQ1
Reverse Primer	TQR	CTTAACTCCCTGGCTCATCATG

Reagent	Comments	Amount
Sample	DNA extracted from eye swabs	2 µl
Forward Primer	TQF – 500 nM	1 µl
Reverse Primer	TQR – 500 nM	1 µl
Probe	100 nM	1 µl
PerfeCTa qPCR Toughmix	Cat# 95112 - Quanta Biosciences	10 µl
	Total Volume	15 µl

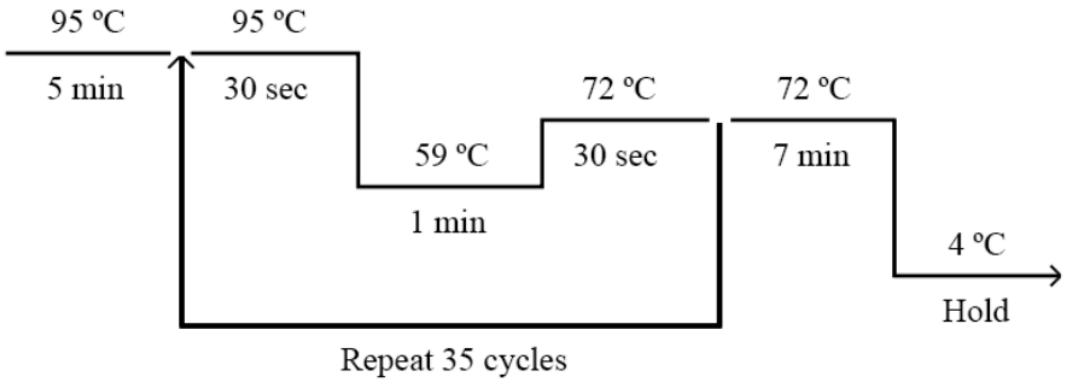


PCR analysis was run on Applied Biosystems® 7500 Fast Real Time PCR System (ThermoFisher Scientific).

APPENDIX 4: *Mycoplasma conjunctivae* PCR protocol

Primer	Name	Sequence (5'- 3')
Forward Primer	LPPS-TM-L	CAGCTGGTGTAGCACTTTTTGC
Reverse Primer	LPPS-TM-R	TTAACACCTATGCTCTCGTCTTTGA

Reagent	Comments	Amount
Positive Control	*Mycoplasma conjunctivae*	2 µl
Negative Control	DEPC Water	2 µl
Sample	DNA extracted from eye swabs	2-5 µl
Forward Primer	LPPS-TM-L	1 µl
Reverse Primer	LPPS-TM-R	1 µl
Jump Start Red Taq Ready Mix	Cat# P-0982 - Sigma	10 µl
DEPC Water	Up to a total volume of 25 µL	6-9 µl
	Total Volume	25 µl

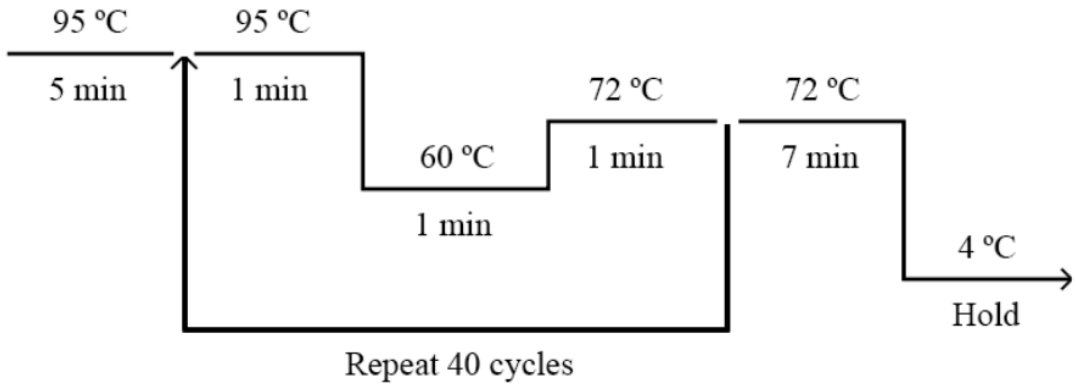


PCR analysis was run on Applied Biosystems® GeneAMP® PCR System 9700 (ThermoFisher Scientific).

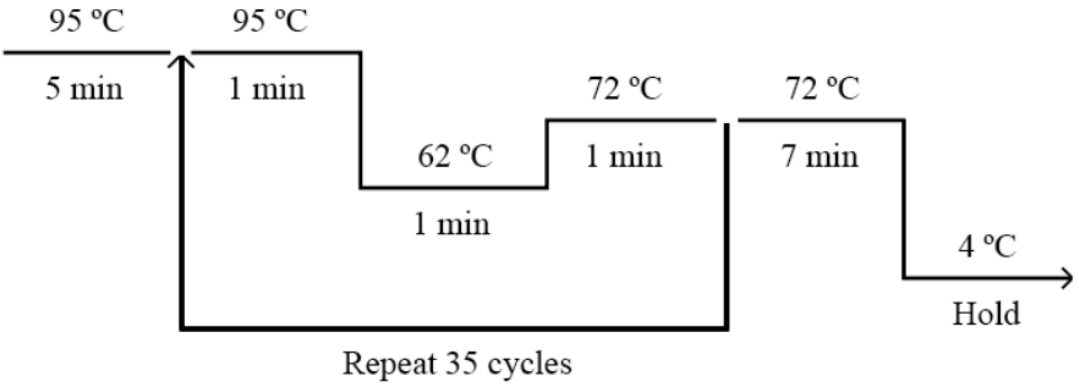
APPENDIX 5: Cervid herpesvirus 2 PCR protocol

Primer	Name	Sequence (5'- 3')	PCR
Forward Primer	CR30	TCGAARGCCGAGTACCTGCG	1st PCR
Reverse Primer	CR31	CCAGTCCCAGGCRACCGTCAC	1st PCR
Forward Primer	CR32	TGGTGGCCTTYGACCGCGAC	2nd PCR
Reverse Primer	CR33	GCTCCGGCGAGTAGCTGGTGTG	2nd PCR

1st PCR		
Reagent	**Comments**	**Amount**
Positive Control	Purified CvHV2 1:100 (strain Salla82, Finland)	5 µl
Negative Control	DEPC Water	5 µl
Sample	DNA extracted from eye swabs	5 µl
Forward Primer	CR30 – 25M	1 µl
Reverse Primer	CR31 – 25M	1 µl
Red Taq DNA Polymerase	Cat# D4309 - Sigma	10 µl
Dymethil sulfoxide	DMSO	2 µl
DEPC Water		6 µl
	Total Volume	25 µl



2nd PCR		
Reagent	**Comments**	**Amount**
Sample	PCR product from the 1st PCR	2 µl
Forward Primer	CR32	1 µl
Reverse Primer	CR33	1 µl
Red Taq DNA Polymerase	Catalog nr: D4309 - Sigma	10 µl
Dymethil sulfoxide	DMSO	2 µl
DEPC Water		9 µl
	Total Volume	25 µl



PCR analysis was run on Applied Biosystems® GeneAMP® PCR System 9700 (ThermoFisher Scientific).

APPENDIX 6: Post-processing of the PCR products

1. MinElute gel extraction protocol

MinElute Gel Extraction Kit (Qiagen, Hilden, Germany)

1. Excise the DNA fragment from the agarose gel.
2. Weight the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel.
3. Incubate at 50 ºC for 10 min. To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 volume of isopropanol and mix by inverting the tube.
6. Place a MinElute column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the MinElute column and centrifufe for 1 min.
8. Discard the flow-through and place the MinElute column in the same collection tube.
9. Add 500 µl of Buffer QG to the spin column and centrifuge for 1 min.
10. Repeat 8.
11. To wash, add 750 µl of Buffer PE to the MinElute column and centrifuge for 1 min.
12. Discard the flow-through and centrifuge the MinElute column for an additional 1 min at 17.900 *x g* (13.000 rpm).
13. Place the MinElute column into a clean 1.5 ml microcentrifuge tube.
14. To elute DNA, add 10 µl of Buffer EB or water to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.

2. Purification of the PCR product

USB® Exo-SAP-IT® PCR Product Cleanup Kit (Affymetix® Inc, Cleveland, USA)

Reagent	Comments	Amount
Sample	Final PCR product	2 µl
USB® Exo-SAP-IT® PCR Product Cleanup	Cat# 78200 – Affymetrix® Inc	5 µl
	Total Volume	7 µl

1. Remove ExoSAP-IT reagent from -20 ºC freezer and keep on ice throughout this procedure.

2. Mix 5 µl of a post-PCR reaction product with 2 µl of ExoSAP-IT reagent for a combined 7 µl reaction volume

3. Incubate at 37 ºC for 15 min to degrade remaining primers and nucleotids.

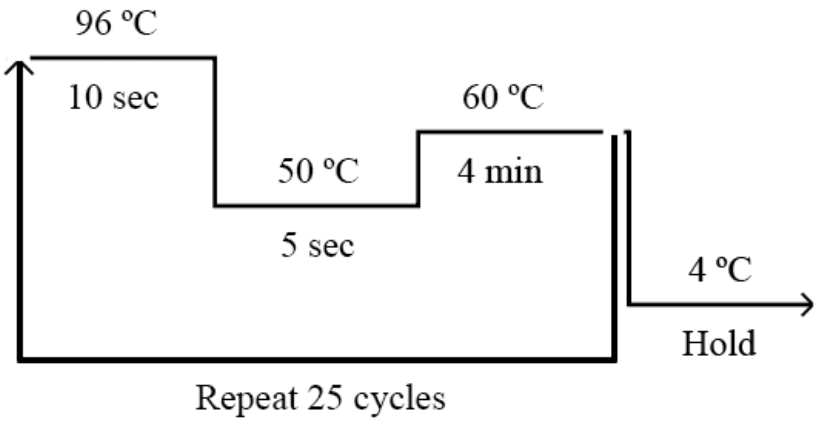
4. Incubate at 80 ºC for 15 min to inactivate ExoSAP-IT reagent.

5. The PCR product is now ready for use in DNA cycle sequencing reaction.

3. Cycle sequencing reaction using BigDye 3.1

Big Dye Terminator v.3.1 kit (Applied Biosystems®, ThermoFisher Scientific)

Reagent	Comments	Amount
Sample	Purified PCR product	2-4 µl
Ready Reaction Premix	Cat# 4337455 – Applied Biosystems®	3 µl
Big Dye Sequencing Buffer	Cat# 4337455 – Applied Biosystems®	2 µl
Forward Primer	3 pmol/µl	1 µl
Reverse Primer	3 pmol/µl	1 µl
DEPC Water	Up to a total volume of 20 µl	10-12 µl
	Total Volume	20 µl



Cycle sequencing reaction was run on Applied Biosystems® GeneAMP® PCR System 9.700 (ThermoFisher Scientific).