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THE ARCTIC
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OF NORWAY

Faculty of Biosciences, Fisheries and Economics, Department of Arctic and Marine Biology

Effect of Antiviral Drugs against Cervid Herpesvirus 2 (CvHV2) *in vitro*

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Emily Elizabeth Magnuson

BIO-3950 Master Thesis in Biological Science, November 2018

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Supervisors

Morten Tryland, UiT Arctic University of Norway

Javier Sanchez Romano, UiT Arctic University of Norway

Anett Kristin Larsen, UiT Arctic University of Norway

Cover photo:

Reindeer calves in northern Sweden, March 2017
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Overview:

Cervid herpesvirus 2 (CvHV2) is an alphaherpesvirus found in *Rangifer* subspecies throughout most of the circumpolar Arctic and the causative agent of infectious keratoconjunctivitis (IKC) in semi-domesticated Eurasian tundra reindeer *(Rangifer tarandus tarandus)*. IKC occurs as regular outbreaks, affecting dozens of reindeer in a herd, and is most common and severe among calves and young animals. IKC often appears as mild clinical signs from which the animals often recover, but the disease can progress to more advanced stages where the eye is severely damaged, resulting in blindness or death. Development of an antiviral therapy for CvHV2 could improve animal welfare conditions and reduce economic losses within reindeer herding industry in Fennoscandia. To our knowledge, only one pilot study has previously tested the effectiveness of antiviral drugs against CvHV2 and indicated that the nucleoside analog drug Acyclovir, commonly used as an antiherpetic treatment in humans and other species, was not successful inhibiting viral replication. This master's study aimed to further investigate the effect of antiviral drugs against CvHV2 by testing Ganciclovir and Cidofovir, and to evaluate their potential use as part of a treatment for IKC in semi-domesticated reindeer in Fennoscandia. An *in vitro* experiment which used Madin-Darby bovine kidney (MDBK) cell cultures was used as a preliminary model for natural CvHV2 infection in reindeer. A negative dose-response relationship was found for both Ganciclovir and Cidofovir, however, neither drug was able to completely inhibit the viral replication even at the highest drug concentrations and lowest viral titers tested. These findings indicate that both drugs are tentative candidates for the development of an antiviral treatment for CvHV2, but further studies to attempt to increase the therapeutic index of either drug should be strongly considered before testing in live animals.

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1. INTRODUCTION

1.1 HOST: *Rangifer tarandus*

With an estimated global population of three million animals, *Rangifer tarandus*, known as either caribou or reindeer, is the most populous cervid in the circumpolar Arctic and Subarctic zones (Gunn 2016). The majority of *Rangifer* live in North America, with smaller populations distributed throughout Fennoscandia, Russia, Iceland, Svalbard, Greenland**.** In North America, all subspecies are wild and free-ranging caribou, with the exception of a small population of semi-domesticated Eurasian tundra reindeer, of Russian origin, living in Alaska (Lantis 1950). *Rangifer* in Fennoscandia, Russia, Iceland, Greenland are predominantly semi-domesticated reindeer, with small, localized populations of wild reindeer and caribou. The taxonomy of *Rangifer* subspecies has been debated, but it is generally accepted that there are seven extant subspecies of *Rangifer*: Woodland caribou (*R. t. caribou)*, Canadian barren-ground caribou (*R. t. groenlandicus*), Peary caribou (*R. t. pearyi)*, Alaska tundra or Porcupine caribou (*R. t. granti*), Eurasian tundra reindeer (*R. t. tarandus*), wild forest reindeer (*R. t. fennicus*), and Svalbard reindeer (*R. t. platyrhynchus*) (Banfield 1961).

While *Rangifer* are not considered an endangered species, the total global population is thought to be declining significantly, and certain subspecies and populations are protected due to reduced size and habitat changes (Valkenburg et al. 1996; McLoughlin et al. 2003; Vors and Boyce 2009; Hervieux et al. 2013). It is estimated that *Rangifer* have declined by 2,000,000 individuals or 40% of the total global population in the last 10-30 years (Gunn 2016). Current hypotheses of the cause of these declines include effects related to climate change, such as phenology shifts, increases in extreme weather events, and range shifts of other species, as well as anthropogenic causes like habitat fragmentation related to development in rural areas (Cameron et al. 2005; Schaefer 2003; Vors and Boyce 2009; Uboni et al. 2016). Declines or extinctions could lead to substantial monetary and cultural losses for circumpolar communities, as well as ecosystem damages through the disruption of current species interactions (Johnson et al. 2015).

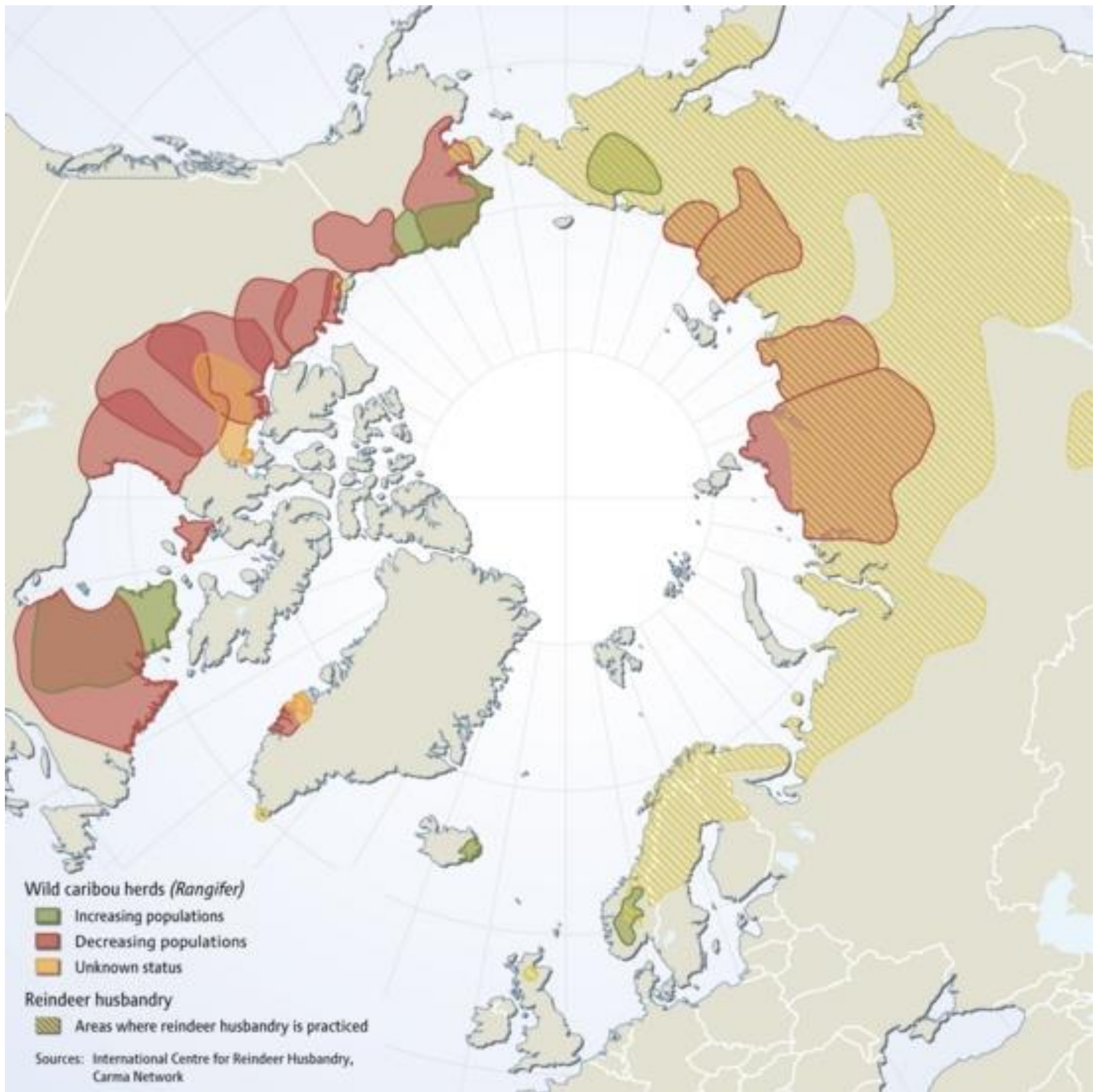


Figure 1: Circumpolar distribution of *Rangifer* herds through the Arctic, illustrating the current dynamics of wild populations, overlaid with the geographic regions of reindeer husbandry (Pravettoni 2010). Used with permission for educational purposes.

1.1.1 Biology and Ecology

All *Rangifer* subspecies have homologous adaptations for survival in the harsh and variable environmental conditions of the Arctic and sub-Arctic. These include broad, four-toed hooves that assist in walking and digging for forage in deep snow, and a pelage composed of a dense underlayer and an outer coat of long, hollow guard hairs, that trap air close to the body aiding both heat retention and flotation when swimming (Blix 2005). Both sexes have antlers, with differing seasonality corresponding to the energetically intensive reproductive periods of the year for either sex. Males typically have larger antlers, grown throughout the summer and used to compete with other males during autumn mating, while females retain their antlers through the winter season, giving them a foraging advantage while gestating and shed them in the spring after their calves are born (Palmer 1934).

Rangifer subspecies are diverse in other aspects their morphology due to local adaptations during the glaciation period of the Pleistocene (Yannic et al. 2013). Forest dwelling subspecies typically have longer, thinner legs, better adapted to outrunning predators (Nieminen and Helle 1980; Klein et al. 1987). In contrast, the high Arctic subspecies, Svalbard reindeer and Peary caribou, have exceptionally short-legs and a stout abdomen, having evolved in predator-free island environments and thus adapted to more sedentary, energy-conserving movements (Gravlund et al. 1998).

Rangifer live in regions of the world with low plant diversity and productivity, where forage is covered by snow and ice most of the year. Because of this, forage availability is a significant ecological pressure in *Rangifer* and can determine population distribution and migration patterns. Diet can vary substantially between seasons and regions, but typical diet items include grasses, sedges, lichens, mushrooms and woody shrubs (Bergerud 1972; Rominger and Oldemeyer 1990). Lichens are a particularly important food item for semi-domesticated reindeer and can compose a majority of their diet in the winter (Inga 2007).

With the exception of Svalbard reindeer, wild *Rangifer* populations have long seasonal migrations between winter and summer ranges. The Porcupine caribou herd in Alaska migrates more than 5000km (Fancy 1989). These migrations are driven by forage availability, weather

conditions, and avoidance of predators and insect harassment. Winter movements are typically related to depth of snow and ability to reach snow covered forage, while summer movements can be affected by avoidance of parasitic insects (Bevanger and Jordhoy 2004). Semi-domesticated reindeer are not allowed to migrate freely as wild *Rangifer* populations do but are still herded seasonally between summer and winter pastures (Pape and Loffler 2012).

1.1.2 Semi-Domesticated Reindeer in Norway

Semi-domesticated reindeer are unique among all *Rangifer* populations because they are free-ranging but herded and actively managed by humans. Reindeer have been utilized by the Saami, the indigenous people of Fennoscandia, and others for thousands of years through hunting, but as early as 800 A.D., the Saami began domestication of reindeer (Bjorklund 2012). Reindeer husbandry in Fennoscandia began with the herding of small groups of reindeer (<25 animals) by single families (Hultblad 1968). Over time, these reindeer herds became bigger and family groups started to work collectively in traditional *Siida* units in order to manage larger numbers of animals. Reindeer husbandry is thought to have become a primary livelihood for Saami people in Norway in the early 18th century (Leem 1975).

Reindeer husbandry continues to be an economically and culturally significant activity for Saami people and others in present-day Fennoscandia. In 2017 there were an estimated 650,000 semi-domestic reindeer in Norway, Sweden, and Finland (Landbruksdirektoratet 2017). The annual revenue of reindeer herding can vary dramatically depending on the specific herding conditions of each year, but the value of semi-domestic reindeer in Fennoscandia ranged from 338-345 million Norwegian kroner (NOK) in 2015-2016 (Landbruksdirektoratet 2017).

In Norway, there are 6 designated reindeer pasture areas, covering approximately 140,000 km^2 or nearly 40% of the surface area of the country (Ulvevadet and Klokov 2004). Only Saami people are allowed to own reindeer in Norway, except within small 'Concession Areas' (*Tamreinlag*) in southern Norway, outside of the designated reindeer pasture areas.

Traditional earmarks of a unique pattern of knife cuts or modern electronic microchips, are used to identify ownership of each reindeer. Herds are gathered 1-2 times annually for ear-marking or

tagging, anti-parasite treatment, and slaughter. Saami herders use all-terrain vehicles, snowmobiles, and helicopters to direct the movement of a herd during controlled seasonal migrations. Due to habitat fragmentation and issues of land ownership and pasture rights, some reindeer herds are transported in trucks from one seasonal pasture area to another (Vistnes and Nellemann 2007).

Yearly loses of semi-domesticated reindeer can be fairly high with 18-20% reported lost by herders (Nybakk et al 2002; Landbruksdirektoratet 2017). Starvation, predation by large carnivores, extreme weather events, and accidents, including vehicle strikes, are all common causes of reindeer loss (Nybakk et al 2002, Tveraa et al. 2014). In the past, disease outbreaks have been a serious challenge to reindeer herding, killing thousands of animals, however current health conditions of reindeer in Norway are favorable for survival and meat production (Tryland 2012). Outbreaks of infectious keratoconjunctivitis and contagious ecthyma can occur, but do not cause significant mortality annually (Tryland et al 2001a; Tryland et al. 2009). Semi-domesticated reindeer are also impacted by a wide variety of parasites including helminths, arthropods, and protozoa (Josefsen et al. 2014). Warble flies and the nematodes *Setaria tundra* and *Elaphostrongylus rangiferi* are examples of the most commonly found parasites (Helle 1980; Handeland and Slettbakk 1994; Laaksonen et al. 2009; Åsbakk et al. 2014)

It is anticipated that the impacts of disease among semi-domesticated reindeer may become more severe with the progression of climate change (Tryland 2012). In addition to possible exposure to novel parasites and pathogens, climate change conditions such as increased mid-winter freeze-thaw cycles and rain on snow events may cause reindeer to become more susceptible to enzootic diseases due to starvation related stress (Tryland 2012). The formation of ice on top of snow or buried vegetation following episodes of above-freezing temperatures in winter, significantly reduces forage availability for reindeer (Tyler 2010; Forbes et al. 2016). Increased levels of glucocorticoids caused by emaciation can lead to immunosuppression and higher incidence of disease (Nathan et al. 1977, Coutinho and Chapman 2011). Such conditions could leave *Rangifer* more vulnerable to common pathogens present in the environment or reactivation of latent infections.

Due to increased rain on snow events and decreased winter forage, it has also become necessary for some herders to provide supplemental feed to maintain their herd overwinter, which may create further health problems. Aggregation of animals at feeding stations can lead to increased disease transmission due to increased herd density and hygiene challenges such as dirty corrals or airborne feed particulates (Rehbinder and Nilsson 1995). Necrobacillosis, parapoxvirus infections, pasteurellosis, and infectious keratoconjunctivitis are diseases of particular concern under such circumstances (Kummeneje 1976; Rehbinder and Nilsson 1995; Tryland et al. 2001b, Tryland et al. 2009; Handeland et al. 2010).

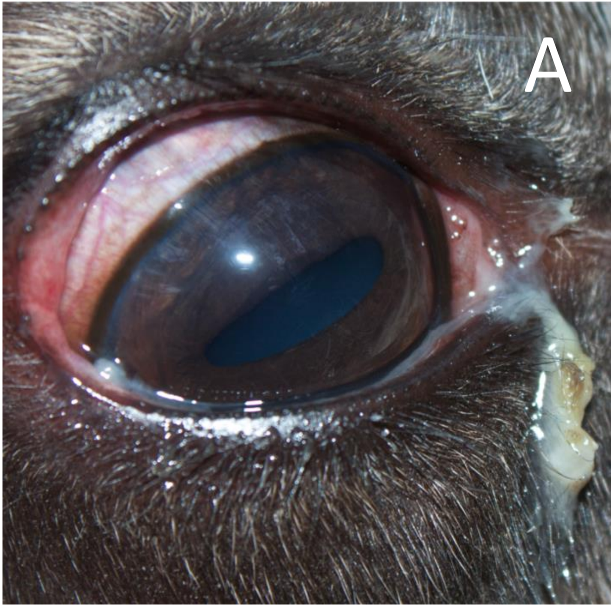
1.2 DISEASE: Infectious Keratoconjunctivitis

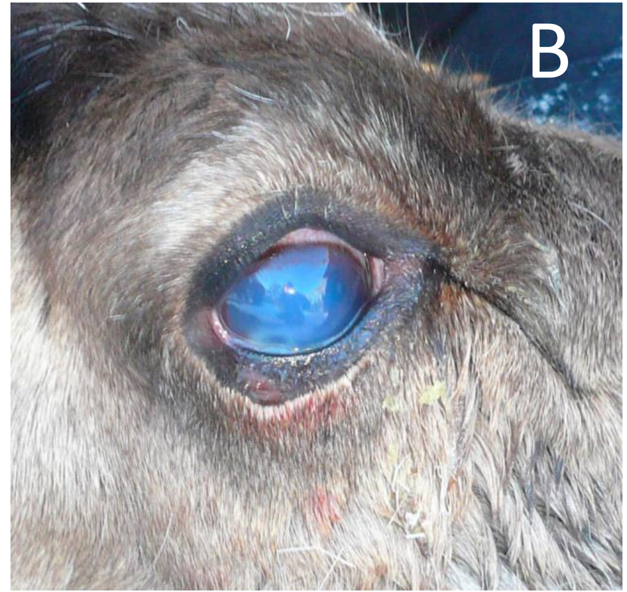
Infectious keratoconjunctivitis (IKC) is a multifactorial, transmissible eye disease common among both domestic animals and wildlife. IKC does not typically cause high mortality as infections are often limited to subclinical or mild clinical signs, but it can result in significant morbidity due to its highly infectious nature, particularly among herd species where animal density can increase transmission (Bergman 1912). Infectious bovine keratoconjunctivitis (IBK) is considered the most important ocular diseases among bovines and can result in significant economic losses due to emaciation and loss of productivity in meat or dairy production animals (Angelos 2010; Angelos 2015). IKC has also been well documented in a variety of wild ruminants including moose, alpine chamois, ibex, red deer, mule deer, pronghorn antelope, caribou, and semi-domesticated reindeer (Dubay et al. 2000; Giacometti et al. 2002; Gortazer et al. 1998; Evans et al. 2008; Bergman 1912; Thorne 1982).

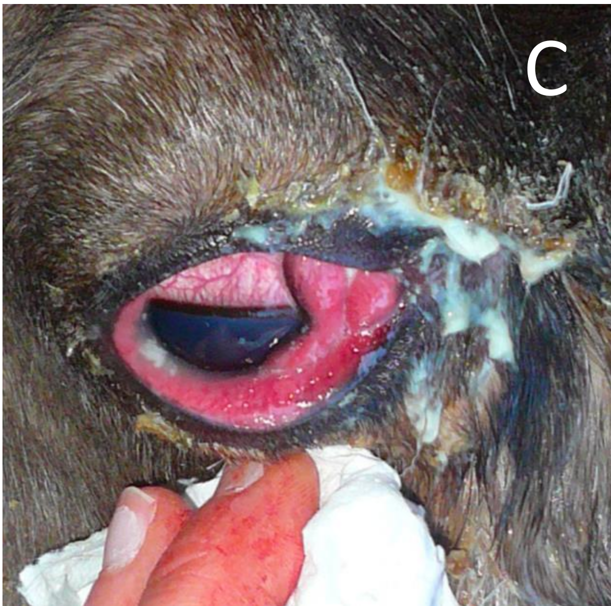
IKC was first documented in semi-domesticated reindeer at the beginning of the 20th century, and continues to occur throughout Fennoscandia in sporadic outbreaks primarily affecting calves, which often display the most severe clinical signs (Bergman 1912; Tryland et al. 2009). While no scientific study has reported the current prevalence of IKC in Norway, a questionnaire survey of reindeer herders in Norway and Sweden showed that IKC is likely common as 55% (n=63) reported observing IKC in their animals within the previous year (Tryland et al. 2016). Herders additionally responded that they most often observed IKC affecting only 1-5 animals at a time,

but there are also cases of IKC causing larger outbreaks (Rehbinder and Nilsson 1995; Tryland et al. 2009).

Staining and clumping of the fur below the eye caused by increased lacrimation and pus is often the first clinical sign (Fig. 3A) observed by herders during an outbreak of IKC (Tryland et al. 2017). This may occur either unilaterally or bilaterally (Winqvist and Rehbinder 1973). During the progress of a mild to moderate infection, inflammation of the conjunctiva and cornea occurs, and the eye may appear cloudy, opaque or blueish due to corneal edema (Fig. 3B) (Winqvist and Rehbinder 1973). In the final stages panophthalmitis occurs accompanied by accumulation of periorbital edema, blood and purulent secretions (Fig. 3C). Ocular ulcers can lead to loss of the lens or other eye structures (Fig. 3D) (Rehbinder 1977). At the early stages of IKC, many animals spontaneously recover without permanent ocular damage, but when the disease progresses to the most severe clinical signs, blindness is likely to occur (Ryser-Degiorgis et al. 2009; Tryland et al. 2009). Long-term survival of a partially or fully blind reindeer is critically impacted as the ability to locate food and avoid environmental obstacles and predators diminishes with the loss of sight (Tryland et al. 2017). Even early stages of IKC are likely to be highly painful for affected animals, and these infections are of concern from the perspective of both animal welfare and economic losses for reindeer herders. Indirect mortality can also occur because herders may choose to slaughter infected animals as a means to control the spread of an outbreak (Tryland et al. 2016).







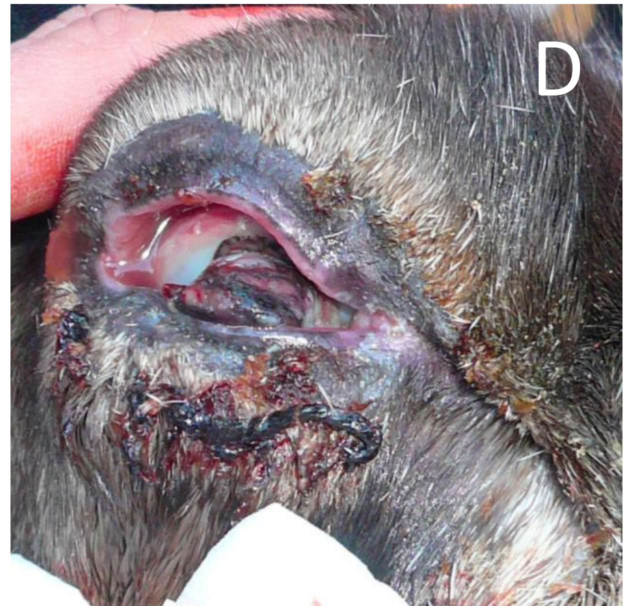


Figure 2. Clinical signs of infectious keratoconjunctivitis in semi-domesticated reindeer at increasing stages of the disease: (A) conjunctivitis with increased lacrimation and purulent secretions, (B) corneal opacity caused by corneal edema. (C) severe conjunctivitis with periorbital edema, purulent secretions and bleeding, (D) severe corneal ulceration that led to corneal rupture. Adapted from Tryland et al. 2009 (B,C, D) and Tryland et al. 2017 (A) with permission.

IKC is considered a multifactorial disease because both environmental conditions and presence of pathogens affect the development of the disease. UV exposure, dust and other airborne particles can irritate the fibrous tunic and conjunctiva, leaving the eye more vulnerable to colonization by pathogens (Rehbinder et al. 1978). A variety of pathogens have been associated with IKC in wild ruminants including species of the bacterial families *Moraxellaceae*, *Mycoplasmataceae* and *Chlamydiaceae*, and a diversity of alphaherpesviruses (Rehbinder et al. 1975; Nettleton et al. 1986; Tryland et al. 2009; Smits et al. 2013; Giangaspero et al. 2010; Dubay 2000). Because of the multifactorial nature of IKC, it was initially unclear which infectious agent was the cause for the development of IKC in reindeer. During a severe outbreak of IKC in 1993 among Swedish semi-domesticated reindeer, bacteriology of eye swab samples and serological tests detected the presence of several bacterial species in the eyes, including *Moraxella sp.* and *Pasteurella multocida* (Rehbinder and Nilsson 1995).

Cervid herpesvirus 2 (CvHV2) was determined to be the primary pathogen in a 2009 outbreak of IKC in Norwegian semi-domesticated reindeer (Tryland et al. 2009). Both affected and unaffected animals were seropositive for CvHV2 antibodies, but the virus could only be isolated from the eyes of reindeer with active infections (Tryland et al. 2009). Viral activity of isolates was investigated by assessing virulence in cell culture. Severity of damage to the culture cells, known as cytopathic effect (CPE), increased with severity of clinical signs in sampled reindeer, apart from those with the most advanced IKC infections. In the most advanced IKC cases, CvHV2 could not be detected and bacteria, including *Moraxella sp.,* appeared to cause secondary infections. CvHV2 was further implicated in IKC occurrence during an experimental inoculation study with CvHV2, *Moraxella bovoculi*, or both CvHV2 and *M. bovoculi* in semi-domesticated reindeer calves (Tryland et al. 2017). Calves inoculated with CvHV2 or both, CvHV2 and *M. bovoculi,* rapidly developed severe cases of IKC, while those inoculated only with *M. bovoculi* did not develop any clinical signs. It is concluded through these two studies that while IKC may be a multifactorial disease, CvHV2 is unquestionably a causative agent in reindeer in Norway.

1.3 PATHOGEN: Cervid Herpesvirus 2

1.3.1 Characterization of Alphaherpesviruses

Herpesviridae is a large family of enveloped, double-stranded DNA (dsDNA) viruses with complex genomes, capable of establishing life-long latent infections in mammals, birds and reptiles. The genomes of herpesviruses are large, with 125-290 kb containing 60-120 genes and form a torus configuration within the viral capsid when not undergoing replication (Furlong et al. 1972; Davison et al. 2009). The virion is composed of an icosahedral capsid core containing the double-stranded DNA genome, surrounded by a protein matrix called the tegument, and enclosed by a lipid bilayer envelope, embedded with surface glycoproteins (Fig. 3). The capsid of the virion is in an icosahedral shape approximately 100-150nm in diameter with 162 capsomeres (Pellett and Roizman 2006). Between the capsid and viral envelope, the tegument is an amorphous and asymmetrically thick mass of proteins and enzymes utilized in the early stages of host cell infection and protect against the cell's initial immune defenses (Jenkins and Hoffman 2000). Lastly, the viral envelope is composed of proteins originating from the endoplasmic reticulum of the host cell and contains a variety of viral glycoproteins responsible for the virion's ability to bind to and enter host cells (Jenkins and Hoffman 2000).

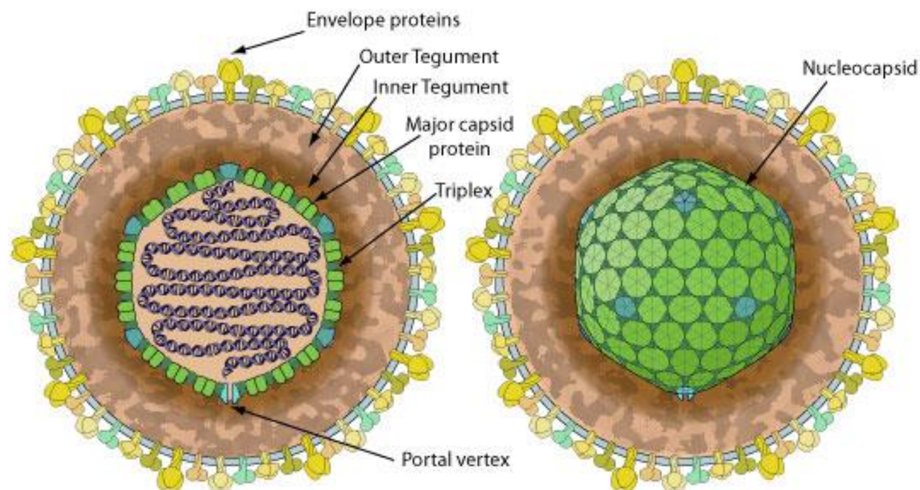


Figure 3. Structure of herpesvirus virion, highlighting the viral envelope, surface glycoproteins, tegument, and nucleocapsid. Used with permission from ViralZone© 2009 – Swiss Institute for Bioinformatics (www.expasy.org/viralzone).

Herpesviridae is divided into three subfamilies: *alphaherpesvirinae* (α-herpes), *betaherpesvirinae* (β-herpes), and *gammaherpesvirinae* (γ-herpes). Taxonomy of the subfamilies is based on genetic and biological characteristics including host cell tropism, length of reproductive cycle, behavior in cell culture and ability to establish latent infections in sensory nerve ganglia or other tissues in the body (Davison et al 2009; Knowles 2011). Alphaherpesviruses, referred to as neurotropic herpesviruses, infect a large variety of hosts, exhibit a wide range of tissue tropisms, replicate rapidly, and establish latency in sensory nerve ganglia (Knowles et al. 2011). Human herpes simplex viruses 1 and 2 (HSV1/HSV2) and varicella zoster virus (VZV) are common alphaherpesviruses that effect humans (Davison et al. 2009). A variety of alphaherpesvirus infect domestic and wild ruminants such as bovine herpesvirus 1 (BoHV1), caprine herpesvirus 1 (CpHV1), bubaline herpesvirus 1 (BuHV1) and cervid herpesviruses 1 (CvHV1) and 2 (CvHV2) (Thiry et al. 2006).

Alphaherpesviruses undergo rapid replication in the host cell nucleus during a lytic infection (Roismann et al. 1992; Knowles et al. 2011). The virion utilizes a variety of glycoproteins (gC, gD, gB and gH) to quickly attach to the cellular membrane, fuse, and enter a host cell (Tikoo et al. 1995). Immediately after entry, the virion signals for cessation of host cell protein production and the intact capsid moves along the cytoskeleton to inject the viral genome into the host nucleus (Fields et al. 2001).

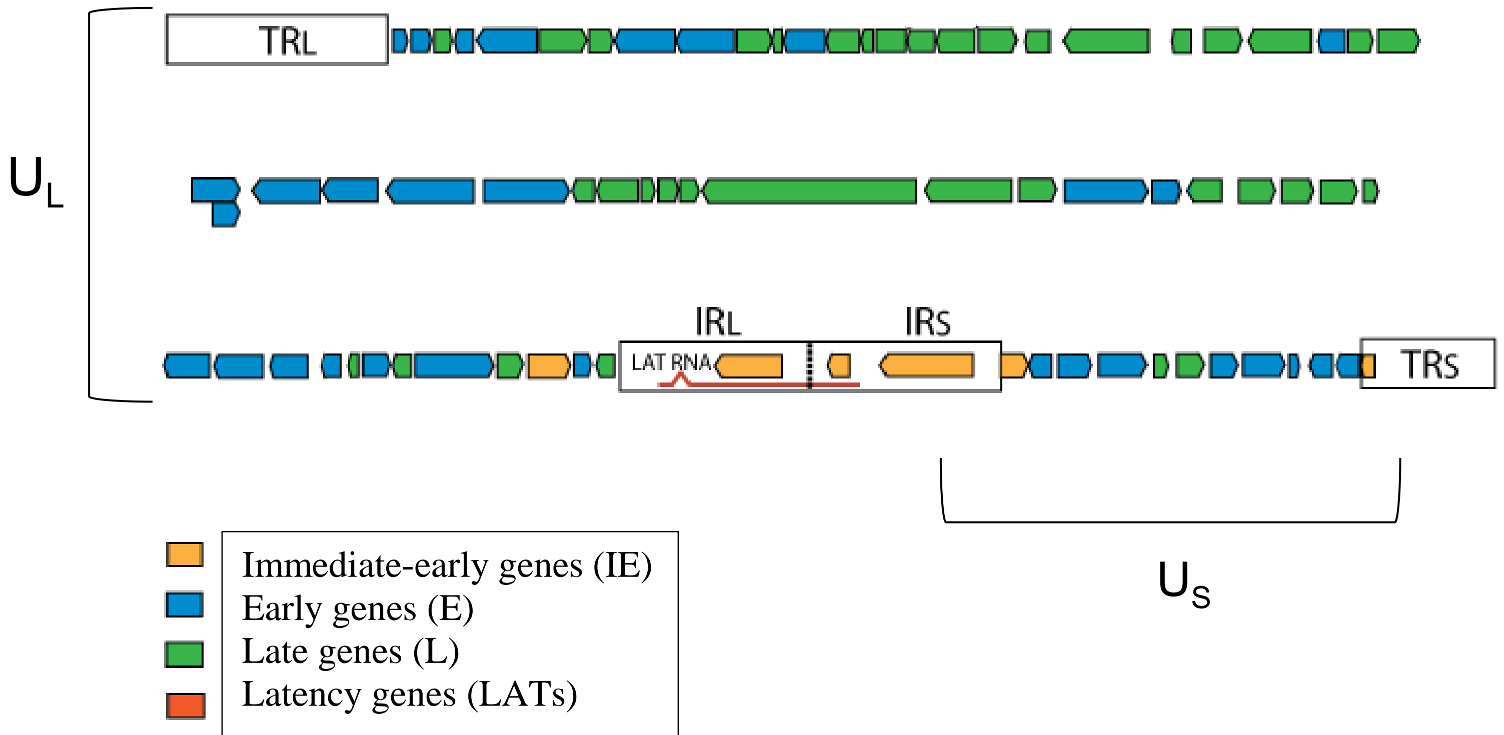


Figure 4. Organization of herpesvirus genome: Two segments, the long unique unit (U_L) and the short unique unit (U_S), each surrounded by a set of a terminal (TR_L/TR_S) and an internal repeat sequence (IR_L/IR_S). Gene classes are described in the figure legend. Adapted with permission from ViralZone© 2009 – Swiss Institute for Bioinformatics (www.expasy.org/viralzone).

The dsDNA linear molecule is composed of two main segments (Fig. 4): a long unique unit (U_L) and a short unique unit (U_S), each capped by two internal repeat sequences, an internal repeat (IR) and a terminal repeat (TR) (Schwyzer and Ackermann. 1996). Viral gene expression is divided into three classes: immediate-early (IE), early (E), and late (L). As soon as the genome is injected into the nucleus of the host cell, IE genes code for creation of regulatory proteins, to take over control of the host cell's replication mechanisms (Thiry et al. 2006; Thellman and Trizenberg 2017). E genes produce enzymes, such as viral kinases and DNA polymerase, and increase the available pool of nucleotides (Levings et al. 2013). Lastly L genes function to produce the structural proteins of the new viral particles (Thellman and Trizenberg 2017).

When the viral DNA is transcribed by cellular RNA polymerase into mRNA, it moves into the cytoplasm for translation (Fields et al. 2001). The proteins produced during translation disperse into the nucleus, endoplasmic reticulum, and cytoplasm. In the nucleus, the viral DNA is shaped, and capsid structural proteins are formed. The tegument and envelope are initially formed as transformed sections of the cell's nuclear envelope. Budding is triggered when the nucleocapsids bind to this section of the envelope. Once released from the nucleus, the enveloped viral particles gather in the endoplasmic reticulum before being expelled from the cell by exocytosis.

The most characteristic aspect of the *Herpesviridae* family is the ability to establish latency after a primary lytic infection. In a latent alphaherpesvirus infection, virions travel from the site of the primary infection, usually epithelial cells of mucosal tissues, into the sensory ganglia neurons (Engels and Ackermann 1986; Jones et al. 2006). The virions enter the neuron cell in the same manner as during active replication: glycoproteins bind to the cell surface and the virion fuses with the membrane until the nucleocapsid is able to enter the cell. Once inside the neural cell, the virion injects its genome in the nucleus of the cell and activates a small set of genes called latency associated transcript (LATs). LATs maintain latency by suppressing IE and E genes and protecting the cell from apoptosis (Jones et al. 2006). The specific mechanism viral reactivation and return to a lytic infection is not well-known. In ruminants, alphaherpesvirus reactivation can occur either under conditions of physiological stress such as starvation or mating, or spontaneously (Engels and Ackermann 1996; Thiry et al. 2006)

1.3.2 Prevalence and Detection of CvHV2

Cervid herpesvirus 2 is an alphaherpesvirus known to infect reindeer and caribou in all regions of the circumpolar Arctic with the exceptions of Svalbard and Iceland (Dieterich et al. 1981; Elazhary et al. 1981; Ek-Kommonen 1982; Rehbinder et al. 1992; Hyllseth et al. 1993, Stuen et al. 1993, Evans et al. 2012). CvHV2 was first described in Finland in 1980 during a BoHV1 serology study of semi-domesticated reindeer (Ek-Kommonen et al. 1982). When BoHV1 antibody neutralization tests showed a seroprevalence of 23% in Finnish reindeer sampled in a region with no seropositive cattle, it was hypothesized that a novel alphaherpesvirus was circulating in the reindeer population (Ek-Kommonen et al. 1982). Identification of CvHV2 as an alphaherpesvirus distinct from BoHV1 was later confirmed when it was isolated in 1986 (Ek-

Kommonen et al. 1986). Antigenic similarities between alphaherpesviruses, including CvHV2 and BoHV1, reduce the specificity of some serology tests (Deregt et al. 2005; Lyaku et al. 1992; Thiry et al. 2006; Das Neves et al. 2010). ELISA kits most commonly used for identification of alphaherpesviruses in ruminants utilize glycoprotein B has the antigen, coded by the mostly highly conserved gene in the genome of ruminant alphaherpesviruses (Ross and Belak 2002). This means that not all ELISA kits will be able to identify which alphaherpesvirus is present. Viral isolation and PCR analysis are more specific alternatives (Ros et al. 1999; Das Neves et al. 2010), while ELISA tests detect previous exposure to the virus, viral isolation and PCR detect active infections with actual presence of viral particles.

Cervid Herpesvirus 2 (CvHV2) is enzootic in reindeer throughout Fennoscandia (Das Neves et al. 2009e; Romano et al. 2018; Tryland et al. 2018). A large serological study of 3026 serum samples from semi-domesticated reindeer in Finnmark, Norway, found seroprevalence of CvHV2 varied from 7.6-90.7% between herding districts, and extrapolated an estimated prevalence of 48% among all reindeer in the region (Das Neves et al. 2009e). A study of wild reindeer in southern Norway (n=831) showed a seroprevalence of 28.5% (Lillehaug et al. 2003). The conditions most highly correlated with CvHV2 seroprevalence are age and animal density (Lillehaug et al. 2003; Das Neves et al. 2009). Das Neves et al. (2009e) also found that increased reindeer densities within herding districts are significantly associated with higher CvHV2 occurrence, similar to what has been documented in extensive cattle studies of BoHV1 (Miller 1991; Van Wuijckhuise et al. 1998). Seroprevalence of CvHV2 is highly variable between age classes, with markedly higher prevalence among adult animals than calves (Das Neves et al. 2009e; Lillehaug et al. 2003; Ek-Kommonen et al. 1982). This reflects the characteristic nature of herpesviruses that establish life-long infections with periods of latency and reactivation. Adult animals are more likely than calves to have been exposed to CvHV2 and become carriers of the virus, further spreading the virus to other individuals.

1.3.3 Transmission and Pathogenesis

Alphaherpesviruses in ruminants, including CvHV2, are spread by close contact with infected animals and contact with contagious aerosols and fomites (Engels and Ackermann 1996; Muylkens et al. 2007). CvHV2 infections are known to originate in the mucosa and epithelium of

the upper respiratory tract, genital tract, and conjunctiva (Das Neves et al. 2009a; Tryland et al. 2009; Tryland et al. 2017). Having gained entry to a host, CvHV2 initiates a lytic infection which causes epithelium damage leading to lesions and erosions in the eye, conjunctiva, oral and nasal cavities and genital tract (Das Neves et al. 2009a, Tryland et al. 2009, Tryland et al. 2017). Movement of viral particles from the initial infection site is poorly understood, but viremia has been shown to occur in experimental studies of both primary infections (inoculation) and upon reactivation of latent infections (Das Neves et al. 2009a; Das Neves et al. 2009b). CvHV2 was detected in a wide variety of tissues including lung, liver, lymph nodes, testicles, mammary glands, and spleen (Das Neves et al. 2009a). CvHV2 has also been detected in fetal tissues removed from adult females in experimental infection studies, indicating that vertical transmission occurs (Das Neves et al. 2009a). Observation of naturally occurring reactivations and experimental studies treating seropositive reindeer with the immunosuppressing drug dexamethasone confirmed that CvHV2 establishes latent infections as is characteristic of all herpesviruses (Rockborn et al. 1990; Das Neves et al. 2009b). CvHV2 has been detected in the trigeminal ganglion in experimental studies, suggesting that latency establishes in those nervous tissues (Das Neves et al. 2009a).

The impacts of CvHV2 are not well understood due to lack of scientific investigation and difficulty observing free-ranging reindeer year-round. . Even though CvHV2 was considered to cause asymptomatic infections (Nettleton et al. 1988, Thiry et al. 2006), experimental studies have shown that CvHV2 may be associated with abortion and neonatal diseases and it is well documented that CvHV2 causes respiratory and genital tract infections, and eye disease of varying severity (Rockborn et al. 1990; Das Neves et al. 2009 exp inf; Tryland et al. 2009; Tryland et al. 2017). CvHV2 was described as the causative infectious agent in a 2009 outbreak of infectious keratoconjunctivitis (IKC) in Troms County, Norway (Tryland et al. 2009). A 2014 experimental study further confirmed the connection between CvHV2 and IKC, by documenting the development of severe infections in reindeer calves ocularly inoculated with CvHV2 (Tryland et al. 2017).

1.4 Antiviral drugs

Because of fundamental differences between the nature of bacterial and viral replication, antiviral drugs rely on pointedly different mechanisms of inhibition than antibiotics. Viruses are not capable of autonomous replication like bacterial binary fission, and instead, must invade host cells and utilize cellular mechanics to replicate (Forterre 2006). Therefore, the challenge of antiviral drug development is that the compound must be effective in inhibiting viral replication, but not cause toxicity by damaging host cells, or minimize the toxic effect compared to the antiviral effect (Fields et al. 2001). There are two general classes of antiviral drugs: drugs which target the virus directly and drugs which target the host cell in order to inhibit viral replication. Antiviral drugs that target cellular mechanisms are more likely to result in greater cell toxicity than drugs that target viral mechanisms (Westreenen and Boucher 2002).

One of the largest families of antiviral drugs are nucleoside and nucleotide analogs which employ mechanisms of inhibition that cause termination of elongating DNA or RNA strand during replication of the viral genome (Clercq and Neyts 2009). Nucleosides are unphosphorylated sugar bases of nucleotides, the organic monomers that represents building blocks for constructing the double strands of DNA. Nucleoside analogs are molecules that share a similar composition and structure to naturally occurring nucleosides and after undergoing multiple intracellular phosphorylation steps, can be incorporated into the elongating DNA or RNA strand as in place of natural nucleotides during transcription (Clercq and Neyts 2009). Nucleotide similarly incorporated into the elongating DNA or RNA strand, but do not require the first initial phosphorylation step as nucleoside analogs do (Clercq and Neyts 2009). Once either molecule is incorporated into the growing DNA or RNA strand, termination occurs because analog does not contain the 3'-hydroxyl function at the (2'-deoxy)riboside moiety, and therefore does not have the correct configuration to bind with the subsequently added nucleotide (Clercq and Neyts 2009).

No antiviral drug is currently known to be effective in blocking CvHV2 replication. Acyclovir, nucleoside analog and common antiherpetic treatment used in humans, is the only drug previously tested against CvHV2 (Roche 2016). Acyclovir showed limited inhibition of CvHV2 when tested *in vitro* with Madin-Darby bovine kidney cells. This experiment contrasted with

other acyclovir studies of closely related ruminant alphaherpesviruses. Acyclovir has been shown to successfully block BoHV1 *in vitro* infections, and in combination with the imidazole nucleoside Mizoribine, acyclovir was found to be effective against CpHV1 (Collins 1983; Enan et al. 2012; Elia et al. 2015; Camero et al. 2017). Ganciclovir, an acyclovir homologue, is known to inhibit equine herpesvirus 1 and 3 (Smith et al. 1983; Enan et al. 2012). Outside of *in vitro* studies, CpHV1 infections have been successfully treated with cidofovir, a cytidine nucleoside analogue (Tempestra et al. 2007; Tempestra et al. 2008). Based on their documented effectiveness of other ruminant alphaherpesvirus inhibition in previous studies, ganciclovir and cidofovir were chosen to be tested against CvHV2 in this project.

1.4.1 Ganciclovir

Developed in the 1982, ganciclovir (Cymevene®, Cytovene®; Fig. 5) is a guanosine nucleoside analogue that has primarily been used in human medicine to treat cytomegalovirus diseases in AIDS patients (Ovilvie et al. 1982; Pape 1988; Jabs et al. 1989, Clercq and Neyts 2009). Oral bioavailability of ganciclovir is <10% and the drug is typically administered in humans by intravenous injection (Kimberlin and Whitley 2007). Antiherpetic ability of ganciclovir has been demonstrated and is highly effective in treating HSV-1 and HSV-2, though it is rarely used for this purpose due to concerns of carcinogenicity and reproductive toxicity (Jacbonson et al. 1987).

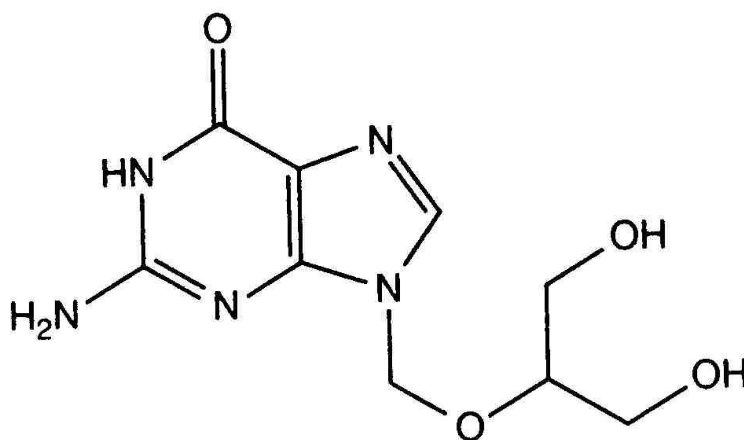


Figure 5. Chemical structure of Ganciclovir. Adapted with permission from Clercq and Neyts 2009.

The mechanism of inhibition of ganciclovir (Fig. 6) blocks viral replication by inhibiting viral DNA polymerases as it competitively blocks incorporation of guanosine nucleotides and by terminating DNA chain elongation (Crumpacker at el. 1979; Chen et al. 2014). Ganciclovir converted to ganciclovir triphosphate in the cell's cytoplasm, phosphorylated once by viral thymidine kinase (TK) and twice by cellular enzymes, before it is incorporated into the DNA strand (Martin et al. 1983). Ganciclovir contains an equivalent structure of a 3'-hydroxyl group that allows nucleotides to be added to the DNA strand in some viral species, but when treating herpesviruses, the molecule does not have the correct conformation to bind to an incoming nucleotide (Markham and Faulds 1994; Clercq and Neyts 2009; Chen et al. 2014).

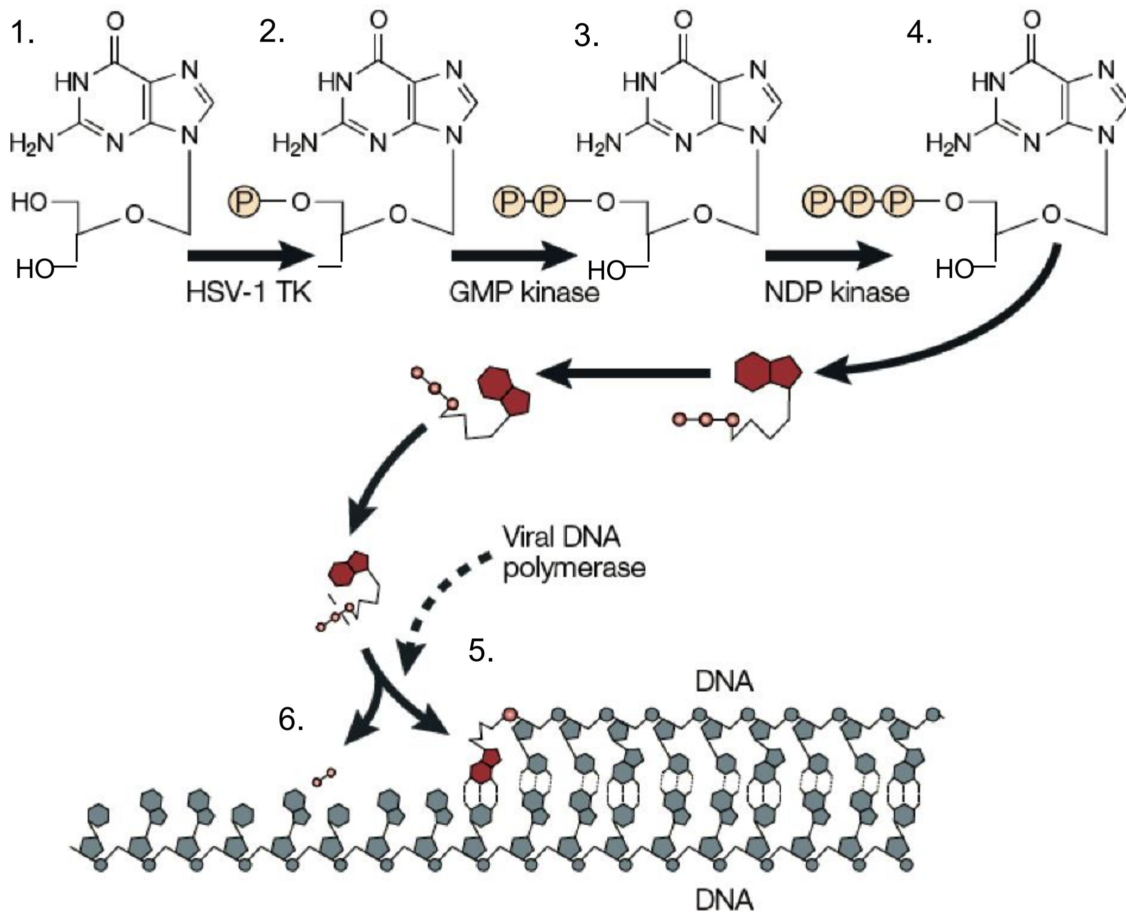


Figure 6. Ganciclovir's mechanism of inhibition of HSV-1, including the tri-phosphorylation, once by viral TK (2) and twice by cellular enzymes (3/4), and incorporation into the DNA strand (5) leading to termination of strand elongation (6). Adapted with permission from Clercq and Neyts 2009.

1.4.2 Cidofovir

Cidofovir (Vistide®; Figure 7) is a monophosphate acyclic nucleotide analog, specifically an acyclic cytosine analogue, with a broad spectrum of antiviral activity, known to be effective against herpesviruses, adenoviruses and papillomaviruses (De Clercq et al. 1986). In the same nucleoside family of antiviral drugs as ganciclovir, cidofovir is a cytosine nucleoside analogue rather than a guanosine nucleoside analog like ganciclovir. Cidofovir is a highly important treatment for CMV retinitis in human AIDS patients (Kirsch et al. 1995). During initial animal testing when cidofovir was being developed, it was found that the drug was successful in treating HSV1 infections in mice and rabbits, including localized therapy of herpetic keratitis (De Clercq et al. 1986). Cidofovir tested in veterinary *in vivo* studies have also shown success of topically applied cidofovir cream solutions against CpHV1 and canine herpesvirus-1 (CHV1) (Tempesta et al. 2008; Ledbetter et al. 2015).

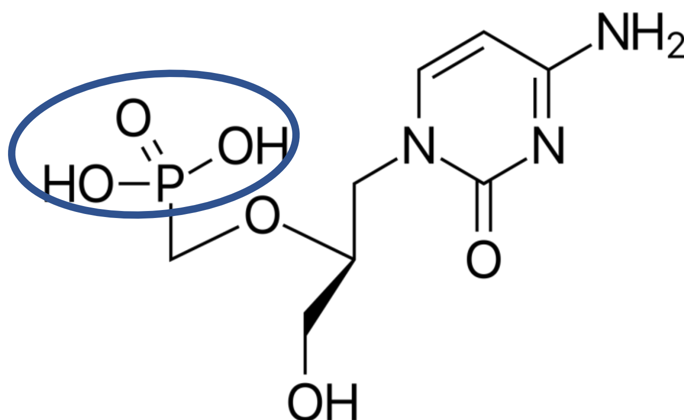


Figure 7. Chemical structure of Cidofovir, with the monophosphate group highlighted in a blue circle. Adapted with permission from Clercq and Neyts 2009.

The structure of cidofovir molecules causes relatively poor uptake into infected cells as its negatively charged configuration slows its entry endocytosis (Connelly et al. 1993). The monophosphate group slows cellular uptake as the negatively charged moiety is repelled by the similarly negatively charged heads of the phospholipid bilayer of the cell. However, despite poor uptake of the drug, once the drug has entered the cell, the phosphorylated forms of cidofovir

have relatively long half-lives (17-65 hours in humans) and allows for prolonged antiviral activity (Ho et al. 1992; Aduma et al. 1995).

The mechanism of inhibition of cidofovir (Figure **8**) is similar to that of ganciclovir, but cidofovir is a phosphonate, requiring only two phosphorylation steps to act as a substrate for viral DNA polymerase and the phosphorylation is completely only by cellular enzymes and not viral TK (Ho et al. 1992; Aduma et al. 1995). As cidofovir is incorporated into the growing DNA chain of replicating viral particles, transcription drastically slows down and when two successive cidofovir molecules have been added to the strand, transcription stops (Xiong et al. 1997).

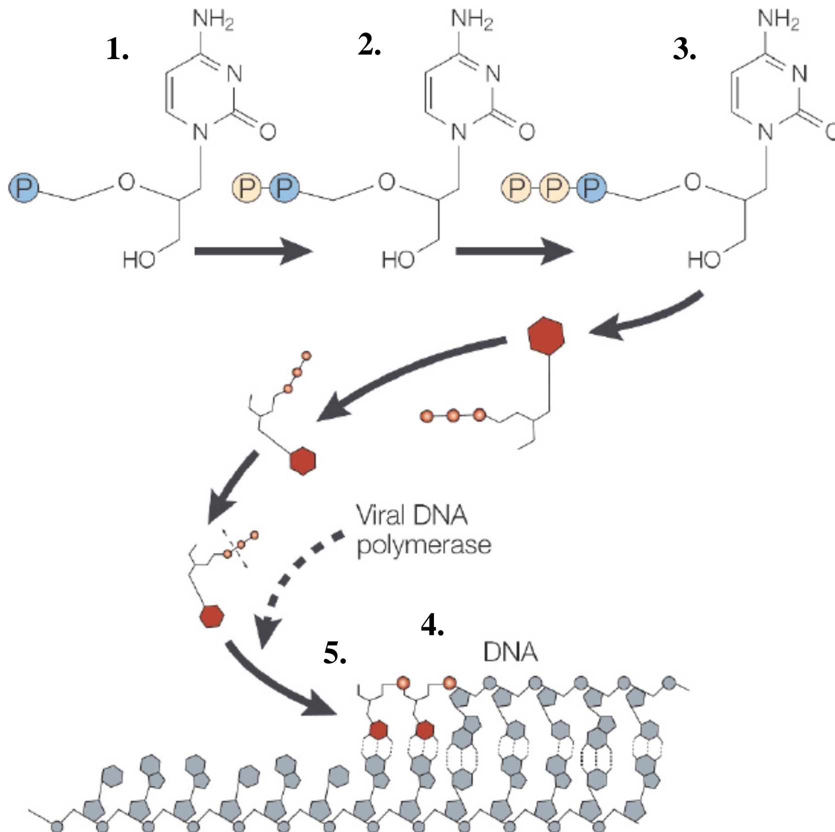


Figure 8. Cidofovir's mechanism of inhibition of HSV-1, including the two step phosphorylation from the monophosphate form (1) by cellular enzymes (2/3) and incorporation into the DNA strand (4) leading to termination of strand elongation (5). Adapted with permission from Clercq and Neyts 2009.

1.4 Aim of Study

The purpose of this study was to investigate the antiviral effect of Ganciclovir and Cidofovir against CvHV2 in a preliminary *in vitro* model with MDBK cells, and to evaluate their potential as a possible treatment for IKC in semi-domesticated reindeer in Fennoscandia.

Research questions:

1. Is there a dose-dependent relationship between Ganciclovir or Cidofovir and estimated CvHV2 titers after 72 hours exposure to the drugs?

2. If yes, what is the strength of that relationship? Could Ganciclovir or Cidofovir be considered for further studies and possible use as a treatment for IKC in semi-domesticated reindeer?

2. Materials and methods

2.1 MDBK-cells: culture conditions and maintenance

A culture of a continuous cell line of MDBK cells (American Type Culture Collection®, CCL-22™; Fig. 9). The cells were grown in $75cm^2$ or $150cm^2$ plastic cell culture flasks (ThermoFisher, Waltham, USA) with Eagle's minimum essential medium (EMEM) (Sigma-Aldrich, St. Louis, USA) supplemented with 10% horse serum (Sigma-Aldrich, St. Louis, USA) and incubated at 37.5°C with 5% CO_2. The cells were subcultured when cell density reached 70-80%,typically after 2-3 days (See Appendix 1). Viability of the culture was confirmed throughout the experiment by use of 0.4% Trypan Blue (Sigma-Aldrich, St. Louis, USA) when counting cells during subculturing and by regular observation under a microscope. Trypan Blue is only absorbed by living cells, so it can be used as a dye exclusion test for viability (Strober 2013).

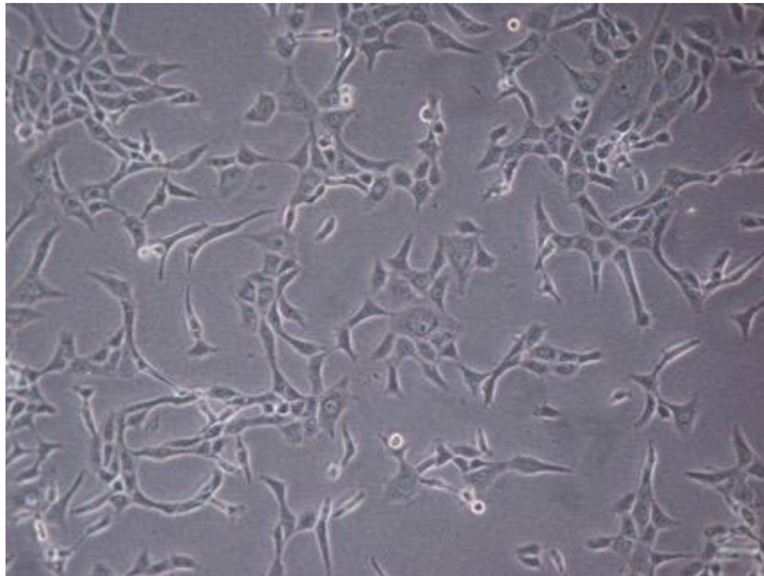


Figure 9. MDBK at medium density (<50% confluence) and actively replicating. Dividing cells appearing slightly luminescent.

2.2 Cytotoxicity assay

A cytotoxicity assay was used to determine the maximum concentration of each drug that could be used in MDBK cell culture. The assay was completed using a MTT Cell proliferation Kit I (Roche Life Science, Mannheim, Germany) following the manufacturer's protocol. The MTT Test Kit assessed metabolic activity by measuring the conversion of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to purple formazan crystals ((E,Z)-5-[4,5-dimethylthiazol-2-yl]-1,3-diphenylformazan) in the mitochondria of cells. This assay assumes normal metabolic function measured by the MTT Test Kit is an indicator of cells experiencing no significant cytotoxic effect from the antiviral drug and can be used as a measure of viability (Van Meerloo et al. 2011).

MDBK cells were seeded into three 96-well cell culture plates (Falcon™, BD Biosciences, Franklin Lakes, USA) at a density of 2.5×10^4 cells per well, incubated for 24-hours, and then exposed to a panel of concentrations of each antiviral drug. Ganciclovir concentrations of 1600 µM, 1200 µM, 800 µM, 600 µM, 400 µM, 200 µM, 100 µM, 50 µM, 25 µM, and 12.5 µM and Cidofovir concentrations of 300 µM, 200 µM, 150 µM, 100 µM, 75 µM, 50 µM, 25 µM, 12,5 µM, 6,25 µM, and 5 µM were tested. Both a negative control (no antiviral drug, + MTT) and a background (no antiviral drug, no MTT) were used on each plate (Fig. 10).

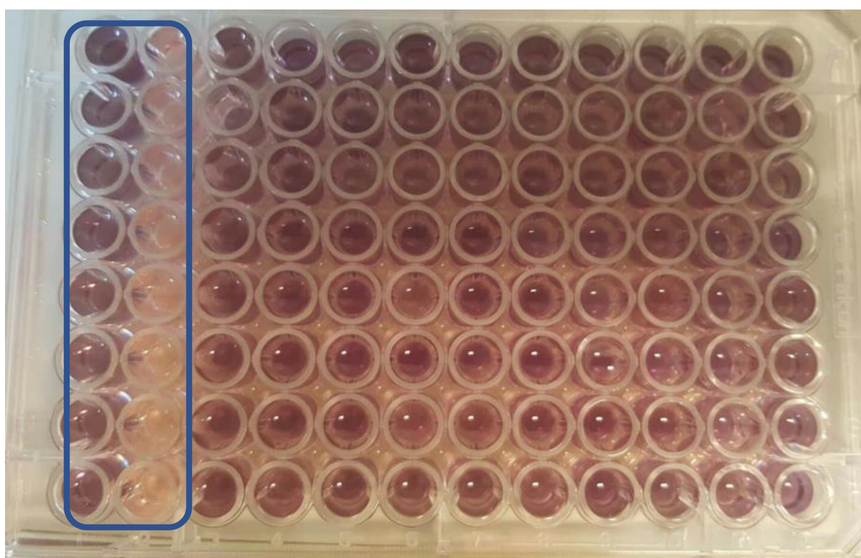


Figure 10. 96-well plate in the MTT cytotoxicity assay. The two controls are highlighted with a blue box: a negative control in the first column and a background control in the second column.

After the plates were incubated for 72-hours, mimicking the experimental conditions of the later antiviral assay, the MTT solution was added and incubated to allow the conversion of the yellow tetrazolium salt to purple formazan crystals to occur. The solubilizing solution was then added to dissolve any crystals present and the plates were incubated for an additional 24-hours. The amount of purple formazan crystals present was quantified by measuring the absorbance (as optical density) of the solutions with a microplate reader (Epoch Microplate Spectrophotometer, BioTek, Winooski, USA) at a wavelength of 650nm. The negative control was used for reference for the absorbance values. The resulting absorbance values were used to calculate the percentage of cytotoxicity .

$$\%\ Cytotoxicity = \frac{ODc - ODt}{ODc}\ x\ 100\%$$

ODc= Optical density of the control, ODt= Optical density of the test.

Equation 1: Calculation of percentage of cytotoxicity.

2.3 Antiviral Assay

The antiviral assay was composed of two different tests to measure the inhibitory ability of the antiviral drugs: an experimental CvHV2 infection of MDBK cells, and a 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay to quantify the lowest infectious titer of the virus which caused CPE. Frozen stock of 4.22 x 10^5 $TCID_{50}$/mL CvHV2 originally isolated from reindeer affected by IKC in Troms County, 2009, was used for the experimental infection (Tryland et al. 2009).

2.3.1 Experimental infection and antiviral challenge

A solution of MDBK cells in EMEM and horse serum (10%) was distributed into 24-well plates (Nunc™, ThermoFisher Scientific, Waltham, USA) at a density of 1.5 x 10^5 cells per well and incubated (37°C, 5% CO_2) for 24 hours to achieve a cell monolayer in each well. Each plate was subsequently inoculated with a single concentration of antiviral drug to be tested (one plate for each concentration), followed by six tenfold dilutions of CvHV2 (10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 $TCID_{50}$/mL). Each combination of drug concentration and viral dilution (example: 200 μM

Ganciclovir and 10^4 $TDCID_{50}$/mL CvHV2) had three replicate wells included on the same 24-well plate. The maximum drug concentrations tested in the antiviral assay were selected based on the results of the cytotoxicity assays. Both a positive (antiviral drug without CvHV2) and negative (only MDBK cells, without antiviral drug or CvHV2) controls were used. While a control plate (MDBK cells, without antiviral drug) was also inoculated with the CvHV2 dilutions as the viral control.

The 24-well plates were incubated for three days (37°C, 5% CO2) and observed with a microscope. Any visible CPE was documented and photographed (Fig. 11). In order to fully lyse the MDBK cells and release intracellular viral particles, the 24-well plates were subjected to three freeze-thaw cycles at -80°C. The supernatant and cell debris were collected in Eppendorf tubes and frozen at -80C until later use in the $TCID_{50}$ assay. The solutions from the three replicate wells for each combination of drug concentration and viral dilution were pooled into the same Eppendorf tube.

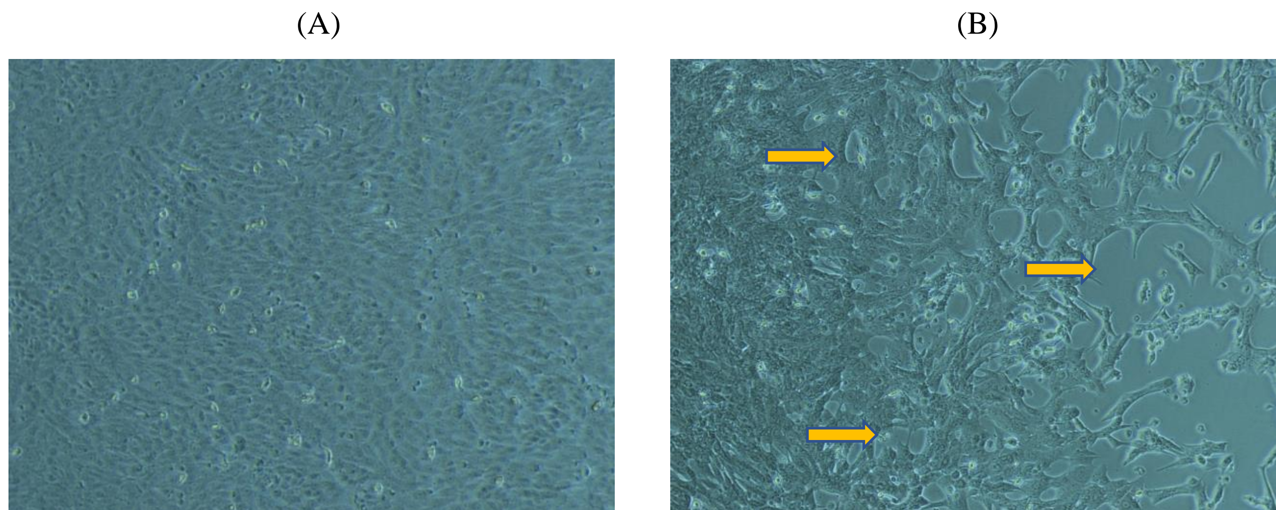


Figure 11. MDBK cell monolayer during the experimental infection and antiviral challenge, showing a healthy unaffected monolayer of cells (A) and presence of CPE (B) highlighted with yellow arrows.

2.3.2 $TCID_{50}$ Viral Quantification Assay

The $TCID_{50}$ assay performed in this experiment is an endpoint dilution assay that quantifies the amount of viable virus after exposure to Ganciclovir and Cidofovir. Using the solutions collected during the antiviral challenge experiments, a 10-fold dilution series (10^{-1} to 10^{-8}) was performed for each drug concentration and viral dilution and pipetted into 96-well plates containing 25,000 MDBK cells per well. The plates were incubated (37°C, 5% CO2) for three days to allow any subsequent CPE to become visible. After the incubation period, the cells were fixed using 100 µL formaldehyde (3.7%) per well, dyed with 50 µL Crystal Violet solution (0.1%), and observed visually for the presence of CPE.

The $TCID_{50}$ values were calculated using the Reed-Muench counting method (Reed and Muench 1938; Hierholzer and Killington 2005). This method involves counting rows of the 96-well plate that have visible CPE and calculating the 50% dilution endpoint. The $TCID_{50}$ value will be between the last row of dilutions in which >50% of the wells show CPE and the first row showing <50% CPE and is a method to quantify the remaining number of viable viral particles after exposure to the antiviral.

2.3.3 Statistics and Analysis

Statistical analysis was completed using R software (Version 0.99.878, 2009-2016). Simple Linear Models were used to analysis the results of the antiviral assay. Results of the Linear Model analysis were considered significant with p-values < 0.05. Descriptive statistics in the form of a percent inhibition were calculate to more further illustrate the magnitude of antiviral effect

3. RESULTS

3.1 Cytotoxicity assay

Cell viability was evaluated after 72-hour exposure (37°C and 5% CO_2) to ganciclovir and cidofovir, in two respective runs of the MTT cytotoxicity assay. Ganciclovir caused a marked drop in cell viability at concentrations >200 µM, reducing cell viability by 20% or greater (Fig. 12). Cell viability dropped to less than 50% for concentrations above 600 µM ganciclovir. Concentrations below the cytotoxicity concentration 20 threshold (CC20), meaning the concentrations that did not cause reduce cell viability by more than 20%, were accepted as candidate ganciclovir concentrations for use in the sequent antiviral assay.

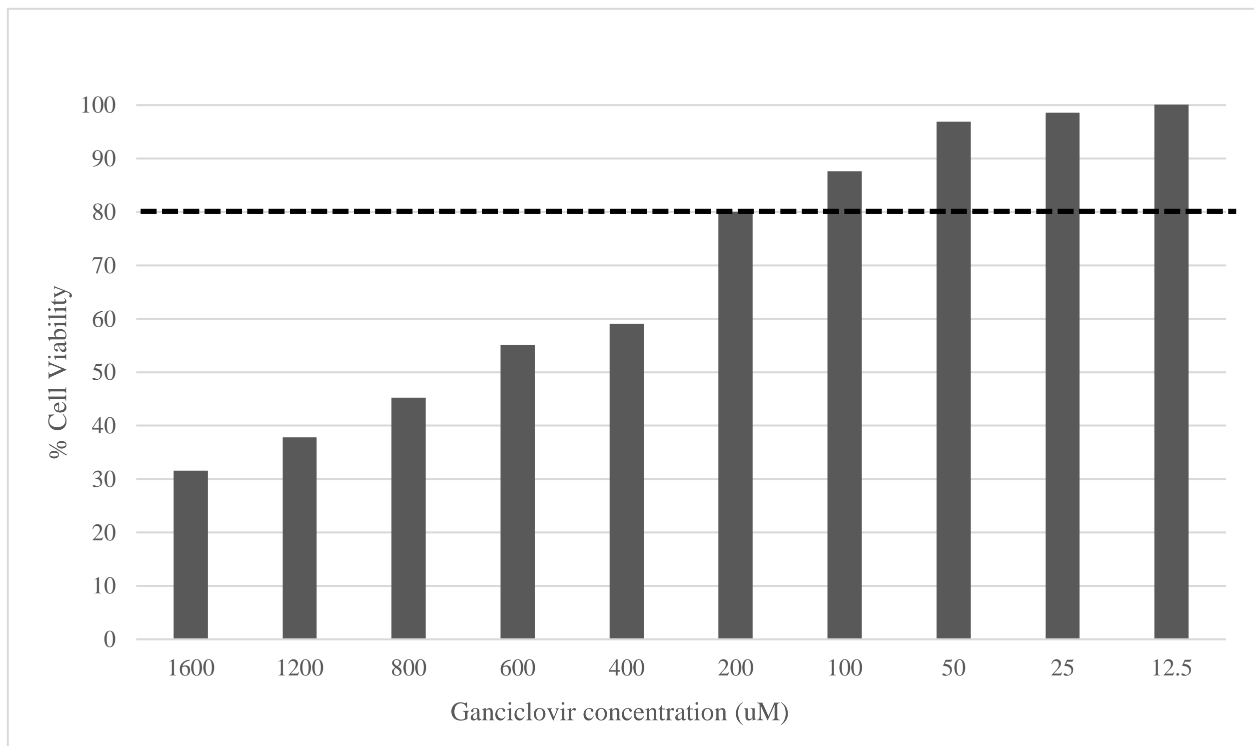


Figure 12. Ganciclovir MTT cytotoxicity assay results: average percentage of viable cultured MDBK cells after 72-hour exposure to a panel of ganciclovir concentrations (n=24 per concentration). The CC20 threshold is marked by a dashed line at 80% cell viability.

No cidofovir concentration tested in the MTT cytotoxicity assay caused a reduction of cell viability greater than 31% (Fig. 13). Cells exposed to cidofovir concentrations 75µM, 100 µM, 150 µM, 200 µM and 300 µM were <80% viable, thus concentrations ≤50 µM were below the CC20 threshold and were considered for use in the later antiviral assay.

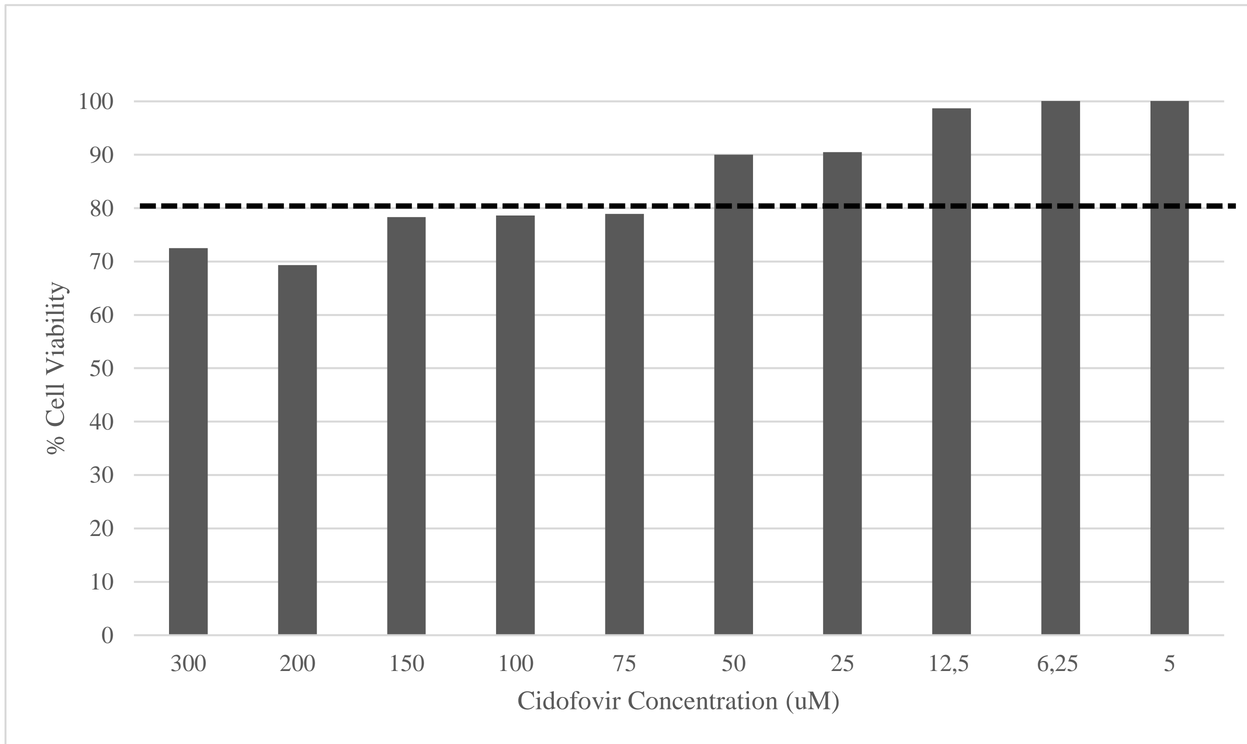


Figure 13. Cidofovir MTT cytotoxicity assay results: average percentage of viable cultured MDBK cells after 72-hour exposure to a panel of Cidofovir concentrations (n=24 per concentration). The CC20 threshold is marked by a dashed line at 80% cell viability.

3.2 Antiviral Assay

Viral titers after 72-hour exposure to ganciclovir and cidofovir were estimated with the Reed-Muench counting method (Reed and Muench 1938) and calculation of $TCID_{50}$ values (Equation 2). The $TCID_{50}$ viral titers decreased with increasing drug concentrations for both ganciclovir and cidofovir, with the exception of the lowest concentrations tested of both drugs, 6.25 µM

ganciclovir and 3.125 cidofovir (Table 2). At these lowest concentrations, titers were seemingly comparable to the titers of the untreated wells. The greatest reduction in viral titers occurred with the 10^0 initial viral load with the highest antiviral concentrations, 200 µM ganciclovir and 50 µM cidofovir. This combination of the 10^0 initial viral load and the highest antiviral concentrations resulted in first degree titers levels of 1.20 and 1.87 log_{10} $TCID_{50}$/mL, for ganciclovir and cidofovir respectively. Reduction in titers with initial viral loads 10^2-10^5 $TCID_{50}$/mL ranged from 2.32 to 3.50 log_{10} $TCID_{50}$/mL with 200 µM ganciclovir and 2.21 to 3.75 log_{10} $TCID_{50}$/mL with cidofovir. Complete inhibition of CvHV2 replication did not occur with any combination of initial viral load and antiviral drug concentration tested.

Table 1. Antiviral activity of ganciclovir (GCV) and cidofovir (CID) shown through $TCID_{50}$/mL values across a panel of initial viral inoculation loads (10^0-10^5 $TCID_{50}$/mL) and antiviral concentrations. All values are expressed as an average of the two experimental runs with $TCID_{50}$/mL units.

Initial viral load ($TCID_{50}$/mL)	GCV Concentration					
	200 µM	**50** µM	**25** µM	**12,5** µM	**6,25** µM	**No GCV**
10^5	$10^{4.40}$	$10^{5.24}$	$10^{6.04}$	$10^{6.62}$	$10^{7.28}$	$10^{7.25}$
10^4	$10^{4.21}$	$10^{5.22}$	$10^{5.72}$	$10^{6.01}$	$10^{6.28}$	$10^{6.53}$
10^3	$10^{3.71}$	$10^{5.21}$	$10^{6.04}$	$10^{6.26}$	$10^{5.43}$	$10^{6.54}$
10^2	$10^{3.55}$	$10^{5.05}$	$10^{5.21}$	$10^{4.82}$	$10^{5.71}$	$10^{6.22}$
10^1	$10^{2.21}$	$10^{4.71}$	$10^{5.23}$	$10^{5.71}$	$10^{6.04}$	$10^{5.71}$
10^0	$10^{1.20}$	$10^{3.26}$	$10^{5.20}$	$10^{3.41}$	$10^{5.24}$	$10^{5.21}$

Initial viral load ($TCID_{50}$/ml)	CID Concentration					
	50 µM	**25** µM	**12,5** µM	**6,25** µM	**3,125** µM	**No CID**
10^5	$10^{4.87}$	$10^{5.32}$	$10^{5.48}$	$10^{6.93}$	$10^{7.37}$	$10^{7.70}$
10^4	$10^{5.24}$	$10^{4.59}$	$10^{5.21}$	$10^{6.20}$	$10^{7.20}$	$10^{7.47}$
10^3	$10^{4.54}$	$10^{5.24}$	$10^{6.21}$	$10^{7.19}$	$10^{7.47}$	$10^{7.47}$
10^2	$10^{3.95}$	$10^{4.41}$	$10^{5.24}$	$10^{6.93}$	$10^{7.70}$	$10^{7.70}$
10^1	$10^{3.99}$	$10^{4.31}$	$10^{4.74}$	$10^{6.20}$	$10^{7.70}$	$10^{6.20}$
10^0	$10^{1.87}$	$10^{3.00}$	$10^{4.37}$	$10^{5.37}$	$10^{7.70}$	$10^{6.39}$

The strength of the relationship between ganciclovir and cidofovir exposure and viral titers estimated by the $TCID_{50}$ values, was tested with simple linear regression analysis. $TCID_{50}$/mL values were logarithmically transformed prior to the regression analysis and passed standard normality and linear model assumption testing.

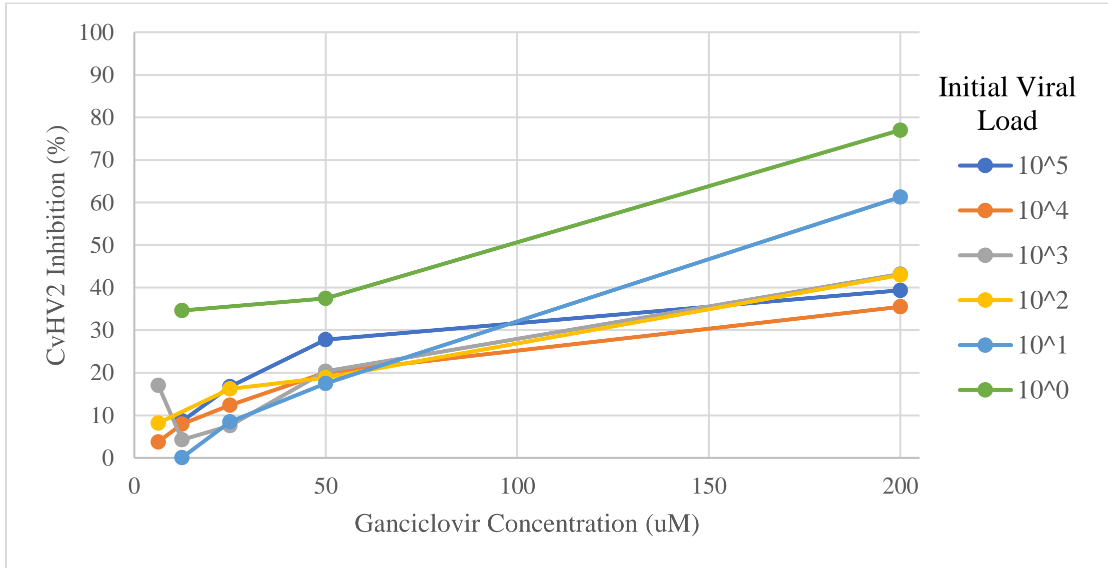
A significant (p-value <0.05) negative relationship was found with ganciclovir and all initial viral loads, and cidofovir with 10^5 and 10^3-10^1 $TCID_{50}$/mL initial viral loads, demonstrating a negative dose-response of CvHV2 to both drugs. The 10^4 initial viral load in the cidofovir experiment was not shown to be significant (p-value >0.05).

Table 2. Linear regression model results of relationship between ganciclovir (GCV) and cidofovir (CID) exposure and estimated viral titers ($TCID_{50}$/mL).

Initial Viral Load	GCV			CID		
	p-value	R^2	*m* (slope)	p-value	R^2	*m* (slope)
10^5	0.02292	0.7042	-0.013209	0.02746	0.6776	-0.055
10^4	0.004864	0.8604	-0.010451	***0.1224***	0.3604	-0.04345
10^3	0.008938	0.8121	-0.012379	0.003394	0.883	-0.06541
10^2	0.01747	0.7403	-0.010637	0.01872	0.7315	-0.07436
10^1	0.0001396	0.976	-0.018373	0.01278	0.7766	-0.08359
10^0	0.02058	0.719	-0.018777	0.01411	0.7656	-0.10354

The percentage of inhibition of CvHV2 by both antiviral drugs was calculated and plotted for comparison of effect of the different initial viral loads (Fig. 14).

(A)



(B)

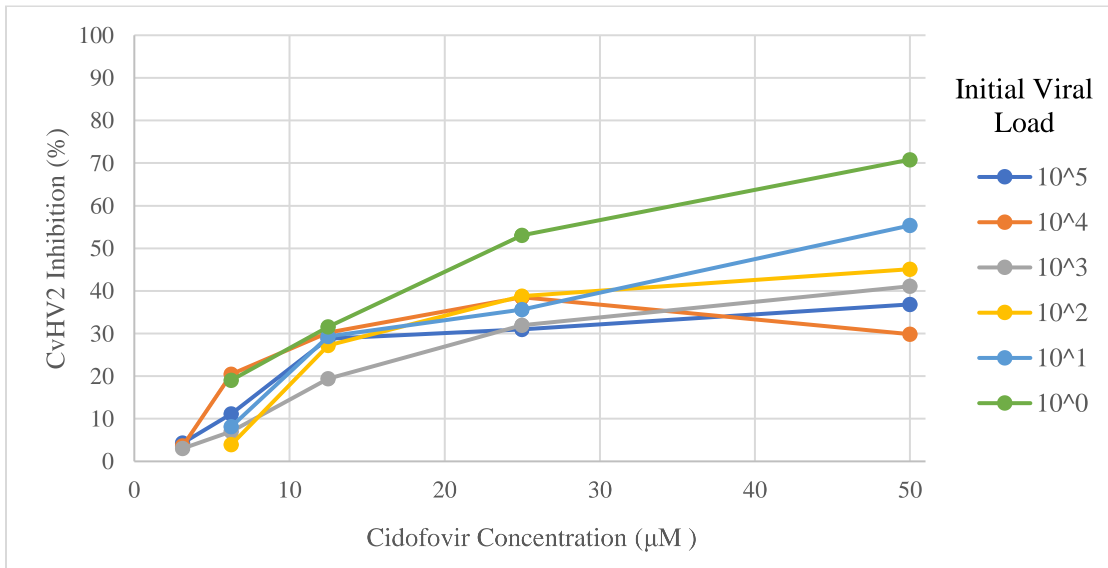


Figure 14. Inhibition of CvHV2 replication shown as a percentage reduction in $TCID_{50}$ values in cells exposed to antiviral compounds (A: ganciclovir, B: cidofovir) compared to the $TCID_{50}$ values of unexposed wells, at all initial viral loads 10^0-10^5 $TCID_{50}$/mL CvHV2.

4. DISCUSSION

No veterinary treatment currently exists for management of CvHV2 infections and the only prior study investigating the effect of antiviral drugs against CvHV2, found that the commonly used antiherpetic drug acyclovir, had limited inhibitory effect (Roche 2016). Due to the indications that CvHV2 is the causative agent for IKC in reindeer in Norway (Tryland et al. 2009; Tryland et al. 2017), this experiment was designed to provide further insight into future possibilities of developing an antiviral treatment for CvHV2, by evaluating the effect of two antiviral drugs, ganciclovir and cidofovir, *in vitro*.

4.1 Cytotoxicity in MDBK cell culture

Prior to the antiviral assay, it was necessary to establish the maximum concentrations of the two antiviral drugs in MDBK cell culture to keep the later antiviral assay unbiased from drug related toxicity. In this experiment, drug concentrations were selected for use in the antiviral assay if the concentration caused no more than a 20% reduction in cell viability and were above the cytotoxic concentration 20 (CC_{20}). It would have been possible to use a stricter selection criteria than CC_{20} because some cytotoxic effect is still occurring at this threshold, however CC_{20} was chosen as it has been previous used as a standard in other similar antiviral drug studies (Elia et al. 2015, Roche 2016, Camero et al. 2017). Furthermore, testing of antiviral drugs *in vitro* using an immortal cell line is the most preliminary step in developing a disease treatment and in this study it was considered tolerable to accept concentrations at the CC_{20} threshold in order to test a wider panel of drug concentrations and gain as much insight as possible into the effect of the drugs.

Because CC_{20} was used to select the drug concentrations tested, it is possible there may have been some bias of drug related toxicity in the concentrations of 200 µM, 50 µM, and 25 µM ganciclovir and 50 µM, 25 µM, and 12.5 µM cidofovir. In the context of use in veterinary medicine, it would likely be necessary to further consider the impacts of these high concentrations to try to minimize potential adverse effects due to toxicity *in vivo*. In humans, ganciclovir is linked to significant myelosuppression leading to anemia and neutropenia in and is of carcinogenic concern (Kimberlin and Whitley 2007, Markham and Faulds 1994). Human

HSV keratitis patients topically treated with 0.15% ganciclovir experienced only local effects, including superficial punctate keratopathy and conjunctival hyperemia, but both were considered highly tolerable by medical observers (Chou and Hong 2014). Systemic adverse effects are infrequent with cidofovir, but local effects of topical eye treatments such as inflammation of conjunctiva and upper eyelid, are in both humans and animals (Hillenkamp et al. 2002; Broekema and Dikkers 2008; Fontenelle et al. 2008).

However, in the context of this preliminary *in vitro* study, 200 µM, 50 µM, and 25 µM ganciclovir and 50 µM, 25 µM, and 12.5 µM cidofovir were considered acceptable to be included in the analysis. The $TCID_{50}$ results of these highest concentrations passed normality and variance testing prior to the linear model analysis and were not considered outliers.

4.2 Antiviral effect against CvHV2

In both the ganciclovir and cidofovir experiments, a statistically significant (p-value <0.05) negative relationship was found in the linear model analysis of drug exposure and resulting viral titer levels, with the initial viral loads of 10^0-10^5 $TCID_{50}$/mL for ganciclovir and 10^0-10^3 and 10^5 $TCID_{50}$/mL for cidofovir. This confirms a negative dose-response relationship between both drugs and CvHV2 titers. However, it appears that the magnitude of antiviral effect is minimal despite the statistically significant dose-response relationship. The resulting slope values range from -0.010451 to -0.018777 log(TCID50/mL)/µM for ganciclovir and -0.04345 to -0.103354 log(TCID50/mL)/µM for Cidofovir. There was no apparent biological explanation for the one single initial viral load was not found to be significant (p-value>0.05), 10^4 $TCID_{50}$/mL in the cidofovir experiment. This may have occurred because the extremely low slope values of the linear regression analysis (-0.04345 log(TCID50/mL)/µM). Small natural variation in the experiment could have overcome the very low magnitude of effect indicated by the slope values and caused the 10^4 $TCID_{50}$/mL to not be significant.

Both ganciclovir and cidofovir showed increases trends in the percentage of inhibition with increasing drug concentration (Fig.14). Both drugs also exhibited a clear trend of increasing CvHV2 inhibition at lower initial viral loads. Overall viral inhibition was low, with only the lowest initial viral loads, 10^1 and 10^0 $TCID_{50}$/mL with the highest concentrations of ganciclovir

and cidofovir causing more than 50% inhibition. Calculation of half maximal inhibitory concentration (IC50) was not calculated in this experiment because 50% inhibition did not occur with such a majority of the initial viral loads.

Based on the linear model results, ganciclovir and cidofovir were more successful in inhibiting CvHV2 than acyclovir, the only other antiviral drug previously tested against CvHV2 (Roche 2016). In the acyclovir study, linear modeling was also used to analysis the strength of the relationship between drug exposure and estimated viral titers and a significant negative dose-response relationship was only found at initial viral loads of 10^1 and 10^2 $TCID_{50}$/ml. In this study of ganciclovir and cidofovir, a significant dose-response relationship was found at initial viral loads of 10^0-10^5 $TCID_{50}$/ml for ganciclovir and 10^0-10^3 and 10^5 $TCID_{50}$/ml for cidofovir. The maximum reduction of estimated CvHV2 titers by acyclovir was 1.67 log_{10} $TCID_{50}$0/ml while the maximum reductions caused by ganciclovir and cidofovir were 3.5 log_{10} $TCID_{50}$/ml and 3.75 log_{10} $TCID_{50}$/ml respectively.

Comparison of ganciclovir or cidofovir to the results of other antiviral drug tests can be made by contrasting viral inhibition values or $TCID_{50}$ results directly. The viral inhibition caused by ganciclovir and cidofovir appears only moderate compared to previous studies of other nucleoside analog antiviral drugs considered successful in treating related alphaherpesvirus. No combination of initial viral load and ganciclovir or cidofovir concentration resulted in complete inhibition of CvHV2. A feline herpesvirus 1 (FHV1) *in vitro* study of a panel of antiviral drugs including ganciclovir and cidofovir, found that both drugs were much more powerful inhibitors of FHV1 at similar concentrations to those tested in this study (van der Meulen et al. 2006). Using a plaque-reduction assay instead of estimating viral titers through $TCID_{50}$ calculations, van der Meulen et al. found <75% reduction in plaque number at a ganciclovir concentration of 27 μM and a cidofovir concentration of 32 μM. CvHV2 was inhibited <40% at similar concentrations of ganciclovir and cidofovir respectively. A paired *in vitro* and *in vivo* of canine herpesvirus 1 (CHV1) found cidofovir to highly successful in inhibiting CHV1 in MDBK cell culture, with a half maximal effective concentration (EC50) of 4.4 ± 2.3 μM and over 75% inhibition occuring with 50 μM (Ledbetter et al. 2015). The lowest concentration of cidofovir tested with CvHV2 was 3.125 μM and caused <10% inhibition of viral activity.

Given the seemingly moderate the antiviral effects of ganciclovir and cidofovir against CvHV2 to other successful studies of related herpesviruses, ganciclovir and cidofovir may be candidates for development as treatments for IKC infections in reindeer only with certain considerations.

While it could be possible to simply test concentrations of ganciclovir and cidofovir to try to achieve greater inhibition of CvHV2, this would cause too much cytotoxicity. An already lenient cytotoxicity threshold (CC_{20}) was chosen for this study order to test a wider panel of drug concentrations in this preliminary stage of treatment testing, and complete inhibition of CvHV2 did not occur even at the highest concentrations tested. Ideal candidate drugs for development as a treatment *in vivo* are have a high therapeutic index, meaning they are highly effective with low toxicity, (Prusoff et al. 1989). Therefore, possibilities to further increase the effect of either drug should be considered, such that complete inhibition of CvHV2 would occur with treatment of fully non-toxic concentrations.

A synergistic antiviral effect of the immunosuppressive drug mizoribine has been found with guanosine nucleoside analogs which could potentially increase the effect of ganciclovir against CvHV2. In treatment of CpHV1, acyclovir alone, in concentrations up to 440 µM, were not successful in significantly reducing viral replication, but with the addition of 154 µM mizoribine, a concentration of 28.5 µM acyclovir was successful in reducing CpHV1 activity by 50% and 440 µM caused complete inhibition of the viral replication (Elia et al. 2015). There are no previous studies of ganciclovir and mizoribine in the treatment of alphaherpesviruses, but the combination has been shown to have a strong synergistic effect in inhibiting cytomegalovirus, a betaherpesvirus, replication *in vitro* (Kuramoto et al. 2010).

As there are no known synergistic combinations of cidofovir with other compounds, it may not be possible to increase the therapeutic index of cidofovir in treating CvHV2, but given the success of *in vivo* cidofovir treatment against other herpesvirus infections, this may be could be considered acceptable. A intravaginal cidofovir treatment in goats experimentally inoculated with CpHV1, was able to prevent development of genital lesions and reduced viral shedding in effected animals (Tempesta et al. 2008). Topical cidofovir solutions have also been found to be successful in the treatment of herpetic eye diseases in domestic dogs and cats, but are associated

with moderate local ocular toxicity effects such as exacerbation of conjunctivitis and ulcerative blepharitis in both species (Fontenelle et al. 2008; Ledbetter et al. 2015).

4.3 Development of a practical treatment

After considering methods to increase the therapeutic index of either ganciclovir or cidofovir, the next steps in developing an antiviral treatment for IKC would include a secondary *in vitro* study in primary reindeer cells, specifically either corneal or conjunctival epithelial cells. Using an immortal cell line such as MDBK is very useful in a preliminary because they are commercially produced and can be easily maintained in a lab setting (Prusoff et al. 1989). However, using a species specific cell type would better address the behavior of CvHV2 in reindeer, its natural host. Developing primary reindeer cell lines would be challenging as no established protocol for reindeer corneal or conjunctival cells exists, but protocols for establishment of similar primary cell lines from humans, dogs, and cats are published and may be a useful starting point (Kim et al. 2004; Sandmeyer et al. 2005; Werner et al. 2007).

The final and most relevant step in developing an antiviral treatment for CvHV2 would be testing the drug as a treatment in live, either in under experimental conditions or through off-label use during a naturally occuring outbreak of IKC. Testing off-label during an outbreak would be a highly convenient to test the treatments as it would require far less costs and planning as a controlled, inoculation study, but this would be less scientifically and statistically rigorous than an experimental inoculation study design (Gazarian et al. 2006). An experimental treatment study would allow for most conditions of the infection to be controlled including timing of the inoculation, observation before and after application of the treatment, and even simply standardization of the initial pathogen. While CvHV2 has been clearly shown to be a causative agent of IKC in Norway, IKC is a multifactorial disease and there are other viruses and bacteria associated with the condition (Tryland et al. 2009; Tryland et al. 2017). It is not possible to know in the field during an outbreak if additional pathogens are contributing to the disease (Tryland et al. 2009; Tryland et al. 2017).

Even with the development of a successful antiviral treatment for IKC, there will be significant challenges in animals that are free-ranging most of the year and experience increased stress when

handled (Wiklund et al. 1995). The most useful treatment of IKC in reindeer would involve a minimal number of applications, preferably one single administration of the drug. Systemic treatment of IKC would likely be more practical than a topical treatment as it could be delivered in a single dose and alleviate the need for affected animals to be corralled and handled multiple times per day for application of the topical drug solution. Cidofovir may be a better candidate for development of a systemic treatment than ganciclovir, given the adverse effects associated with systemic ganciclovir treatment (Jacbonson et al. 1987), as well as the long half-life and extended intracellular retention of cidofovir (Ho et al. 1992; Markham and Faulds 1994; Aduma et al. 1995; Kimberlin and Whitley 2007). A rabbit model study of human CMV retinitis, showed that cidofovir is exceptional well retained in the vitreous humor and continued to exhibit an antiherpetic effect up to ten days following a three day treatment (Flores-Aguilar et al. 1994). Extended intracellular ocular retention of cidofovir could be an ideal quality for a one-time systemic treatment delivery as the antiviral effect would continue longer period.

5. CONCLUSION

This study investigated the antiviral effects of ganciclovir and cidofovir against CvHV2 *in vitro*, with the aim of identifying a candidate drug for development of a treatment for IKC in semi-domesticated reindeer. A significant dose-dependent relationship between exposure to ganciclovir and cidofovir and estimated viral titer levels, however, neither drug caused complete inhibition of CvHV2 and only a moderate percent of CvHV2 inhibition occurred. This indicated that ganciclovir and cidofovir are candidates for further study of candidate drugs for development of a treatment for IKC, only if possibilities to increase the therapeutic index of either ganciclovir or cidofovir is considered. Development of a practical antiviral treatment for IKC in free-ranging semi-domesticated reindeer will be challenging, but would, based on the enzootic distribution of CvHV2 in many reindeer populations and its ability to cause IKC, be a benefit to both the economics and animal welfare of reindeer husbandry in Fennoscandia.

Bibliography

Aduma, P., Connelly, M.C., Srinivas, R.V., Fridland, A. 1995. Metabolic diversity and antiviral activities of acyclic nucleoside phosphonates. Mol Pharmacol. 47: 816-822.

Angelos, J.A., 2010. *Moraxella bovoculi* and infectious bovine keratoconjunctivitis: Cause or coincidence? Vet Clin North Am Food Anim Pract. (26): 73-78.

Angelos, J.A., 2015. Infectious bovine keratoconjunctivitis (pinkeye). Vet Clin North Am Food Anim Pract. 31 (1): 61-79.

Åsbakk, K., Kumpula, J., Oksanen, A., Laaksonen, S. 2014. Infestation by *Hypoderma tarandi* in reindeer calves from northern Finland - Prevalence and risk factors. Vet Parasit. 200: 172-178.

Banfield, A.F. 1961. A revision of the Reindeer and caribou genus Rangifer. Biological Series Report 66. National Museum of Canada, Bulletin 177.

Bergman, A. 1912. Contagious keratitis in reindeer. Scand Vet J. 2: 145-177.

Bevanger, K., Jordhøy, P. (2004). *Reindeer- the mountain nomad*. Oslo: Naturforlaget.

Blix, AS. (2005). *Arctic animals and their adaptations to life on the edge*. Trondheim, Norway: Tapir Academic Press.

Broekema, F., Dikkers, F. 2008. Side-effects of cidofovir in the treatment of recurrent respiratory papillomatosis. Eur Arch Otorhimolgaryngol. 265 (8): 871-879.

Camero, M., Buonavoglia, D., Lucente, MS., et al. 2017. Enhancement of the antiviral activity against caprine herpesvirus type 1 of acyclovir in association with mizoribine. Res Vet Sci. 111: 120-123.

Cameron, R.D., Smith, W.T., White, R.G, Griffith, B. 2005. Central Arctic caribou and petroleum development: distributional , nutritional ,and reproductive implications. Arctic. 58 (1): 1-9.

Chou, T., Hong, B. 2014. Ganciclovir ophthalmic gel 0.15% for the treatment of acute herpetic keratitis: background, effectiveness, tolerability, safety, and future application. Ther Clin Risk Manag. 10: 665-681.

Collins, P. 1983. The spectrum of antiviral activities of acyclovir in vitro and in vivo. J Antimicrob Chemother. 12: 19-27.

Connelly, M.C., Robbins, B.L., Fridland, A. 1993. Mechanism of uptake of the phosphonate analog (S)-1(3-hydroxy-2-phosphonylmethoxy-propyl) cytosine (HPMPC) in Vero cells. Biochem Pharmacol. 46: 1053-1057.

Coutinho A.E., Chapman, K.E. 2011. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol Cell Endocrinol. 335:2–13.

Crumpacker, C.S., Schnipper, L.E., Zaia, J.A., Levin, M.J., 1979. Growth Inhibition by Acycloguanosine of Herpesviruses Isolated from Human Infections. Antimicrobial Agents and Chemotherapy 15, 642-645.

Das Neves, C.G., Mork, T., Godfroid, J., Sorensen, K.K., Breines, E., Hareide, E., Thiry, J., Rimstad, E., Thiry, E., Tryland, M. 2009a. Experimental Infection of Reindeer with Cervid Herpesvirus 2. Clin Vaccine Immunol. 16 (12): 1758-1765.

Das Neves, C.G., Mork, T., Thiry, J., Godfroid, J., Rimstad, E., Thiry, E., Tryland, M. 2009b. Cervid herpesvirus 2 experimentally reactivated in reindeer can produce generalized viremia and abortion. Virus Res. 145: 312-328.

Das Neves, C.G., E. Rimstad, Tryland, M. 2009d. Cervid herpesvirus 2 causes respiratory and fetal infections in semidomesticated reindeer. J Clin Microbiol. 47:1309–1313.

Das Neves, C., Thiry, J., Skjerve, E., Yoccoz, N., Rimstad, E., Thiry, E., Tryland, M. 2009e. Alphaherpesvirus infections in semi-domesticated reindeer: a cross-sectional serological study. Vet. Microbiol. 139, 262-269.

Das Neves, C.G., Roth, S., Rimstad, E., Thiry, E., Tryland, M. 2010. Cervid herpesvirus 2 infection in reindeer: a review. Vet Microbiol. 143: 70-80.

Das Neves, C.G., Ihlebaek, H.M., Skjerve, E., Hemmingsen, W., Li, H., Tryland, M. 2013. Gammaherpesvirus infection in semidomesticated reindeer (*Rangifer tarandus tarandus*): a cross-sectional, serologic study in northern Norway. Journal of Wildlife Disease. 49 (2): 261-269.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., Thiry, E. 2009. The order Herpesvirales. Archives of Virology 154 (1): 171-177.

De Clercq, E., Holy, A., Rosenber, I., Sakuma, T., Balzarini, J. Maudgal, P.C. 1986. A novel selective broad-spectrum anti-DNA virus agent. Nature. 323: 464-467.

Deregt, D., Gilbert, S.A., Campbell, I., Burton, K.M., Reid, H.W., Little-van den Hurk, S.D., Penniket, C., Baxi, M.K. 2005 Phylogeny and antigenic relationship of three cervid herpesviruses. Virus Re. 114: 140-148.

Dubay, S.A., Williams, E.S., Mills, K., Boerger-Field, A.M. 2000. Association of *Moraxella ovis* with keratoconjunctivitis in mule deer and moose in Wyoming. J Wild Dis. 36 (2): 241-247.

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

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

Elia, G., Camero, M., Decaro, N., Lovero, A., Martella, V., Tempesta, M., Buonavoglia, C., Crescenzo, G. 2015. *In vitro* inhibition of caprine herpesvirus 1 by acyclovir and mizoribine. Res Vet Sci. 99: 208-211.

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

Enan, Abdallah, F.M., Sobhy, H. 2012. Effect of Acyclovir on bovine herpesvirus type 1 infection in *in vitro* cultured cells. Int J Virol. 8 (4): 307-312.

Evans, A., Bey, F., Schoster, J.V., Faarder, F.E., Finstad, G.L. 2008. Preliminary studies on the etiology of keratoconjunctivitis in reindeer (*Rangifer tarandus tarandus*) calves in Alaska. J Wild Dis. 44: 1051-1055.

Evans, A., Das Neves, C.G., Finstad, G.F., Beckman, K.B., Skyerve, E., Nymo, I.H., Tryland, M. 2012. Evidence of alphaherpesvirus infections in Alaskan caribou and reindeer. BMC Vet Res. 8 (5).

Fields, B. N., Knipe, D. M., Howley, P. M., & Griffin, D. E. (2001). *Fields virology*. Philadelphia: Lippincott Williams & Wilkins.

Flores-Aguilar, M., Huang, J.S., Wiley, C.A., Clercq, E., Vuong, C. 1994. Long-acting therapy of viral retinitis with (S)-1-(2-Hydroxy-2-Phosphonylmethoxypropyl)cytosine. J Inf Dis. 169: 642-649

Fontenelle, J., Powell, C., Veir, J., Radecki, S. 2008. Effect of topical ophthalmic application of

cidofovir on experimentally induced primary ocular feline herpesvirus-1 infection in cats. Amer J Vet Res. 69 (2): 289-93.

Forbes, B.C., Kumpula, T., Meschtyb, N. 2016. Sea ice, rain-on-snow and tundra reindeer nomadism in Arctic Russia. Biol Lett. 12 (11).

Forterre, P. 2006. The origin of viruses and their possible roles in major evolutionary transitions. Virus Res. 117 (1): 5-16.

Furlong, D., Swift, H., Roizman, B. 1972.Arrangement of herpesvirus deoxyribonucleic acid in the core. J Virol. 10 (5): 1071-1074.

Gazarian, M., Kelly, M., McPhee, J., Gradins, L., Ward, R., Campbell, J. 2006. Off-label use of medicines: consensus recommendations for evaluating appropriateness. Med J Aus. 185 (10):544-549.

Giacometti, M., Janovsky, M., Belloy, L., Frey, J. 2002. Infectious keratoconjunctivitis of ibex, chamois, and other caprinae. Rev Sci Tech. 21: 335-345.

Giangaspero, M., Orusa, R., Nicholas, R. AJ., Harasawa, R., Ayling, R.D., Churchward, C.P., Whatmore, A., Bradley, D., Robetto, S., Sacchi, L., Domenis, L. 2010. Characterization of *Mycoplasma* isolated from an ibex (Capra ibex) suffering from keratoconjunctivitis in northern Italy. J Wild Dis. 46 (4): 1070-1078.

Gortazer, C., Fernandez de Luco, D., Frolich, K. 1998. Keratoconjunctivitis in free-ranging red deer (*Cervus elaphus)*. Population in Spain. Z Jagdwiss. 44: 257-261.

Gravlund, P., Meldgaard, M., Paabo, S., Arctander, P. 1998. Polyphyletic origin of the small-bodied, high-Arctic subspecies of tundra reindeer (*Rangifer tarandus*). Mol Phylogenet Evol. 10 (2): 151-159

Gunn, A., 2016. Rangifer tarandus. The IUCN Red List of Threatened Species 2016: e.T29742A22167140.

Handeland, K., Slettbakk, T. 1994. Outbreaks of Clinical Cerebrospinal Elaphostrongylosis in Reindeer (*Rangifer tarandus tarandus*) in Finnmark, Norway, and their Relation to Climatic Conditions. Zoonoses Pub Heal. 41(10): 407-410.

Handeland, K., Boye, M., Bergsjo, B., Bondal, H., Isaksen, K., Agerholm, JS. 2010. Digital necrobacillosis in Norwegian wild tundra reindeer (*Rangifer tarandus tarandus*). J Comp Pathol. 143 (1): 29-38.

Helle, T. 1980. Abundance of warble fly (*Oedemagena tarandi*) larvae in semi-domestic reindeer (*Rangifer tarandus*) in Finland. Reports from the Kevo Subarctic Research Station. 16: 1-6.

Hervieux, D., Hebblewhite, M., DeCasare, N.J., Russel, M., Smith, K., Robertson, S., Boutin, S. 2013. Widespread declines of woodland caribou (*Rangifer tarandus caribou)* continue in Alberta. Can J Zool. 91 (12): 872-882.

Hierholzer J.C., Killington, R.A. (2005). *Virology Methods Manual.* Cambridge, USA: Academic Press.

Hillenkamp, J., Reindhard, T., Ross, R.S., Bohringer, D. 2002. The effect of cidofovir 1% with and without cyclosporin a 1% as a topical treatment of acute adenoviral keratoconjunctivitis: a controlled clinical pilot study. Opthalomlogy. 109 (5): 845-850.

Ho, H.T., Woods, K.L., Bronson, J.J. De Boeck, H., Martin, J.C., Hitchcock, M.M., 1992. Intracellular metabolism of the antiherpes agent (S)-1(3-hydroxy-2-phosphonylmethoxy-propyl) cytosine. Mol Pharmacol. 41: 197-467.

Inga, B. 2007. Reindeer (*Rangifer tarandus tarandus*) feeding on lichens and mushrooms:

traditional ecological knowledge among reindeer-herding Sami in northern Sweden. Rangifer. 27 (2): 93-106.

Jabs, D.A. Enger C., Bartlett J.G. 1989. Cytomegalovirus retinitis and acquired immunodeficiency syndrome. Arch Ophthalmol. 107: 75-80

Jacbonson, M.A., Miranda, D., Cederberg, D.M. 1987. Human pharmacokinetics and tolerance of oral ganciclovir. Antimicrob Agents Chemo. 31: 1251-1254.

Johnson, C.J., Ehlers, L.W., Seip, D.R. 2015. Witnessing extinction- cumulative impacts across landscapes and the future loss of an evolutionarily significant unit of woodland caribou in Canada. Biol Conserv. 186: 176-186.

Jones, C., Geiser, V., Henderson, G., Jiang, Y., Meyer, F., Perez, S., Zhang, Y., 2006. Functional analysis of bovine herpesvirus 1 (BHV-1) genes expressed during latency. Vet Micro. 113: 199-210.

Josefsen, T.D., A. Oksanen, and B. Gjerde. 2014. Parasiter hos rein I Fennoskandia – en oversikt. *Norsk Veterinærtidsskrift* 2:185–201.

Kim, H.S., Jun Song, X., de Paiva, C.S., Chen, Z., Pflugfelder, S.C., 2004. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. Exp. Eye Res. 79: 41–49.

Kimberlin, D.W., Whitley, R.J. 2007. Antiviral therapy of HSV-1 and -2. *In* Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge University Press.

Kirsch, L, Arevalo, F., Chavez de la Paz, E., Munguia, D., De Clercq, E., Freeman, W. 1995. Intravitreal cidofovir (HPMPC) treatment of cytomegalovirus retinitis in patients with acquired immune deficiency syndrome. Ophthalmology. 102: 533-543.

Klein, D.R., Meldgaard, M., Fancy, S.G. 1987. Factors determining leg length in *Rangifer* tarandus. J Mamm. 68 (3): 642-655.

Knowles, D.P., 2011. Chapter 9 - Herpesvirales, In: *Fenner's Veterinary Virology* (Fourth Edition). Academic Press, San Diego, pp. 179-201.

Kummeneje, K. 1976. Pasteurellosis in reindeer in Northern Norway: A contribution to its epidemiology. Acta Vet Scand. 17: 488–94.

Kuramoto, T., Daikoku, T., Yoshida, Y., Takmoto, M., Oshima, K. 2010. Novel anticytomegalovirus activity of immunosuppressant mizoribine and its synergism with ganciclovir. J Pharmaco Exp Therap. 333 (3): 816-821.

Laaksonen, S., Solismaa, M., Kortet, R., Kuusela, J., Oksanen, A., 2009. Vectors and transmission dynamics for *Setaria tundra* (*Filarioidea*; *Onchocercidae*), a parasite of reindeer in Finland. Parasites & Vectors. 2 (3).

Landbruksdirektoratet, 2017. Totalregnskap for reindriftsnæringen, In: Regnskap 2016 og budsjett 2017. Landbruksdirektoratet, Oslo, p. 175.

Lantis, M. 1950. The reindeer industry in Alaska. Arctic. 3 (1): 27-44.

Levings, R.L., Roth, J.A., 2013. Immunity to Bovine Herpesvirus 1: I. Viral lifecycle and innate immunity. Anim Health Res Rev. 14(1): 88-102.

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

Lyaku, J.S., Nettleton, P.F., Marsden, H. 1992. A comparison of serological relationships among 5 ruminant alphaherpesviruses by ELISA. Arch Virol. 124: 333-341.

Martin, J.C., Dvorak, C.A., Smee, D.F., Matthews, T.R, Verheyden J.H. 1983. 9-[1,3-Dihydroxy-2-propoxy)methyl]guanine: a new potent and selective antiherpes agent. J Med Chem. 26: 759-761.

McLoughlin, PD., Dzus, E., Wynes, B., Boutin, S. 2003. Declines in Populations of Woodland Caribous. J Wildlife Management. 67 (4): 755-761.

Muylkens, B., Thiry, J., Kirten, P., Schynts, F., Thiry, E. 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. Vet Res. 38 (2): 181-209.

Nathan, D.B., Heller, E.D., Perek, M. 1977. The effect of starvation on antibody production of chicks. Poult Sci. 56 (5): 1468-1471.

Nieminen, M., Helle, T. 1980. Variations in body measurements of wild and semi-domestic reindeer (*Rangifer tarandus*) in Fennoscandia. Ann Zool Fennici. 17: 275-283.

Nettleton, P.F., Sinclair, J.A., Herring J.A., Inglis D.M., Fletcher, T.J, Ross, H.M., Bonniwell, MA. 1986. Prevalence of herpesvirus infection in British red deer and investigations of further disease outbreaks. Vet Rec. 188: 267-270.

Nettleton, P.F., Thiry, E., Reid, H.W., Pastoret,P. 1988. Herpesvirus infections in cervidae, Rev. Sci. Tech. Off. Int. Epizoot. 7: 977–988.

Nybakk, K., Kjelvik, O., Kvam, T., Overskaug, K., Sunde, P. 2002. Mortality of semi-domestic reindeer *Rangifer tarandus* in central Norway. Wildlife Biol. 8(1): 63-68.

Ovilvie, K.K., Cheriyan, U.O., Radatus B.K. 1982. Biologically active acyclonucleoside analogues. II. The synthesis of 9-[2-hydroxy-1(hydroxymethyl)ethoxy]methyl]guanine (BIOLF-62. Can J Chem. 60: 3005-3010.

Pape, J.W. 1988. Treatment of gastrointestinal infections. AIDS. 2 (1): 161-167.

Palmer, L.J. 1934. Raising reindeer in Alaska. U.S. Dept. of Agriculture Publ. No. 207.

Pellett, P.E., Roizman, B., 2006. The *Herpesviridae*: a brief introduction, In: *Fields Virology,* 5th ed. Lippincott, Williams & Wilkins, Philadelphia, USA, pp. 2479-2499.

Pravettoni, R. 2010. Protecting Arctic Biodiversity, GRID Arendal.

Prusoff, W.H., Lin, T.S., August, E.M., Wood, T.G., Marongiu, M.E. 1989. Approaches to antiviral drug development. Yale J Biol Med. 62(2): 215-225.

Reed, L.J., Muench, H. 1938. A simple method of estimating fifty percent endpoints. Amer J Epidem. 27 (3): 493-497.

Rehbinder, C., Christensson, D., Glatthard, V. 1975. Parasitic granulomas in reindeer: a histopathological, parasitological and bacteriological study. Nord Vet Med. 27 (10): 499-507.

Rehbinder, C. 1977. Clinical and Epizootiological Studies on Keratitis in Reindeer. Acta Vet Scand 18 (66).

Rehbinder, C., Nilsson, A. 1995. An outbreak of kerato-conjunctivitis among corralled, supplementary fed, semi-domestic reindeer calves. Rangifer. 15 (1): 9-14.

Roche, X. 2016. Effet de l'acyclovir *in vitro* sur la replication de l'herpesvirus des cervides de type 2 dans les cellules MDBK [unpublished dissertation]. [Nantes, France]: Nantes-Atlantic National Collection of Veterinary Medicine, Food Science and Engineering

Rockborn, G., Rehbinder, C., Kilgeborn, B., Leffler, M., Klintevall, K., Nikkila, T., Landen, A., Nordkvist, M. 1990. The demonstration of a herpesvirus, related to bovine herpesvirus 1, in reindeer with ulcerative and necrotizing lesions of the upper alimentary tract and nose. Rangifer, 3: 373-384.

Roizmann, B., Desrosiers, R.C., Fleckenstein, B., Lopez, C., Minson, A.C., Studdert M.J. 1992. The family Herpesviridae- an update. Arch Virol. 123 (3-4): 425-449.

Romano, J.S., Mork, T., Laaksonen, S., Agren, E., Nymo, I.H., Sunde, M., Tryland, M. 2018. Infectious keratoconjunctivitis in semi-domesticated Eurasian tundra reindeer (*Rangifer tarandus tarandus):* microbiological study of clinically affected and unaffected animals with special reference to cervid herpesvirus 2. BMC Vet Res. 14 (15).

Rominger, E.M., Oldemyer, J.L. 1990. Early-winter diet of woodland caribou in relation to snow accumulation, Selkirk Mountains, British Columbia, Canada. J Zoo. 68 (12): 2691-2694.

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

Ryser-Degiorgis, M.P., Bischof, D.F., Marreros, N., Willisch, C., Signer, C., Filli, F., Brosi, G., Frey, J., Vilei, E.M. 2009. Detection of *Mycoplasma conjunctivae* in the eyes of healthy, free-ranging alpine ibex: possible involvement of alpine ibex as carriers for the main causing agent of infectious keratoconjunctivitis in wild caprinae. Vet Microbiol. 134: 368-374.

Sandmeyer, L.S., Keller, C.B., Bienzle, D. 2005. Culture of feline corneal epithelial cells and infection with feline herpesvirus-1 as an investigative tool. Am. J. Vet. Res. 66: 205–209.

Schaefer, J.A. 2003. Long-term range recession and the persistence of caribou in the Taiga. Conserv Biol. 17 (5): 1435-1439.

Smith, K.O., Galloway, K.S., Hodges, S.L., Ogilvie, K.K., Radatus, B.K., Kalter, S.S., Heberling, R.L. 1983. Sensitivity of equine herpesviruses 1 and 3 in vitro to a new nucleoside analogue, 9-[[2-hydroxyl-1-(hydroxymethyl) ethoxy] methyl] guanine. Amer J Vet Res. 44: 1032-1035.

Smits, S.L. Schapendonk, C.E., Van Leeuwen, M., Kuiken, T., Bodewes, R., Stalin Raj, V., Haagmans, B.L., Das Neves, C.G., Tryland, M., Osterhaus, A.E. 2013. Identification and characterization of two novel viruses in ocular infections in reindeer. Plos One. 8 (7).

Strober, W. 2013. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. Appendix 3.

Stuen, S., Krogsrud, J., Hyllseth, B., Tyler, N.C., 1993. Serosurvey of three virus infections in reindeer in northern Norway and Svalbard. Rangifer 13, 215–219.

Schwyzer, M., Ackermann, M., 1996. Molecular virology of ruminant herpesviruses. Vet Micro (53): 17–29.

Tempesta, M., Crescenzo, G., Camero, et al. 2008. Assessing the efficacy of cidofovir against herpesvirus-induced genital lesions in goats using different therapeutic regimens.

Thellman, N.M., Triezenberg, S.J. 2017. Herpes simplex virus establishment, maintenance, and reactivation: in vitro modeling of latency. Pathogens. 6 (28).

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

Thorne, E.T. 1982. Infectious keratoconjunctivitis. *In* Diseases of wildlife in Wyoming, E.T. Throne, N. Kingston, W.R. Jolley, and RC Bergstorms (eds.). Wyoming Game and Fish Department, Cheyenne, Wyoming, pp 81-84.

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

Tryland, M., Josefsen, T. D., Oksanen, A., & Aschfalk A. 2001a. Contagious ecthyma in Norwegian semi-domesticated reindeer (*Rangifer tarandus tarandus*). Veterinary Record 149: 394-395.

Tryland, M., Josefsen, T.D., Oksanen, A., Aschfalk, A. 2001b. Parapoxvirus infection in Norwegian semi- domesticated reindeer (*Rangifer tarandus tarandus*). Vet Rec. 149(13):394–5.

Tryland, M., Das Neves, C.G., Sunde, M., Mork, T. 2009. Cervid herpesvirus 2, the primary agent in an outbreak of infectious keratoconjunctivitis in semidomesticated reindeer. J Clin Microbiology. 47 (11): 3707-3713.

Tryland, M. 2012. Are we facing new health challenges and diseases in reindeer in Fennoscandia? Rangifer. 32(1): 35-47.

Tryland, M., Stubsjoen, S.M., Agren, E., Johansen, B., Kielland, C. 2016. Herding conditions related to infectious keratoconjunctivitis in semi-domesticated reindeer: a questionnaire-based survey among reindeer herders. Acta Vet. Scand. 58 (22).

Tryland, M., Romano, J.S., Marcin, N., Nymo, I.H., Josefsen, T.D., Sørensen, K.K., Mørk, T. 2017. Cervid herpesvirus 2 and not *Moraxella bovoculi* caused keratoconjunctivitis in experimentally inoculated semi-domesticated Eurasian tundra reindeer. Acta Vet. Scand. 58 (23).

Tyler, N.C. 2010. Climate, snow, ice, crashes and declines in populations of reindeer and caribou (*Rangifer tarandus L.*). Ecol Monogr. 80 (2): 197-219.

Tveraa, T., Stien, A., Brøseth, H., Yoccoz, N.G., 2014. The role of predation and food limitation on claims for compensation, reindeer demography and population dynamics. Journal of Applied Ecology 51, 1264-1272

Uboni, A., Horstkotte, T., Karrlejarvi, E., Seveque, A., Stammler, F., Olofsson, J., Forbes, BC., Moen, J. 2016. Long-term trends and role of climate in the population dynamics of Eurasian reindeer. Plos One.

Ulvevadet, B., Klokov, K. (2004). *Family-based reindeer herding and hunting economies, and the status and management of wild reindeer/caribou populations.* Tromso, Norway: Centre for Saami Studies, University of Tromso.

Valkenburg, P., Davis, J.L., Ver Hoef, J.M., Boertje, R.D., McNay, M.E., Eagan, R.M., Reed, D.J., Gardner, C.L., Tobery, R.W. 1996. Population decline in the Delta Caribou Herd with reference to other Alaskan Herds. Rangifer. 9: 53-62.

Van der Meulen, K.M., Garre´, B., Croubels, S., Nauwynck, H.J. 2006. In vitro comparison of antiviral drugs against feline herpesvirus 1. BMC Vet. Res. 2:13.

Van Meerloo, J., Kaspers, G.K., Cloos, J. 2011. Cell sensitivity assays: the MTT assay. *In:* Cancer Cell Culture. Methods in Molecular Biology (Methods and Protocols), vol 731. Humana Press.

Van Westreenen, M., Boucher, C.A. 2002. Classes of antiviral drugs. *In:* Practical Guidelines in Antiviral Therapy. 1-12.

Vistnes, I., Nellemann, C. 2007. The matter of spatial and temporal scales: a review of reindeer and caribou response to human activity. Polar Biol. 31 (4): 399-407.

Vors, L.S., Boyce, M.S. 2009. Global declines of caribou and reindeer. Glob Change Biol. 15: 2626-2633.

Werner, A., Braun, M., Kietzmann, M. 2008. Isolation and cultivation of canine corneal cells for in vitro studies on the anti-inflammatory effects of dexamethasone. Vet. Ophthalmol. 11:67–74.

Wiklund, E., Andersson, A., Malmfors, G., Lundstrom, K. 1995. Muscle glycogen levels and blood metabolites in reindeer (*Rangifer tarandus tarandus L.*) after transport and lairage. Meat Sci. 42(2): 133-144.

Winqvist, G., Rehbinder, C. 1973. Fine structure of the reindeer cornea in normal conditions and in keratitis. Acta Vet Scand. 14: 292-300.

Xiong, X.F., Smith, J.L., Chen, M.S. 1997. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob Agents Chemother. 41: 594-599.

Appendix 1: Maintenance of MDBK Cell Culture

DESCRIPTION: Establishment and care of Madin-Darby bovine kidney (MDBK) cell line for use in cell culture.

EQUIPMENT NEEDED:

Plastic cell culture bottles (T25 and T75 or T150)

Eagle's Minimal Essential Medium (EMEM) supplemented with:

Horse serum (HS) 10%.

50 mL Falcon tubes

1.5 mL Eppendorf tubes

Trypan blue

Counting chamber

Pipettes, pipette-boy, tips

PBS (phosphate buffered saline)

Trypsin solution

ESTABLISHING A NEW CULTURE:

1. Pre-heat a small volume (20 mL) of water to 37 °C.
2. Remove cryovial of MDBK cells from liquid nitrogen storage.
3. Suspend cryovial in pre-heated water bath and place inside a 37 °C incubator.
4. Pre-heat medium (EMEM with 10% HS)

<u>All work with the cells must be done inside a laminar flow cabinet, wearing protective gloves and coat to prevent contamination of the cell culture.</u>

5. Once cell suspension is thawed, remove cryovial from water bath and wipe outside of vial with alcohol solution to evaporate any remaining water.
6. Transfer the content of cryovial to a T25 bottle with 5 mL of preheated medium.
7. Incubate at 37 °C with 5% CO_2 for 24 hours
8. Remove medium and replace with fresh, pre-warmed EMEM + 10% HS

MAINTENCE AFTER ESTABLISHMENT:

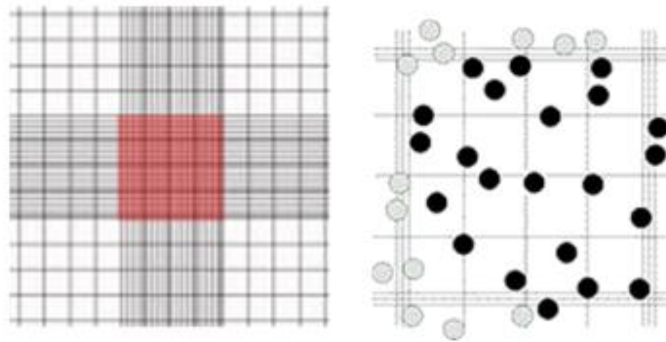
Incubate the established culture at 37°C with 5% CO_2 until cell density requires subculturing. The density and morphology of the cells can be controlled by observing the culture in a microscope. The cells should be subcultured when the cells have reached a confluence of approximately 70-80% within the cell culture bottle. Subculturing typically is required every 3-4 days. If the cells are growing at a slower rate, such that subculturing is not required after 4 days, the medium in the culture bottle should be changed with fresh, pre-heated medium.

SUBCULTURING:

1. Remove medium from the culture bottle using an aspirator
2. Gently wash cell layer with 10 mL PBS
3. Remove PBS from culture bottle with aspirator
3. Add 4mL Trypsin (For T150 bottle. Use more or less depending on bottle volume)
4. Incubate at 37 °C for 2 – 5 minutes
5. Collect cells by gently tapping culture bottle against a solid surface (to fully detach all cells after Trypsin exposure)
6. Transfer the content of the bottle to a 50 mL Falcon tube (with a pipette-boy)
7. Centrifuge tube at 1200 rpm for 5 minutes
8. Observe that the cells have formed a pellet in the bottom of the tube
9. Remove supernatant with aspirator, leaving pellet of cells intact
10. Resuspend pellet with 2 mL EMEM

11. Count the cell density to determine volume of cell solution to be transferred to the new culture bottle:

a. Pipette 180 µL Trypan blue to an Eppendorf-tube
b. Transfer 20 µL of the cell suspension to the tube with Trypan blue and mix thoroughly (1:10 dilution)
c. Transfer 20 µL of colored cell suspension to counting chamber
d. Count cells at magnification 10x in the microscope



e. Count cells inside the central 0.1mm^2 square (show above in red). **The cells on the right bottom border are counted as inside, while the cells on the top and left borders are not count.**

Calculation of counted cells

Every small square in the middle has an area of 0,004 mm^2

25 squares with the volume of 25 x 0.004 = 0,1 mm^2

If 30 cells are counted in a volume of 0,1 mm^2 – that means 30/0.1 = 300 cells/mm^2

There are 1000 mm^2 in one cm^2 (same as 1 mL)

The particle count in the trypan blue solution is 300 x 1000 = 300 000 cells/mL

The trypan blue solution is a 1:10 dilution of original suspension – 300 000 x 10 = 3 000 000 cells/ml

Dividing **x** (counted number of cells pr. 25 squares) by 0.1 and multiplying by 1000 and 10 will be the same as multiplying **x** by 100 000

12. After counting, prepare a new bottle to be inoculated (5 mL EMEM + HS for a T25 bottle, 12 mL EMEM + HS for a T75 bottle or 30 mL EMEM + HS for a T150 bottle)

13. Determine the volume to transfer to the new bottles to obtain the desired density of the cells. MDBK cells should be seeded out at 250,000 cells/T25 bottle, 750,000 cells/T75 bottle or 3,000,000 cells/T150 bottle.

Transfer volume to be calculated as:

$$\frac{number\ of\ cells\ desired\ in\ new\ bottle}{number\ of\ cells\ is\ original\ suspension} = volume\ to\ be\ transferred\ (in\ mL)$$

14. Transfer appropriate volume to the new bottle(s) and carefully tip bottle a couple of times to make sure the cells are evenly distributed to avoid clumping as the cells attach to the bottle surface and grow.

Appendix 2: MTT Cytotoxicity Assay

DESCRIPTION: Cytotoxicity Assay using Roche Diagnostics Cell Proliferation Kit 1

EQUIPMENT NEEDED:

MTT Cell Proliferation Kit I (Sigma Aldrich, St. Louis, USA)
96-well plates (Nunc Edge 2.0, ThermoFisher Scientific, Waltham, USA)
MDBK cells
Antiviral drug solution
Eagle's Minimal Essential Medium (EMEM) supplemented with 10% HS
Pipettes, filtered tips
ELISA plate reader (Epoch^{TM}, Microplate Spectrophotometer, Biotek, Winooski, USA)

MTT Protocol

1. Subculture MDBK cells and count cell density (as described in Maintenance of MDBK Cells)
2. Plate 25,000 cells/well into three 96-well plates. Use 100 uL EMEM + 10% HS for each well aliquot.
3. Incubate plates for 24 hours at 37°C with 5% CO_2
4. After 24 hours, prepare the concentrations of antiviral drug to be tested and incubate to 37°C
5. Remove medium from 96-well plates by turning upside down and gently tapping over waste container.
6. Add 100 uL of antiviral solution in each well for columns 3-12 (treatment columns)
7. Add 100 uL of EMEM + 2% HS in each well for columns 1-2 (controls columns)
8. Incubate for 72 hours at 37°C and 5% CO_2
    a. Check cells every 24 hours for adherence and proper growth
9. Add 10 uL MTT Salt (from Cell Proliferation Kit 1) to wells, skipping column 2 in each plate

10. Incubate for 4 hours at 37°C and 5% CO_2
11. Add 100 uL MTT Buffer to all wells
12. Measure absorbances on ELISA plate reader (wavelength 570 nm)
13. Calculate cell viability with the following formula:

$$\% \; Cytotoxicity = \frac{ODc - ODt}{ODc} \; x \; 100\%$$

ODc= Optical density of the control, ODt= Optical density of the test.

Appendix 3: Experimental CvHV-2 Infection

DESCRIPTION: Experimental infection of MDBK cell culture with CvHV2 as *in vitro* model of naturally occurring CvHV2 infections.

EQUIPMENT NEEDED:

24-well plates (Nunc Edge 2.0, ThermoFisher Scientific, Waltham, USA)
Eagle's Minimal Essential Medium (EMEM) supplemented with 10 % Horse Serum (HS)
MDBK cells
CvHV2 Viral Stock (4.22 x 10^5 $TCID_{50}$/mL, Salla strain)
Pipettes, pipette-boy, tips

VIRUS INFECTION :

1. Distribute 500 uL of MEM + 10% HS, containing 150,000 cells/well to 24-well plates
2. Incubate cells for 24 hours to achieve monolayer of MDBK cells
3. Decant medium from 24-well plates and replace with 500 uL of MEM + 2% HS and the concentration of drug to be tested, in all but three wells.
4. Add 500 uL of MEM to these remaining three wells as a **no drug control**
5. Incubate 24-well plates for 1 hour
6. Create a 10-fold dilution (10^5-10^0) of viral stock solution
7. Create a 10-fold dilution of MEM as a **negative control**
8. Inoculate wells with viral dilutions 10^5-10^0 and the negative control with three replicate wells per dilution
7. Incubate for 72-hours
8. Lys cells by freezing 24-well plates at -80C and melting at room temperature.
9. Repeat 3 times and collect supernatant for use in $TCID_{50}$ assay.

Appendix 4: $TCID_{50}$ Viral Quantification Assay

DESCRIPTION: Determination of viral titers through 50% Tissue Culture Infective Dose endpoint dilution assay ($TCID_{50}$).

EQUIPMENT NEEDED:

96-well plates (Nunc Edge 2.0, ThermoFisher Scientific, Waltham, USA)
Eagle's Minimal Essential Medium (EMEM) supplemented with 10 % Horse Serum (HS)
50 mL Falcon tubes
1.5 mL Eppendorf tubes
Trypan blue
Counting chamber
Pipettes, pipette-boy, tips
PBS (phosphate buffered saline)
Trypsin solution
3.7 % Formaldehyde
0.1 % Crystal violet
Sterile tray for cell suspension

$TCID_{50}$ Protocol:

Prepare a tenfold dilution from the supernatant of each of the initial viral load solutions (10^0-10^5 TCID50/mL) and negative control solution collected at the end of the experimental infection:

1) Mark 8 Eppendorf tubes with 1 – 8
2) Pipette 1080 µL complete EMEM in each tube
3) Transfer 120 µL of the viral stock (10^0) to tube 1 (10^{-1}) – mix thoroughly and change the tip of the pipette
4) Then transfer 120 µL from tube 1 to tube 2 and mix.

5) Continue until reaching tube 8 (10^{-8}).
6) Add 100 µL of your diluted virus-suspensions, negative control solution, and EMEM (internal control) to the 96 well plate following the layout:

Plate 1:

	1	2	3	4	5	6	7	8	9	10	11	12
A (10^{-1})	Negative	Control	Internal	Control	10^5	10^5	10^5	10^5	10^4	10^4	10^4	10^4
B (10^{-2})												
C (10^{-3})												
D (10^{-4})												
E (10^{-5})												
F (10^{-6})												
G (10^{-7})												
H (10^{-8})												

Plate 2:

	1	2	3	4	5	6	7	8	9	10	11	12
A (10^{-1})	Negative	Control	Internal	Control	10^3	10^3	10^3	10^3	10^2	10^2	10^2	10^2
B (10^{-2})												
C (10^{-3})												
D (10^{-4})												
E (10^{-5})												
F (10^{-6})												
G (10^{-7})												
H (10^{-8})												

Plate 3:

	1	2	3	4	5	6	7	8	9	10	11	12
A (10^{-1})	Negative	Control	Internal	Control	10^1	10^1	10^1	10^1	10^0	10^0	10^0	10^0
B (10^{-2})												
C (10^{-3})												
D (10^{-4})												
E (10^{-5})												
F (10^{-6})												
G (10^{-7})												
H (10^{-8})												

7) Prepare cells by subculturing and counting as described in Maintenance of MDBK Cell Culture Protocol.
8) Seed cells in all wells of the 96-well plates at a concentration of 25,000 cells/well in aliquots of 100 µL EMEM + 10% HS
9) Place 96-well plates in the incubator and incubate for 3 hours.
10) After 3 hours: observe the cells in the microscope to control confluence and adherence.
11) Incubate for 3 days and then fix and stain.

FIXING AND STAINING:

1) Remove medium by carefully tipping the 96-well plate over a garbage can and gently tapping the back of the plate to fully empty the liquid.
2) Fix the cells by adding 100 µL/well of a 3.7 % formaldehyde solution for 1 min.
3) Remove the formaldehyde as in step 1.
4) Wash with 200 µL PBS/well.
5) Remove the PBS as in step 1.
6) Stain the cells by adding 50 µL/well of the crystal violet solution and incubate for 10 min.
7) Wash the tray by carefully lowering it in a container full of water and gently moving the plate vertically up and down within the water.
8) Change water and repeat washing until the water no longer turns purple.
9) Allow plate to completely air draw over several days .
10) Count using the Reed-Muench method (following page).

The Reed-Muench method for estimating $TCID_{50}$

Virus dilution	Infections per number inoculated	Observed values		Cumulative values[1]		Infection ratio[2]	% infection[3]
		Positive	Negative	Positive	Negative		
10^{-1}	8/8	8 ↑	0	24	0	24/24	100
10^{-2}	7/8	7	1	16	1	16/17	94
10^{-3}	5/8	5	3	9	4	9/13	69
10^{-4}	3/8	3	5	4	9	4/13	31
10^{-5}	1/8	1	7	1	16	1/17	6
10^{-6}	0/8	0	8 ↓	0	24	0/24	0

[1]The cumulative values are derived by adding up the observed values in the direction of the arrows.
[2]The infection ratio is the number of positives for the cumulative value out of the total for the cumulative value.
[3]The % infection is the infection ratio converted to a percentage.

It had already been determined that the dilution of virus that contains one $TCID_{50}$ lies between 10^{-3} and 10^{-4}, so the end point can be expressed as $10^{-(3+x)}$, where x is the value to be estimated.

$$x = \log_{10} \text{dilution factor} \left(\frac{\text{\% infection at next dilution above 50\%} - 50}{\text{\% infection at next dilution above 50\%} - \text{\% infection at next dilution below 50\%}} \right)$$

$$= 1\left(\frac{69 - 50}{69 - 31}\right)$$

$$= 0.5$$

End point $= 10^{-(3+0.5)} = 10^{-3.5}$.
i.e. 1 ml of a $10^{-3.5}$ dilution contains one $TCID_{50}$ of virus
i.e. 1 ml of a 1/3200 dilution contains one $TCID_{50}$ of virus.
(3.2 is the antilogarithm of 0.5)– $10^{0.5}$
The concentration of virus in the undiluted suspension is 3.2×10^{3} $TCID_{50}$/ml.