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# Gammaherpesvirus in semi-domesticated reindeer (*Rangifer tarandus tarandus*) in Finnmark County, Norway

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In collaboration with

The Norwegian School of Veterinary Science
Section of Arctic Veterinary Medicine

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## Abstract

Climate change can have an increasing effect on ecosystems and wildlife species with many emerging pathogens expanding northwards in the future and it is therefore valuable to document the presence of infectious agents in the Norwegian Arctic. Gammaherpesvirus causes the disease, malignant catarrhal fever (MCF), in wild and domestic ruminants worldwide. In Norway MCF occurs sporadically in wild and domestic ruminants. The present study examined the extent that semi-domesticated reindeer (*Rangifer tarandus tarandus*) in Finnmark, Norway, are exposed to gammaherpesvirus. A serological screening was performed to determine the prevalence of gammaherpesvirus antibodies on slaughtered reindeer in Finnmark. The overall seroprevalence was 3.5% (n=3339). The effect of geography, animal density, age, gender and carcass weight on the risk of exposure to gammaherpesvirus (i.e. seropositive) was assessed using a logistic regression model. The seroprevalence was significantly higher for adult reindeer (> 1 year) than calves (5.2% versus 1.6%). Reindeer in east Finnmark had a higher chance of being infected than in west Finnmark. Reindeer density also had a significant effect on seroprevalence.

Polymerase chain reaction (PCR) was performed, both a specific OvHV2 protocol, targeting the FGAM synthase in ORF 73, and a consensus PCR to detect a wider range of gammaherpesviruses, targeting the polymerase gene. Amplified products from both PCR methods were sequenced. Results from the OvHV2 PCR showed 35% homology (48 nucleotides) to OvHV2, however the match did not correlate to the expected sequence location between the second set primers (555/556). Alignment of sequences obtained from the consensus PCR to other known gammaherpesviruses in GenBank, showed greater but still restricted homology. This suggests that reindeer in Finnmark may be infected by an unknown gammaherpesvirus.

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# 1 Introduction

The semi-domesticated reindeer (*Rangifer tarandus tarandus* L.) is central to the ecosystem in Finnmark, the northernmost county of Norway. The total population of semi-domesticated reindeer in Finnmark was approximately 167 811 animals in 2005/2006 (Anonymous, 2008). In Norway, reindeer husbandry is an exclusive right for the Saami people (indigenous heritage) in which their former herding structure was based on "Siida" (traditional grouping of reindeer flocks). Today it has been replaced by reindeer herding districts and units. In spring and fall, most reindeer herds are moved between inland winter pastures and coastal summer pastures via specific migratory routes. There is a distinct border between the ranges of reindeer in east and west Finnmark and the reindeer seldom co-mingle.

Reindeer in Finnmark usually range freely within the herding districts and contact with the owner or herder is often limited, therefore mortality in these animals can often be a difficult parameter to evaluate. According to the Norwegian Reindeer Husbandry Authority 2008, official numbers of reindeer loss are partly based on reindeer herders' own declarations of predatory loss through financial compensation applications to the authority. According to these data, predation is the main cause of reindeer loss in Finnmark, whereas lynx (*Lynx lynx* L.), wolverine (*Gulo gulo* L.) and the golden eagle (*Aquila chrysaelos* L.) accounted for the majority of losses in 2007. Winter climate and food limitation may increase risk of predation due to the prey's poor nutritional status (Tveraa et al., 2003). Calves are especially vulnerable to mortality due to both starvation and predation if the mother must favour her own growth and survival after harsh winters with limited food (Tveraa et al., 2003). Another important mortality factor in reindeer in Finnmark is infectious disease (Anonymous, 2008). Several virus infections have been documented in Finnmark reindeer including alphaherpes-, parapox- and pestiviruses (das Neves et al., 2009; Klein and Tryland, 2005; Tryland et al., 2005). These infections may impact mortality rates in reindeer. A previous serology survey of wild reindeer documented the presence of gammaherpesvirus (Vikøren et al., 2006), but no studies have detemined which gammaherpesvirus infects reindeer. This study addresses the presence and possible impact of gammaherpesvirus infections in semi-domesticated reindeer in Finnmark.

*Gammaherpesvirinae* is one of three subfamilies in the family *Herpesviridae,* and consists of a large group of viruses that can infect both humans and animals (vertebrates). After a recent reorganization of gammaherpesviruses, these viruses are now subdivided into four genera,

two of which are recently established; *Macavirus* and *Percavirus*, and the two well known *Lymphocryptovirus* and *Rhadinovirus* (Davison et al., 2009; Davison, 2010). The genus *Macavirus* comprises 9 different viruses that are known to cause the disease malignant catarrhal fever (MCF). The first MCF virus identified under this genus was *Alcelaphine herpesvirus 1* (AlHV1) and the common host of AlHV1 is the African wildebeest (*Connochaetes* spp.; Plowright et al., 1960). *Alcelaphine herpesvirus 2* (AlHV2) causes MCF in hartebeest (*Alcelaphus buselaphus* P) and topi (*Damaliscus korrigum* O; Mushi et al., 1981). Another well known virus recognized to cause MCF worldwide is *Ovine herpesvirus 2* (OvHV2; Reid and Buxton, 1989). OvHV2 is enzootic worldwide in domestic sheep (Baxter et al., 1993) and causes sheep-associated MCF (SA-MCF) when transferred to a wide variety of domestic and wild ruminants, including pigs (Li et al., 2001b; Løken et al., 1998; Syrjælæ et al., 2006; Syrjälä et al., 2006; Vikøren et al., 2006). *Hippotragine herpesvirus 1* (HiHV1) has been detected in the roan antelope (*Hippotragus eguinus* D) and oryx (*Oryx gazella* L; Li et al., 2003a; Reid and Bridgen, 1991). *Caprine herpesvirus 2* (CpHV2) is enzootic in domestic goats (Chmielewicz et al., 2001; Li et al., 2001a) and can cause MCF disease in several ruminants, including sika deer (*Cervus nippon* T), white-tailed deer (*Odocolieus virginianus* Z), moose (*Alces alces* L) and roe deer (*Capreolus capreolus* L; Crawford T.B, 2002; Crawford et al., 2002; Keel et al., 2003; Li et al., 2003a; Vikøren et al., 2006). Sheep and goats are recognized as carrier species and do not develop the disease. The species *Bovine herpesvirus 6* (BoHv6), known as the bovine lympotrophic herpesvirus (Rovnak et al., 1998) infects cattle. Three new porcine lympotropic herpesviruses are also placed under the new genus *Macavirus*: *Suid hepesvirus 3, Suid herpesvirus 4* and *Suid herpesvirus 5* (SuHV3, SuHV4 and SuHV5 respectively; (Chmielewicz et al., 2003; Ehlers and Lowden, 2004; Ehlers et al., 1999).

MCF has a worldwide distribution and occurs sporadically and in outbreaks, affecting many species in the family Artiodactyla (even-toed ungulates) including cattle, bison, deer, moose, as well as pigs. The disease is also known to occur in ruminant species in petting zoos, and several reports confirm the disease in ruminant species which have contact with sheep and goats. One outbreak of MCF has been confirmed in a petting zoo where two reindeer died of MCF (Kiupel et al., 2004). In this case OvHV2 was confirmed by PCR. Another petting zoo outbreak has been reported where MCF in reindeer was suspected, although not confirmed (Li et al., 1999). Løken et al. (2009) refers to undocumented reports of annual MCF outbreaks in more than 100 cattle and an unknown number of pigs in Norway. Vikøren et al. (2006)

identified the presence of two types of gammaherpesvirus in Norwegian ruminant cervids; OvHV2 and CpHV2 DNA in moose and roe deer, and OvHV2 DNA in red deer. MCF has also been reported in Norwegian semi-domesticated reindeer housed next to sheep (Vikøren et al., 2006). Gammaherpesvirus serosurveys of sheep and goats in Norway indicated a prevalence of approximately 100% in both species (Løken et al., 2009).

Members of the *Herpesviridae* family produce life-long infections with establishment of latency. Gammaherpesviruses seem to favour establishment of latency more so than do other herpesviruses (Ackermann, 2006). Today it is a common belief that significant levels of subclinical or latent infections are present in a wide range of ruminant species, including cattle, bison, moose, reindeer, roe- and red deer (*Cervus elaphus* L; (Li et al., 2001b; Vikøren et al., 2006). Ackermann (2006) gives a detailed description of the gene expression of well studied gammaherpesviruses including the Epstein Barr virus (EBV), however no common pattern of establishment, maintenance and reactivation of latency is known among gammaherpesviruses. The patterns of gene expression during latency among the viruses in the MCF group, including OvHV2, are less understood than EBV. According to Ackermann (2006), lethal MCF disease occurs when the immune responses of the host are no longer in control of the number of infected cells. Animal species that are most vulnerable to develop lethal disease are those that did not participate in a co-evolution process with the virus (Ackermann, 2006).

The disease MCF occurs in different forms; 1) a head and eye form, 2) a peracute form, 3) an alimentary tract form and 4) a mild form (Radostits et al., 2000). Typical signs of the head and eye form are opacity of the cornea, with a narrow grey ring at the corneoscleral junction. Also severe symptoms such as extreme dejection, agalactia, high fever (41– 41.5°C), rapid pulse rate, dyspnea due to nasal cavity obstruction, anorexia, lymphadenopathy, blepharospasm and congestion of scleral vessels are characteristics of the head and eye form. Early stage may involve nervous signs with weakness in legs, incoordination, muscle tremor and demented appearance, whereas in the final stages headpressing and paralysis are typical. The second form usually lasts for only one to three days and typical signs are gastroenteritis, high fever and dyspnea. The third form has several of the typical symptoms of head and eye form except that there are only minor eye changes and pronounced diarrhoea (Radostits et al. 2000). The mild form is, according to Radostits (2000), rare, and occurs most commonly in experimental animals. In these cases mild erosions may be seen on the oral and nasal mucosa

which can be followed by complete recovery, recrudescence or become chronic. Chronic cases have been described in cattle and bison (O'Toole et al., 1995; Schultheiss et al., 1998), whereas recovery of MCF have been reported in cattle that remained persistently infected afterwards (Baxter et al., 1993; O'Toole et al., 1997). No vaccine is currently available for MCF.

Gammaherpesviruses replicate and persist in lympoid cells (Ackermann, 2006). Knowledge of the latency sites for OvHV2 are based on experiments of interleukin-2 (IL-2) dependent cell lines from MCF affected cattle, which showed that CD4+ and CD8+ lymphocytes were able to persistently harbour the virus. Ackermann (2006) refers to two other experiments (unpublished data) where CD4+ lymphocytes from sheep were confirmed to host latent OvHV2.

Viruses within the herpes family generally share a common structure with icosahedral capsid symmetry and a relatively large double stranded, linear DNA genome encoding 100 – 200 genes. The complete OvHV2 genome, derived from culture of T-lymphoblastoid cell line (BJ1035) from a clinically affected cow, has been cloned and sequenced (Hart et al., 2007). Sequence analyses showed that the unique region of the OvHV2 genome consists of 130 kbp with 73 open reading frames (ORFs). Three ORFs are entirely unique to OvHV2. Comparison studies of genome sequences of OvHV2 isolated from nasal secretion of domestic sheep and the corresponding sequence from cattle with MCF, showed an amino acid identity of 94 – 100% between the predicted ORFs, but 83% for ORF 73 (Taus et al., 2007). These OvHV2 sequences showed no significant changes in the genome, which could indicate that the virus does not change during transmission from sheep to cattle.

The transmission route of OvHV2 between sheep and susceptible ruminants is not fully understood, but previous studies suggest that the virus is shed in nasal secretion from infected sheep and is transmitted through respiration of aerosols both among sheep and to other susceptible ruminants (Li et al., 2004; Taus et al., 2006). Li et al. (2004) showed that OvHV2 is shed in larger amounts and more frequently in lambs between 6 and 9 month than in adults. A strong association between OvHV2 outbreaks in different susceptible animals and exposure to sheep has been documented repeatedly in the litterature (Baxter et al., 1993; Hussy et al., 2002; Løken et al., 1998). However, other studies have documented spread of OvHV2 from sheep to bison over distances of up to 5 km (Li et al., 2008). Different alternative or additional

modes of transmission have been suggested, such as via birds and insects (Li et al., 2008; Li et al., 2001b). According to Ackermann (2006), animals with clinical MCF can not transmit the virus further and are so called dead-end-hosts.

The objectives of this thesis were to determine if semi-domesticated reindeer in Finnmark are exposed to gammaherpesviruses, and if so, to compare seroprevalence between reindeer in east and west Finnmark and between the different reindeer herding districts in Finnmark included in this study. Further aims were to determine whether variables including geography, age, gender, slaughterweight and animal density influence the risk of being infected. Finally, this study aimed to identify which type of gammaherpesvirus the seropositive reindeer were exposed to through PCR, amplicon sequencing and phylogeny.

# 2 Material and methods

## 2.1 Sampling areas

The reindeer herding districts in Finnmark cover approximately 55 100 km² and are divided into two regions; east and west Finnmark. These regions are again divided into 44 reindeer herding districts; 15 in the eastern and 26 in the western part of Finnmark, the latter also including 3 summerpasture districts in norhern Troms. This study encompassed 15 reindeer herding districts in Finnmark (Figure 1).

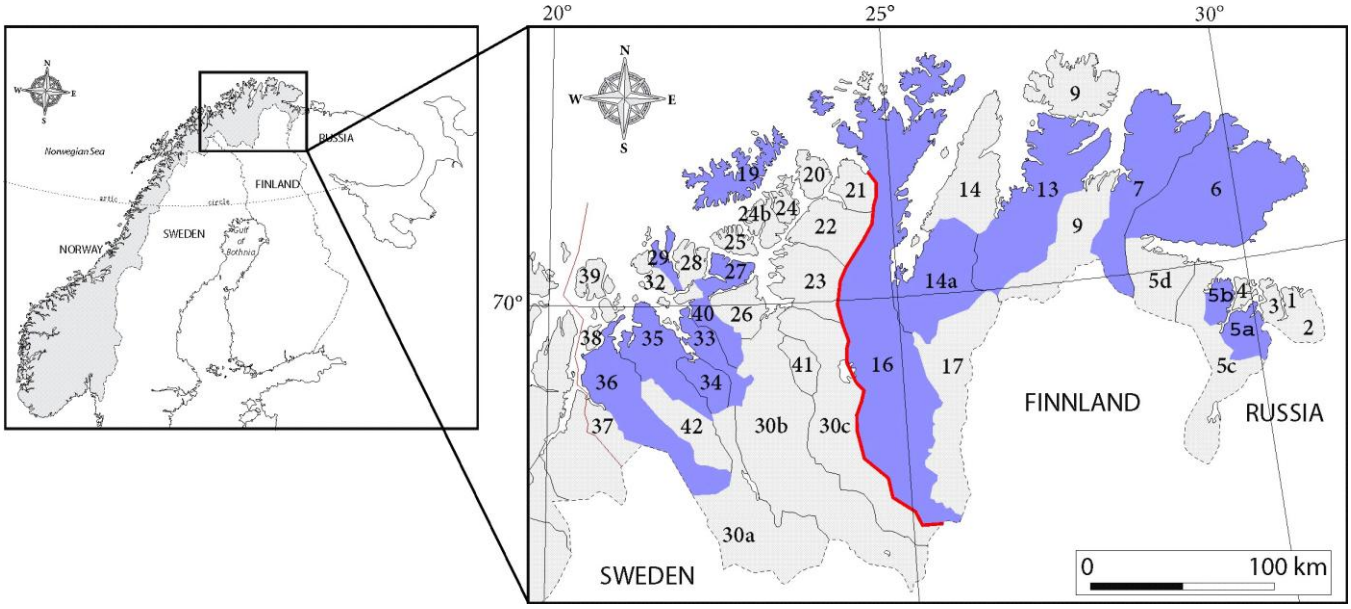


**Figure 1.** The map shows reindeer herding districts in Finnmark County, Norway. Blue colour indicates the districts that are represented in this study. The red line left of district 16 represents the border between east and west (map produced by Norwegian reindeer husbandry authority, 2008 and modified by Tove Midttun, University of Tromsø, Norway).

The number of reindeer and areal size from each region and district (Table 1) were obtained from 2005/2006 statistics (Anonymous, 2008).

**Table 1.** Summary of area (km²), number of reindeer per district and reindeer density (n/km²) on reindeer herding district level. The size of the reindeer herding districts are presented for summer pastures only.

| Region | District | Area km² | Reindeer number | Density n/km² |
|---|---|---|---|---|
| | 5A | 2363 | 2404 | 1,0 |
| E | 5B | 423 | 1756 | 4,2 |
| A | 6 | 5003 | 10532 | 2,1 |
| S | 7 | 2538 | 3603 | 1,4 |
| T | 13 | 3192 | 13932 | 4,4 |
| | 14A | 2003 | 8523 | 4,3 |
| | 16 | 3764 | 29625 | 7,9 |
| | 3 other districts | 5285 | 10751 | |
| *Subtotal* | | *24571* | *81126* | *3,3* |
| | 19 | 818 | 3 851 | 4,7 |
| | 27 | 396 | 5 917 | 14,9 |
| W | 29 | 189 | 1 127 | 6,0 |
| E | 33 | 609 | 6 793 | 11,2 |
| S | 34 | 483 | 5 565 | 11,5 |
| T | 35 | 1 253 | 6 900 | 5,5 |
| | 36 | 2 079 | 8 052 | 3,9 |
| | 40 | 416 | 6 629 | 15,9 |
| | 15 other districts | 7267 | 41851 | |
| *Subtotal* | | *13510* | *86685* | *6,4* |
| **Grand total** | | **38081** | **167811** | 4,4 |

## 2.2 Data collection

A majority of the reindeer samples were collected in the period 2004 – 2006 from four different slaughterhouses in Finnmark located in Kautokeino, Karasjok, Šuoššjávri and Varangerbotn. In addition, 285 blood samples were collected from an outdoor slaughterhouse in Sørøya, Finnmark, October 2009. Table 2 summarizes the yearly distribution of the sampled reindeer sera from Finnmark. An additional 40 blood samples were collected from semi-domesticated reindeer during an outbreak of infectious keratoconjunctivitis in Troms County, Norway, winter 2009 (Tryland et al., 2009). Another 134 samples previously collected (2002 to 2004) from Hattfjelldal, Valdres and Røros, in Nordland, Oppland and Hedmark counties respectiviely, were included in this serological study. Although these samples were treated separately from the Finnmark samples in the statistical analysis, they

were included for comparing results from reindeer in Finnmark with other parts of the country.

**Table 2.** Distribution of reindeer samples analyzed in this study per year, by region (east and west) and reindeer herding districts in Finnmark.

| | | Year | | | | Grand |
|---|---|---|---|---|---|---|
| **Region** | **District** | **2004** | **2005** | **2006** | **2009** | **total** |
| | 5A | 97 | 88 | 0 | 0 | 185 |
| | 5B | 73 | 0 | 0 | 0 | 73 |
| E | 6 | 0 | 119 | 0 | 0 | 119 |
| A | 7 | 0 | 73 | 0 | 0 | 73 |
| S | 13 | 0 | 256 | 0 | 0 | 256 |
| T | 14A | 66 | 0 | 0 | 0 | 66 |
| | 16 | 94 | 157 | 337 | 0 | 588 |
| *Subtotal* | | *330* | *693* | *337* | *0* | *1360* |
| | 19 | 0 | 0 | 0 | 283 | 283 |
| | 27 | 0 | 452 | 0 | 0 | 452 |
| W | 29 | 43 | 0 | 0 | 0 | 43 |
| E | 33 | 170 | 0 | 64 | 0 | 234 |
| S | 34 | 101 | 125 | 136 | 0 | 362 |
| T | 35 | 224 | 0 | 0 | 0 | 224 |
| | 36 | 57 | 0 | 145 | 0 | 202 |
| | 40 | 0 | 0 | 179 | 0 | 179 |
| *Subtotal* | | *595* | *577* | *524* | *283* | *1979* |
| **Grand total** | | **925** | **1270** | **861** | **283** | **3339** |

The distribution of the collected samples between reindeer herding districts depended on the order in which animals came in for slaughtering during the collection period. The slaughterhouses provided slaughter lists with information of animal identity number, gender, age, carcass weight and district of origin for each reindeer. Age was divided into two groups; adults and calves (≤ 1 year old). The total number of reindeer samples analysed in this study was 3513, of these 3339 samples were from Finnmark and 174 samples from Troms, Hattfjelldal, Valdres and Røros.

The distribution of reindeer samples from Finnmark varied between area and districts (Table 2). The number of samples was higher in west (n =1979) than in east (n =1360) Finnmark. The proportion of males versus females was significantly higher in west (67%) than in east Finnmark (55%). The proportion of adult reindeer between east and west showed no

significant difference. The distribution of age and gender varied markedly at district level (Table 3).

**Table 3.** Summary of reindeer samples distributed within each region and district by age groups (adults versus calves) and gender. Unknown animals are those for which gender and age information were not available.

| | | Adults (>1 years) | | | Calves (≤1 year) | | | Un- | Grand |
|---|---|---|---|---|---|---|---|---|---|
| **Region** | **District** | **M** | **F** | **Total** | **M** | **F** | **Total** | **known** | **total** |
| | 5A | 8 | 25 | 33 | 60 | 30 | 90 | 62 | 185 |
| | 5B | 28 | 25 | 53 | 13 | 7 | 20 | 0 | 73 |
| E | 6 | 5 | 0 | 5 | 62 | 48 | 110 | 4 | 119 |
| A | 7 | 10 | 13 | 23 | 29 | 16 | 45 | 5 | 73 |
| S | 13 | 40 | 33 | 73 | 142 | 33 | 175 | 8 | 256 |
| T | 14A | 30 | 26 | 56 | 8 | 2 | 10 | 0 | 66 |
| | 16 | 158 | 268 | 426 | 108 | 42 | 150 | 12 | 588 |
| *Subtotal* | | *279* | *390* | *669* | *422* | *178* | *600* | *91* | *1360* |
| | 19 | 26 | 12 | 38 | 197 | 48 | 245 | 0 | 283 |
| | 27 | 75 | 166 | 241 | 136 | 75 | 211 | 0 | 452 |
| W | 29 | 36 | 6 | 42 | 1 | 0 | 1 | 0 | 43 |
| E | 33 | 88 | 57 | 145 | 1 | 9 | 10 | 79 | 234 |
| S | 34 | 131 | 32 | 163 | 41 | 22 | 63 | 136 | 362 |
| T | 35 | 15 | 13 | 28 | 161 | 33 | 194 | 2 | 224 |
| | 36 | 120 | 11 | 131 | 48 | 1 | 49 | 22 | 202 |
| | 40 | 91 | 86 | 177 | 2 | 0 | 2 | 0 | 179 |
| *Subtotal* | | *582* | *383* | *965* | *587* | *188* | *775* | *239* | *1979* |
| **Grand total** | | 861 | 773 | 1634 | 1009 | 366 | 1375 | 330 | 3339 |

## 2.3 Blood sampling and preparation

Blood was collected from the reindeer when they were bled by cutting Arteria and Vena jugularis. Evacuated blood containers (Venoject, 10 ml glass with 0.1 ml EDTA; Terumo Europe, Leuven, Belgium) were used to prepare plasma, whereas similar blood containers without EDTA were used to prepare serum samples. The samples were centrifuged at 3500 g for 15 minutes. Serum and plasma was transferred to cryotubes. Peripheral blood leucocytes (PBLs; buffy coat) were transferred from the EDTA containers into eppendorf tubes. All samples were stored frozen at -20°C until analysis.

## 2.4 Analysis

### *2.4.1 Serological screening*

A direct competitive-inhibition enzyme-linked immunosorbent assay (ciELISA) was used to detect antibodies against gammaherpesvirus in reindeer sera or plasma. The assay was performed according to the protocol (Appendix). Microtiter plates (96 wells; Immulon 4, Dynatech labs, Chantilly, VA., USA) pre-coated with dried MCFV antigens (Li et al., 2001b), reagents, controls and protocol were kindly provided by Dr. Hong Li (Washington State University, Washington, USA). The positive control provided with the kit was serum from a sheep infected with OvHV2, and the negative control was pooled sera from 19 MCF virus-free sheep (Li et al, 2001b). Due to a slight inconsistency during initial testing of the plates, the producer recommended all samples to be tested in duplicates to eventually prevent false results. Samples that scored inconsistently in the duplicates (one positive and one negative) were retested until both were classified either positive or negative.

The binding between the HRP-labeled monoclonal antibody (15-A Mab HRPO conjugate, VMRD, Inc., Pullman, Washington, USA) and the MCFV antigen precoated in the plate was detected by adding a substrate (TMB One Component HRP Microwell Substrate, BioFX Laboratories, Owing Mills, USA) for the HRP which enabled the development of a blue colour product. Strong colour development indicated little or no inhibition of HRP-labeled monoclonal antibody binding and indicated that MCF antibodies were absent from the sample serum. Weak colour development indicated presence of MCF antibodies in the serum sample due to inhibition of the monoclonal antibody binding to the antigen.

Based on the ability of each serum sample to inhibit binding of the HRP- labeled monoclonal antibody to the AlHV-1 antigen in the plates, the samples were scored according to the colour development, by reading Optical Density (OD) with a wavelength set to 450 nm and filter 3 according to the protocol. The kit was evaluated by including 4 positive and 6 negative control wells for each plate. For test validation the mean OD of the 6 negative control wells was calculated by excluding any well where the OD value was beyond one standard deviation of the mean from all 6 wells. The mean OD of the negative control wells should then range from 0.40 to 2.10. The positive control should cause ≥ 25% inhibition and was given by the formula: 100 – [mean ($OD_{450}$) of positive control x 100/ mean ($OD_{450}$) of negative control].

The results of the serum samples were expressed as percentage inhibition derived by the formula: 100 – [mean ($OD_{450}$) of test serum x 100 / mean ($OD_{450}$) of negative control]. Serum samples were considered positive when the percentage of inhibition was ≥ 25% (Li et al., 2001b). The mean inhibition value of the two duplicates was used in the statistical analysis.

### *2.4.2 Extraction of DNA*

DNA was extracted from a total of 115 peripheral blood leukocytes (PBLs), 76 from seropositive animals and 39 from animals from which no antibodies against gammaherpesviruses were detected. DNA extraction was performed according to the Spin-Column Protocol for Purification of Total DNA from Animal Blood or Cells using DNeasy Blood & Tissue Handbook (Qiagen, USA, 2006).

### *2.4.3 Polymerase Chain Reaction*

Polymerase chain reaction (PCR) is an effective technique to rapidly generate large amounts of genetic material from a slight trace which otherwise would be too small to detect. For diagnostic purposes the technique is commonly used to amplify short sequences of a specific gene. A PCR generally proceeds in three major steps governed by temperature. Denaturation of DNA at temperatures around 93–98°C split the double-stranded template DNA helix in two complementary single strands. This makes it possible to access the sequence hidden inside the double stranded DNA. The annealing step at 50–65°C allows the DNA primers (chemically synthesized oligonucleotides with a length of about twenty bases) to hybridize to the homologue sequences of ssDNA of the template. The extension step at 72°C is the ideal working temperature for the DNA polymerase and the DNA will be synthesized efficiently. The thermostable DNA polymerase (enzymes that catalyze replication) will start replication in the 3`-end of the primer, and synthesize a DNA sequence complementary to the template. dNTPs (referring to the four deoxyribonucleotides that are included in the redTaq: dATP, dCTP, dGTP and dTTP) are added from 5` to 3`, reading the template in the 3` to 5` direction. The high amount of copies of the amplified DNA (amplicons) is separated by size based on electric field.

### *2.4.4 Semi-nested PCR specific for OvHV2*

A semi-nested PCR was used to detect OvHV2 DNA in extracted DNA samples from seropositive and seronegative animals. This method is based on two separate amplifications, each with different sets of primers. One primer is however used in both amplifications, hence the semi-nested design. The first set of primers yields a product of 422 nucleotides and is used as a template for the second amplification. The second set of primers produces a smaller product of 238 nucleotides within the sequences of the initial product. This two-step PCR amplification (Figure 2) was performed using the outer primer set 755 (5`-AAGATAAGCAC CAGTTATGCATCTGATAAA-3`) and 556 (5`- AGTCTGGGGTATATGAATCCAGATGG CTCTC-3`) in the first step, and the inner primer set 555 (5`-TTCTGGGGTAGTGGCGAGC GAAGGCTTC-3`) and 556 in the second step The primers were initially developed by Baxter et al. (1993).

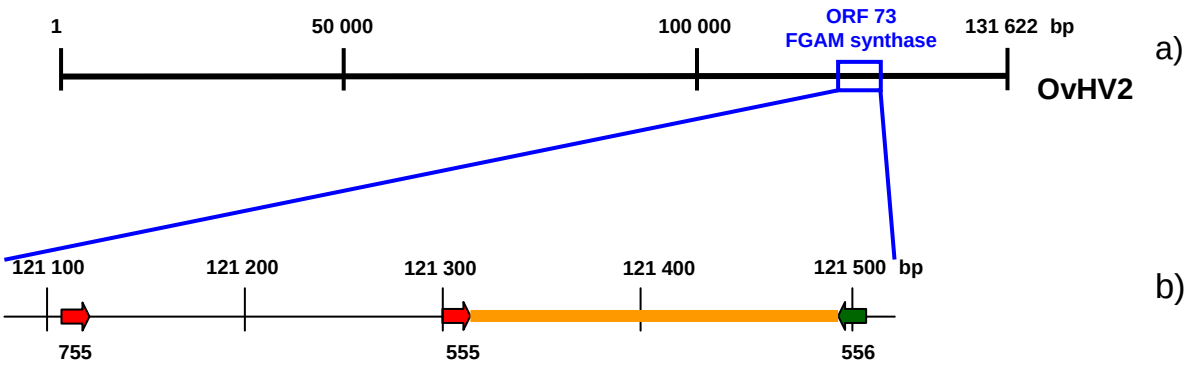


**Figure 2.** The illustration describes the semi-nested amplification process of the OvHV2 specific primers. a) The primers are targeting the FGAM synthase gene located in ORF 73 of the OvHV2 genome; b) amplifying sequences of 422 bp with the first primer set (755/556) and a shorter sequence of 238 bp in the second PCR with primer pair 555/556 (yellow line).

The procedure was carried out as described by (Li et al., 1995). The positive control used in this OvHV2 specific semi-nested PCR was DNA extracted from PBLs from a cow with clinical signs of MCF (kindly provided by the National Veterinary Institute, Oslo, Norway). The enzyme mix used for this PCR was JumpStart™REDTaq®ReadyMix™PCR Reaction Mix for High Throughput PCR (Sigma-Aldrich, St. Louis, USA). This ready reagent mix contained 20 mM Tris-HCL, pH8.3, 100 mM KCl, 4 mM $MgCl_2$, 0.002% gelatine, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), inert dye, 0.003 unit/µL of Taq DNA polymerase,

JumpStart Taq antibody and stabilizers. A 1 kb+ ladder (Invitrogen Tech-Line, St. Louis, USA) was used as a comparison tool for evaluating the size of the fragments.

PBL-samples obtained from 76 animals with, and 39 animals without gammaherpesvirus antibodies, were tested for the presence OvHV2 DNA. As target for the amplification in the first step 2–5 µl of extracted DNA was used and 2–5 µl of the PCR product from the first step was used as target in the second step. Each reaction mixture in both PCR steps contained 8 µl RedTaq and 40 pM of each primer and were diluted with sterile $H_2O$ up to a volume of 25 µl. For both steps the amplification consisted of an initial 5 minute step at 94°C followed by 34 cycles with the following steps: 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes. In the end an elongation step at 72°C was performed for 7 minutes and the temperature was lowered and held at 4°C for storage. Ten µl of the amplified secondary PCR products were loaded on a 1.5% agarose gel (UltraPure™ Agarose, Invitrogen, Carlsbad, USA, made up in 1 x TBE buffer). Electrophoresis was run at 110 Volt for one and a half hour. The gel was stained in approximately 2% ethidium bromide (Invitrogen, Carlsbad, USA) for half an hour and at last put in a water-bath for approximately half an hour to get rid of surplus ethidium bromide.

Several factors regulate the optimalisation of PCR: magnesium concentration, primer design, dNTP, annealing temperature and template DNA concentration. To assess the sensitivity of the semi-nested specific OvHV2 PCR, several factors were accounted for. Positive controls were tested in a gradient of 12 different annealing temperatures from 53–68°C in both steps. The temperature closest to 60°C gave best fragment visualisation on the gel although the temperatures between 56 and 68°C gave only one distinct band. The PCR amplifications were therefore set with annealing temperature of 60°C according to the original protocol. Dimethyl sulfoxide (DMSO) is useful in PCR for template DNA with a high GC content. Due to a high degree of unspecified bands at start, 2.5 µl DMSO (Sigma Chemical Co., St. Louis, USA) was added to the PCR mix in both steps to inhibit secondary structures in the DNA template or the DNA primers and to possibly minimize interfering reactions. This however gave no significant results. The effects of different concentrations of RedTaq were also checked for, without any significant effect on the positive control tested. Ethidium bromide staining gave considerably stronger visualisation of the amplified DNA fragments compared to the SYBR®Safe DNA gel stain, and were thus chosen for the following procedure.

### *2.4.5 Nested consensus PCR*

To detect a wider range of rhadinoviruses, including CpHv2, a nested PCR method based on degenerate (consensus) primers was used in this study (VanDevanter et al., 1996). Based on degenerate primers targeting a highly conserved gene region of the DNA polymerase gene (ORF 9), this assay has proved to be able to detect a wide range of members within the *Herpesviridae* family and is considered to be a powerful tool for identification of previously unreported herpesviruses (Li et al., 2001a; Rovnak et al., 1998; VanDevanter et al., 1996). The consensus PCR used in this study was performed as described by VanDevanter (1996) using the same five degenerate primer sets. Primary PCR was performed with the two forward primers DFA (5`-GAYTTYGCNAGYYTNTAYCC-3`) and ILK (5`-TCCTGGACAAGC AGCARNYSGCNMTNAA-3`) and the reverse primer KG1 (5`-GTCTTGCTCACCAGN TCNACNCCYTT-3`). Secondary PCR were performed with forward primer TGV (5`-TGTA ACTCGGTGTAYGGNTTYACNGGNGT-3`) and reverse primer IYG (5`-CACAGAGTCC GTRTCNCCRTADAT-3`). The size of amplification fragments obtained by this PCR method is expected to range from 215 to 315 base pairs, as previously reported by VanDevanter, 1996. The amplification process is described in figure 3.

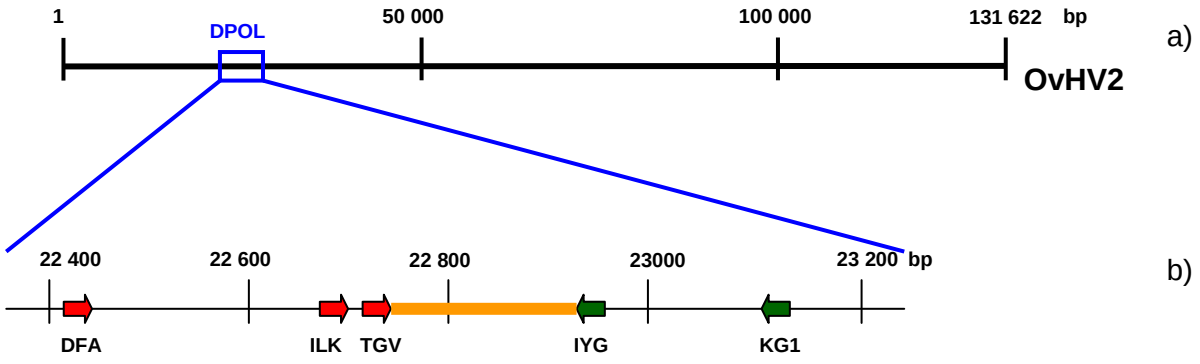


**Figure 3**. Illustration of the consensus PCR amplification process with the three first primers a) targeting the polymerase gene of the OvHV2 genome. b) The first PCR using forward primers (DFA and ILK) and reverse primer KG1 yields sequences with two different sizes. These will be the targets for the second PCR product primers (TGV/ IYG) yielding a smaller sequence (215 - 315 bp) shown as the yellow line.

A total of 20 DNA samples obtained from seropositive reindeer were tested in this PCR method. Added to reaction mixtures containing 10 µl RedTaq, 40 pM of the respective primer and 1.25 µl (2.5%) DMSO, 5 µl DNA template in first step and 5 µl PCR product from the first PCR step as target DNA for the second step, were amplified in a volume of 25 µl. The thermocycler profile used in this study is the same as described by VanDevanter (1996) except an initial step for 5 minutes at 94°C (protocol from Li) followed by 45 cycles with the following steps: 94°C for 30 sec, 46°C for 1 minute and 72°C for 1 minute. After cycling, the reaction was finished by an incubation period for 7 minutes at 72°C before chilling to 4°C. Secondary PCR products were further analyzed as described above for the OvHv2 specific PCR.

### *2.4.6 Sequencing*

To determine the nucleotide sequences of the PCR amplicons, 9 PCR products from the specific OvHV2 PCR and 6 PCR products from the consensus PCR were sequenced in both directions, either directly from the amplified PCR product (if strong signal) or from fragments sliced out of the gel. Prior to the sequencing, 10 µl of the second PCR products from OvHV2 PCR was purified with 1.2 µl ExoSAP-IT® (USB Corporation, Cleveland, USA) to remove primers and dNTPs. This was conducted using the thermocycler program: 37°C for 60 minutes, 80°C for 15 minutes before a hold at 4°C. DNA amplicons from consensus PCR were, after being sliced out from the gel, purified according to the MinElute Gel Extraction Kit Protocol in MinElute Handbook (Qiagen, 2006).

To increase the reliability of the sequencing results, each PCR product was sequenced in both directions with the secondary PCR forward and reverse primer sets for the OvHV2 specific PCR (556/556) and the consensus PCR (TGV/IYG) respectively. Two equal mixtures were made but with different primers, one with forward and one with reverse primer. The amount of the PCR products was adjusted according to the strength of visibility on the gel. Two to four µl PCR product was mixed with 1 µl (3 pmol/ µl) primer and reagents included in the sequence kit (Big Dye Terminator v3.1); 3 µl Ready Reaction premix and 2 µl Big Dye Sequencing buffer. The mixture was adjusted with sterile water up to 20 µl. The thermocycler sequence program was set to 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes. In the end the temperature was held at 4 °C.

### *2.4.7 Sequence analysis*

The DNA sequences were displayed as chromatogram files in Chromas (version 2.33; Technelysium Pty Ltd, Queensland, Australia). The web-based search engine BLAST was used to search for related sequences in the GenBank, National Center for Biotechnology Information (NCBI, USA). To obtain a hint of the evolutionary history of the amplified DNA, a phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). A bootstrap, inferred from 1000 replicates, was performed to estimate the probability of evolutionary history between the analyzed taxa (Felsenstein, 1985). The branch lengths in the tree were drawn in the same units as the evolutionary distances derived from the bootstrap replicates. Phylogenetic analyses were obtained by MEGA4 (Tamura et al., 2007).

## 2.5 Statistical analysis

The serological dataset provides information about region (east and west), district number, animal density, weight, age and gender. These variables were used to evaluate risk factors for being exposed to gammaherpesvirus. The composition of age and gender in some districts differed markedly. The dataset was therefore divided into east and west Finnmark in order to make a more equal distribution of the variables. Reindeer density, i.e., number of reindeer per square kilometre (n/km$^2$) within each summer herding district, was calculated using statistical information available from the Reindeer Husbandry Authority Report (Anonymous, 2008). For the statistical analysis, animal densities which ranged from 1.0 to15.9 animals/km$^2$, were divided into four groups (quartiles) according to level of density. Statistical analysis was performed using the statistical programs JMP 8.0 (SAS, USA) and STATA 11.0 (STATA Corp., College Station, USA).

The likelihood ratio test was used for statistical significance testing with a threshold of P = 0.05. To account for confounding factors a logistic regression model were used to test the association between the response variable (seroprevalence) with the predictor variables (animal density, gender, age and year of sampling). The model assumed a binomial distribution for the response variable. The prevalence was estimated based on the 15 districts that were used as cluster variable. Standard error was adjusted for 15 clusters.

To account for the samples representation of the reindeer population in Finnmark, a weighting/extrapolation was included in the model as a predictor variable. The samples were weighted according to number of reindeer in each district, using the formula: 1/ (sample number per district / total reindeer number per district).

# 3 Results

## 3.1 Seroprevalence

The mean OD of the negative control ranged from 0.78 to 1.86 and was within the recommended values set for test validation. The mean of the positive control caused > 25% inhibition (cut-off value) for all tested ciELISA plates, with 33.5% as the lowest value. The distribution of the inhibition values of the total sample ranged from -110.7 % to 81.9 %, with a mean value of -4.2 % (Figure 4). A change in cut-off value from 25% to 30 % reduced the seroprevalence to 2.7%.

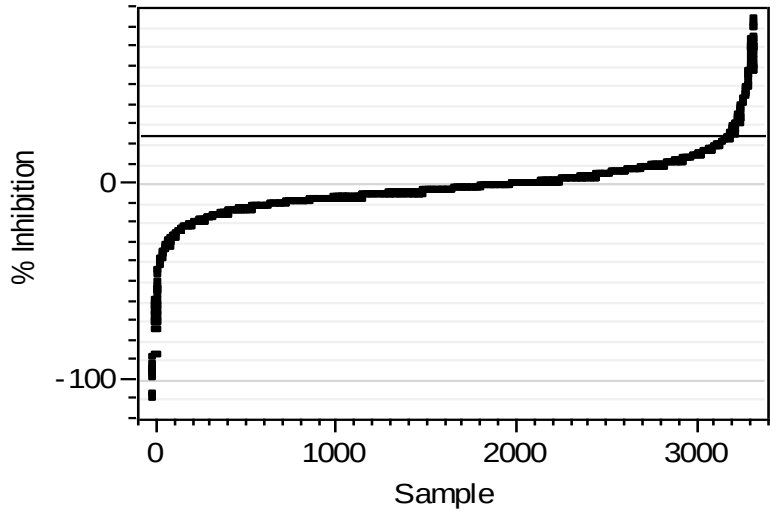


**Figure 4.** The graph shows the distribution of inhibition levels (%) for all samples tested in the direct ciELISA. The cut off value was set at 25% inhibition, initially established by adding 3 SD to the mean OD of 19 negative sheep sera (Li et al., 2001b; Li et al., 1994).

The seroprevalence against gammaherpsevirus among semi-domesticated reindeer in Finnmark was 3.5 % (n=3339). After extrapolation, i.e. the inverse of the number of samples per district divided by the total number of animals in the respective district, the tested samples represented approximately 115 224 of the reindeer population in Finnmark, which accounts for approximately 69% of the total reindeer population in Finnmark. Extrapolation gave a seroprevalence of 3.47% before weighting and 3.54% after extrapolation.

There were distinct differences in seroprevalence between reindeer herding districts (Table 4). Seroprevalence varied from 0 % in district 6 to 6.7 % in district 40.

**Table 4.** Distribution of number of seropositive reindeer, seroprevalence and weight between east and west Finnmark and between reindeer herding districts.

| | | Seroprevalence | | | | Carcass weight | |
|---|---|---|---|---|---|---|---|
| **Region** | **District** | **N pos** | **N tot** | **(%)** | **95%CI** | **Median** | **[Min-Max]** |
| | 5A | 6 | 185 | 3,2 | [1.5-6.9] | 24,0 | [14,4-42,3] |
| E | 5B | 1 | 73 | 1,4 | [0.2-7.4] | 27,6 | [15,2-45,1] |
| A | 6 | 0 | 119 | 0,0 | [0-0] | 19,0 | [12,4-34,7] |
| S | 7 | 1 | 73 | 1,4 | [0.2-7.4] | 23,2 | [17,6-38,7] |
| T | 13 | 9 | 256 | 3,5 | [1.9-6.5] | 19,8 | [10,7-53,5] |
| | 14A | 3 | 66 | 4,5 | [1.6-12.5] | 30,1 | [15,1-58,6] |
| | 16 | 31 | 588 | 5,3 | [3.7-7.4] | 24,4 | [9,7-57,0] |
| *Subtotal* | | *51* | *1360* | *3,8* | *[2.9-4.9]* | *23,4* | *[9,7-58,6]* |
| | 19 | 3 | 283 | 1,1 | [0.4-3.1] | 23,3 | [11,4-69,7] |
| | 27 | 16 | 452 | 3,5 | [2.2-5.7] | 20,7 | [9,2-51,4] |
| W | 29 | 1 | 43 | 2,3 | [0.4-12.1] | 34,9 | [18,6-49,9] |
| E | 33 | 14 | 234 | 6,0 | [3.6-9.8] | 23,5 | [15,2-45,2] |
| S | 34 | 15 | 362 | 4,1 | [2.5-6.7] | 25,4 | [9,9-42,7] |
| T | 35 | 3 | 224 | 1,3 | [0.5-3.9] | 18,6 | [9,9-45,5] |
| | 36 | 1 | 202 | 0,5 | [0.1-2.8] | 25,0 | [13,3-48,5] |
| | 40 | 12 | 179 | 6,7 | [3.9-11.4] | 23,8 | [15,0-39,6] |
| *Subtotal* | | *65* | *1979* | *3,3* | *[2.6-4.2]* | *22,7* | *[9,2-69,7]* |
| **Grand total** | | **116** | **3339** | 3,5 | **[2.9-4.1]** | **22,9** | [9,2-69,7] |

Contingency Analysis showed that adult reindeer had significantly higher seroprevalence (5.2%) than calves (1.6%) (Likelihood ratio test, Chi-square = 30.531, df = 1, Prob>ChiSq< 0.0001). A differentiation between adults and calves, males and females, showed that adult female reindeer had a seroprevalence of 6.6 %, while for adult males it was 3.9 %. For the calves, the seroprevalence was 1.8 % for the males, and only 1.1 % for the females. This distribution in prevalence is approximately the same at region level, except for male calves in east with 3.1% compared to 0.9% in west (Table 5).

**Table 5.** Seroprevalence of gender and age (calves versus adults) between east and west Finnmark.

| Region | Adults | | Calves | | Unknown % | Total % |
|---|---|---|---|---|---|---|
| | M(%) | F(%) | M(%) | F(%) | | |
| East | 4,3 | 5,4 | 3,1 | 1,1 | 3,3 | 3,8 |
| West | 3,8 | 7,8 | 0,9 | 1,1 | 2,5 | 3,3 |
| **Total** | 3,9 | 6,6 | 1,8 | 1,1 | 2,7 | 3,5 |

Contigency table showed that seroprevalence was significantly higher in females (4.8%) than in males (2.8%) (Likelihood ratio test, Chi-square = 8.39, df = 1, Prob>ChiSq < 0.0038), whereas east and west Finnmark showed no significant difference, with 3.8 % in east and 3.3 % in west Finnmark (Likelihood ratio test, Chi-square = 0.517, df =1, Prob>ChiSq = 0.4721). A goodness of fit test indicated a normal distribution of carcass weight (range 9.2 – 69.7 kg), with a cumulation between 15 to 35 kg (std.dev: 7.06). Carcass weight correlated with age, however age represented a better variable in the model. Accordingly, carcass weights were not included in the model.

The logistic regression model showed that region (east vs. west), age and animal density had a significant effects on risk of infection by gammaherpesvirus (Table 6). The model identified significant differences on effect between region (east: P>Z<0.000), age (adults: P>Z<0.001) and animal density (quartiles 2-4: P>Z<0.033 – 0.000) on prevalence. Seroprevalence increased significantly with increasing animal density. On the other hand the variables; year of sampling and gender were not significant in this model (P> 0.05).

Table 6. Logistic regression model of factors associated with infection with gammaherpesvirus. The model is based on a backward procedure where initially all predictor variables were implemented/ integrated. Non-significant variables were removed. The predictor variables, region (east), animal density (3 of 4 quartiles) and age (adults) had a positive correlation with the response variable (seroprevalence).

| γ-hepesvirus seroprevalence | Odds Ratio | Robust Std. Err. | z | P>\|z\| | 95% CI |
|---|---|---|---|---|---|
| Region (east) | 2,246 | 0,413 | 4,4 | <0 | [1,57 - 3,22] |
| Animal density | | | | | |
| 2 | 3,775 | 2,334 | 2,13 | <0,033 | [1,11 - 12,69] |
| 3 | 4,424 | 2,796 | 2,35 | <0,019 | [1,28 - 15,27] |
| 4 | 10,544 | 5,446 | 4,56 | <0 | [3,83 - 29,02] |
| Age (adults>1 year) | 2,033 | 0,443 | 3,26 | <0,001 | [1,33 - 3,12] |

Standard error was adjusted for 13 clusters over districts.
Number of observations = 3009.
Statistically significant P values < 0.05.

## 3.2 Semi-nested PCR specific for OvHV2

PBL-samples frpm 75 seropositive reindeer in Finnmark were tested by the OvHV2 specific semi-nested PCR. The reindeer samples from Troms (n = 40) were also tested for OvHv2 although only one of these animals was seropositive against gammaherpesvirus. A seronegative reindeer from Troms generated a PCR product with a similarity in fragment size (238 bp) to one of the two fragments previously reported by Baxter (1993), whereas all other amplicons generated from samples tested in this study appeared with a slightly lower size on the gel compared to the positive control (OvHV2), as shown in figure 5.

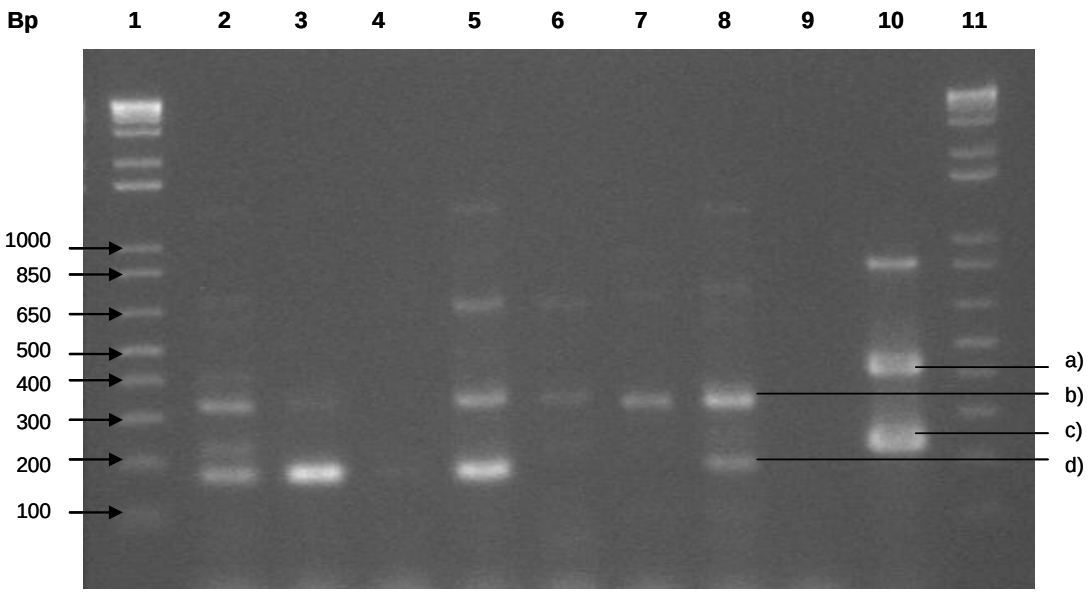


**Figure 5**. Gel electrophoresis of amplified PCR products from PCR using OvHV2 spesific semi-nested primers. Lane 1, 1 Kb plus ladder (Invitrogen Tech-line, USA); lane 2–8, PCR amplicons generated from samples obtained from seropositive animals; lane 9, negative control; lane 10, positive OvHV2 control; lane 11, ladder. Notice that fragments in lane 2–8 had slightly lower size (b and d) than the fragments of the positive control (lane 10) having fragment sizes of 238 bp and 433 bp (c and a respectively).

Alignment of amplicon sequences obtained with forward and reverse primers gave two DNA sequences of 133 and 137 bases respectively. Alignment results comparing the sequences with the matching sequence of the OvHV2 genome in GenBank, did not give the expected match. Figure 6 shows the OvHV2 sequence (obtained from GenBank) above the sequence obtained from the reindeer samples. The obtained sequence did not match with the expected position on the OvHV2 genome, which was between second PCR primer set 555 and 556. However, the bases had best match within primer 755 and further towards primer 555 (Figure 6).

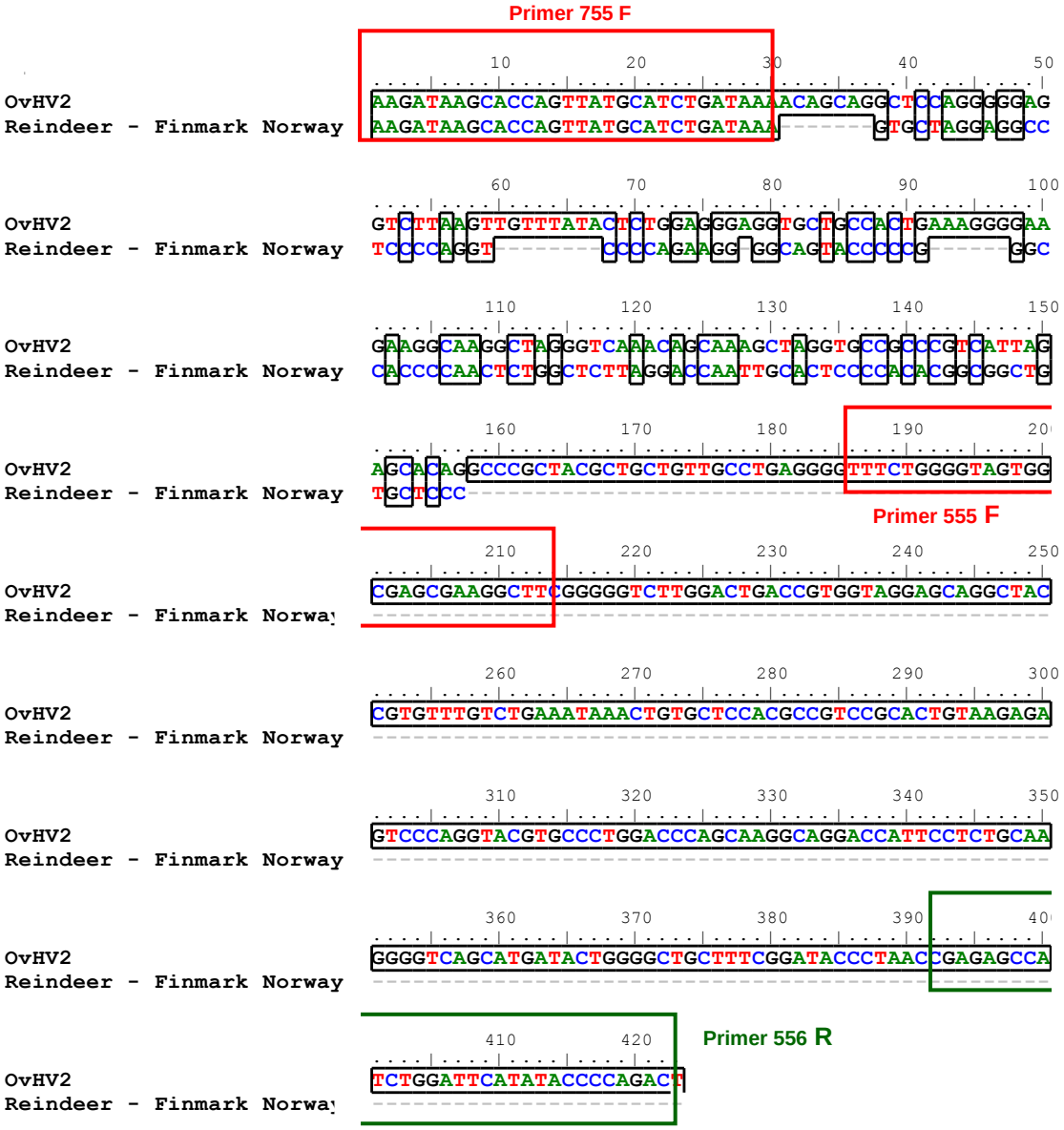


**Figure 6.** The figure illustrates the position of match between the reindeer sequence obtained in this study and FGAM synthetase gene located in ORF 73 on the OvHV2 sequence (obtained from GenBank). The three boxes show the location of the primers within the genome. The bases with homology between the two sequences are boxed with a black line.

## 3.3 Nested consensus PCR

Twenty DNA samples from seropositive reindeer were tested in the nested degenerate consensus PCR. Short fragments of the amplified DNA polymerase gene were strongly visible on the gel as shown in Figure 7, and somewhat identical to those fragments reported by VanDevanter (1996) of approximately 215 bp.

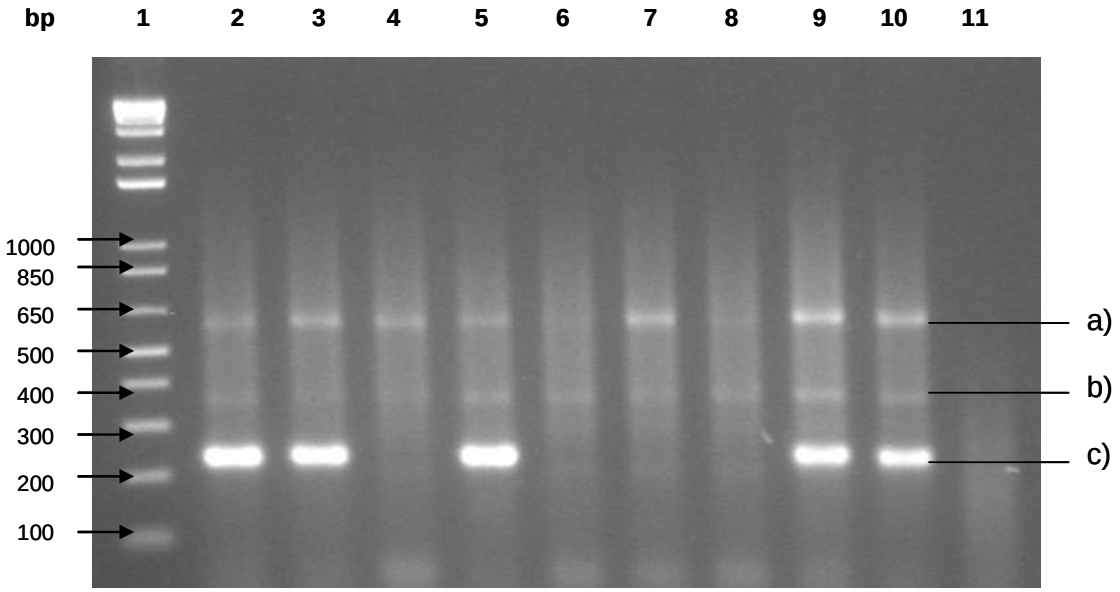


**Figure 7**. Gel electrophoresis of amplified PCR products using nested consensus PCR. The specificities of the consensus PCR products are: Lane 1, 1 Kb plus DNA ladder (Invitrogen Tech-Line, USA): lane 2, 3, 5, 9 and 10: PCR amplicons showing DNA fragments of approximately 215 bp (c), b) is primer products from first step and c) is unspecified fragment : lane 4, 6, 7 and 8: PCR products gave slightly visible bands, lane 11: negative control. Positive control was included, but is not shown on the picture.

The six fragments that were sliced out of the gel yielded long sequences of approximately 230 bp after matching the respective forward and reverse primer. Four of these six nucleotide sequences obtained in this study matched each other with 100% homology, indicating that these reindeer viruses were identical. A subset of the gammaherpesviruses with the closest similarity to the reindeer virus was chosen from GenBank for comparison (Figure 8). Alignment of the polymerase gene sequence between these gammaherpesviruses and the reindeer sequence showed little identity.

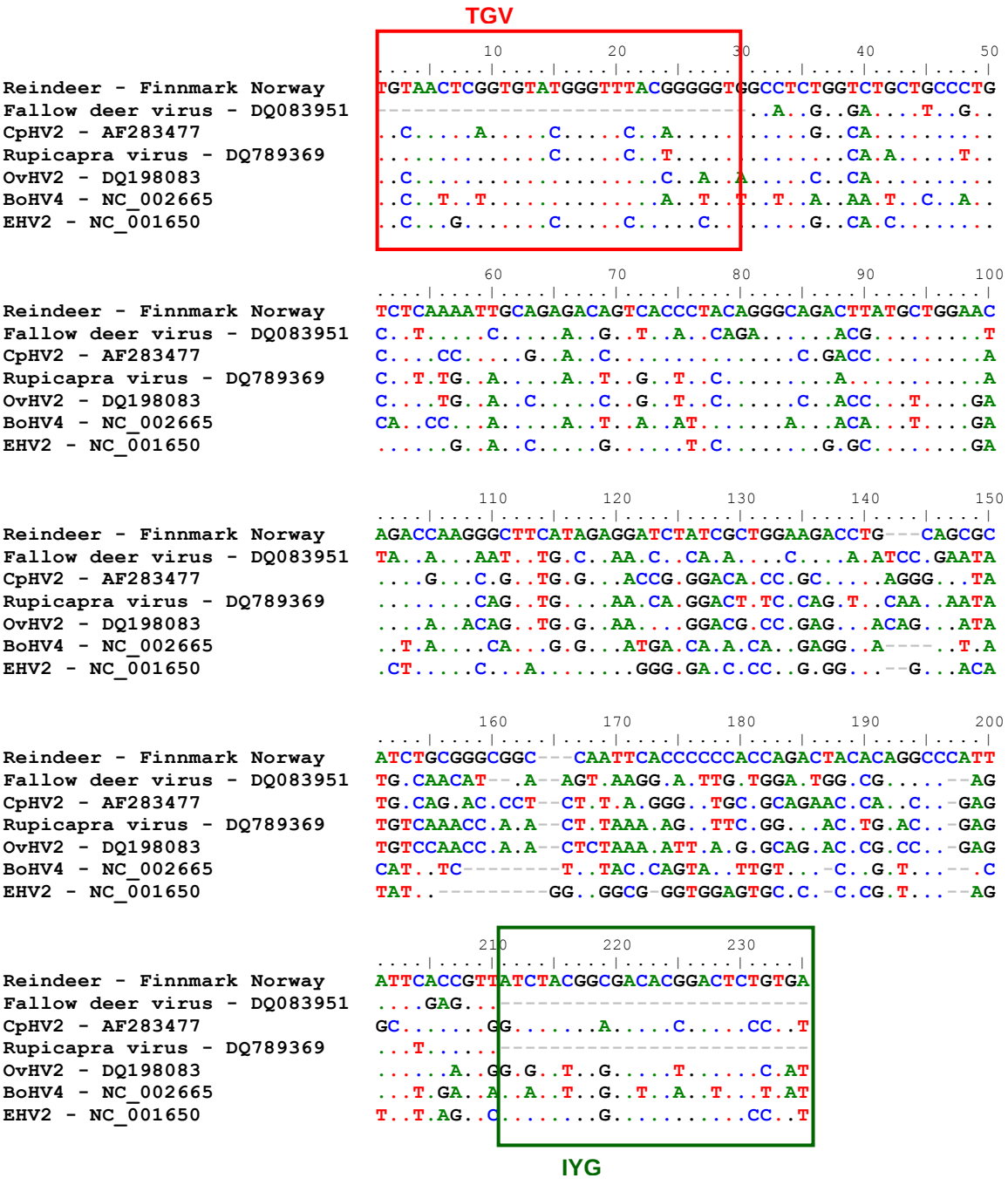


**Figure 8**. Alignment of PCR sequence (nested consensus PCR) obtained froms reindeer (uppermost) and the corresponding sequence of other closely related gammaherpesviruses (sequences obtained from GenBank). The boxes show the location of the second PCR primers (TGV and IYG) of the nested consensus PCR.

The evolutionary relationship between the reindeer gammaherpesvirus and other gammaherpesviruses gives different results according to which taxa one chooses to include. The phylogenetic tree (Figure 9) is only an illustration of one way to see the possible history

and relationship between different gammaherpesviruses with close homology. This tree shows that the Fallow deer herpesvirus is closest related to the reindeer virus, but no such virus is recognized in Norway. This tree also shows that CpHV2 is more closely related to the reindeer virus than OvHV2.

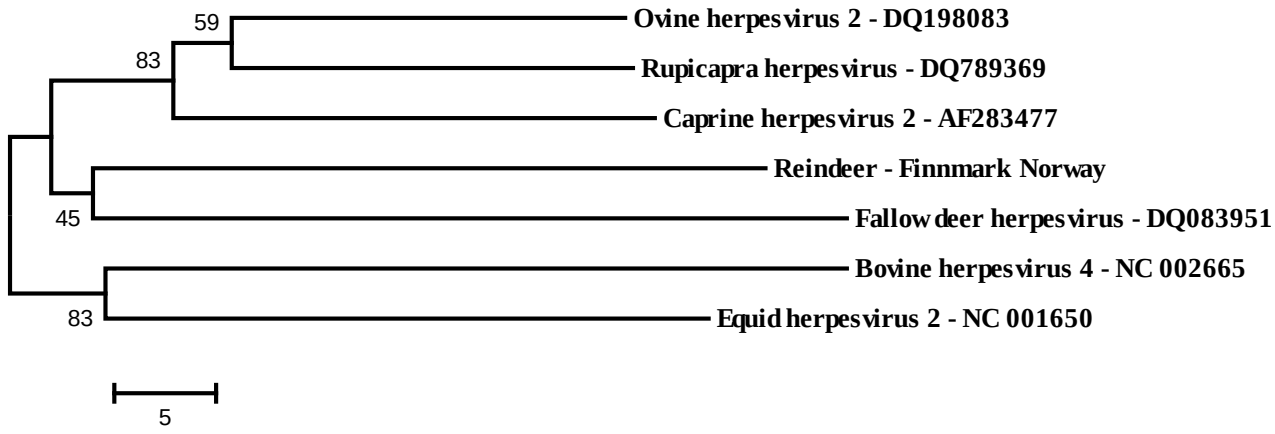


Figure 9. Phylogenetic tree indicating a possible evolutionary relationship between the reindeer virus and other gammaherpesviruses (GenBank) with closest homology. The numbers next to the branches indicate the percentage from the bootstrap test where the taxa clustered together. The scale express 5 nucleotide differences.

# 4 Discussion

This study documented that semi-domesticated reindeer throughout Finnmark are exposed to a gammaherpesvirus. Distribution of seropositive reindeer throughout districts may indicate that low level gammaherpesvirus exposure is widespread in semi-domesticated reindeer in Finnmark. The low prevalence (3.5%) of seropositive reindeer is consistent with the seroprevalence found in wild reindeer in southern Norway and caribou in Alaska, both 4% (Vikøren et al., 2006; Zarnke et al., 2002).

This low prevalence makes the serological screening more vulnerable to random error and inevitably prompts the question of whether the seropositive reindeer in this survey are true positives. The serological assay may have limitations for detecting the real level of antibodies to gammaherpesvirus. The ciELISA has previously been reported to have low sensitivity detecting cattle with clinical MCF, especially if seroconversion occurs after a rapid progression of MCF (Muller-Doblies et al., 1998). However, this report was based on a former ciELISA method (Li et al., 1994), but the direct ciELISA used in the current study may have similar limitations. An unpublished study showed that detectable antibodies in bison experimentally infected with OvHV2 appeared 7 to 30 days before clinical signs (Li et al., 2006). Other studies have shown that the poor sensitivity of the direct ciELISA result in an underestimation of the actual infection rates in cattle and bison (Li et al., 2001b). The sensitivity of the direct ciELISA method was improved for the detection of antibodies in latent or subclinical infected animals in certain ruminant species, and was proved to have high specificity (94%) in bison, cattle and deer (Li et al., 2001b).

No binary classification test was performed to measure sensitivity and specificity of the serological result. Binary classification requires an additional independent immunological or PCR test to act as a gold standard and to confirm the negative ciELISA result for a valid laboratory diagnosis. Additionally, two independent survey populations would be required for a validation and these conditions were not present. The specificity of the direct ciELISA has previously been confirmed in cattle and sheep, but no similar studies have been reported for reindeer. However, the similar seroprevalence found in the current study and the previous surveys in wild reindeer and caribou (Vikøren et al., 2006; Zarnke et al., 2002), may indicate that the specificity is accurate. A change in cut off value from 25% to 30% did not seem to affect the result significantly. Although the prevalence of antibodies detected in this

serosurvey was low, the reliability and power of the ciELISA should be considered strong. The high sample size and the fact that all samples were tested in duplicate are factors that minimize random errors in the assay.

The distinct difference in seroprevalence at reindeer herding district level may have been influenced by differences in the proportion of calves and adults between the districts. For example, district 40 had the highest seroprevalence of 6.7 % with 177 adults but only 2 calves (Table 3), whereas district 6 had no seropositive animals with only 5 adults and 110 calves. The study design did not allow sampling to be completely random as the reindeer owners chose which animals were sent to the slaughterhouse. This may have led to a skewed distribution of samples, not only regarding gender and age, but also on the distribution of reindeer concerning health condition and fitness. This can statistically represent a bias to the model, which expects random sampling.

The logistic regression model accounted for the skewed distribution at district level by integrating this variable into the model. In contrast, the more equal distribution of age and gender between east and west Finnmark seemed to be a result of a representative distribution than at district level. It is unclear why reindeer in east Finnmark had a higher chance of infection of gammaherpesvirus according to the logistic regression model. The reason may be due to a higher presence of carrier species in east that are responsible for the virus transmission. But since both PCR methods failed to indicate OvHV2 and CpHV2 as the causative viruses in the reindeer, sheep and goats seems less likely to be the cause of transmission.

The risk of being infected with gammaherpesvirus increased with increasing animal density. This is in accordance with a previous study on alphaherpesvirus in the same reindeer population in Finnmark (das Neves et al., 2009). It is unlikely that increasing animal density will result in increasing transmission rates among the reindeer since MCF susceptible ruminants are considered not to transmit gammherpesvirus within species (Ackermann, 2006; O'Toole et al., 2002). However horizontal transmission among reindeer cannot be excluded, especially if the causal virus serves as a reservoir in the reindeer. The higher risk of being infected with increasing animal density can more likely be explained by indirect causes following density dependent factors. Resource limitation may influence biological factors directly by affecting life history traits such as body mass, survival and recruitment rates

(Bonenfant et al., 2009; Fowler, 1981). A lower animal fitness and higher stress level are factors that can make the reindeer more susceptible to infections.

The logistic regression model showed a clear association between age and seroprevalence. Again this result was the same as for alphaherpesvirus conducted on the same reindeer material sampled from Finnmark (das Neves et al., 2009). Similar patterns have also been observed for carrier species, including musk ox (*Ovibos moschatus* Z), bighorn sheep (*Ovis Canadensis* S), domestic sheep and goats (Li et al., 1996). No association was found between prevalence of positive cattle and pigs in Norway (by IFAT, PCR and/or ciELISA) and age (Løken et al., 2009), however this study did not include animals under one year. The division of age into only two groups (<1 year and >1 year) can be misleading for the adult group in the current study because it contains both young and old animals, which can have major weight differences. If the age is unequally distributed within the adult group, this may cause a bias in weight. This may be the reason that weight did not have a significant effect on the seroprevalence, in addition to some discrepancies between carcass weight and age in the slaughter list. The most likely explanation for why age was a risk factor for infection is that adult animals have had a longer time to be exposed to the virus and thus to become infected.

Gender had no relevance according to the logistic regression model, although the Contigency table showed that female had a significant higher effect on seroprevalence than males. The different results are due to the effect of counting for several factors in the logistic regression model. Statistically it is correct to account for such confounding factors therefor the results from the logistic regression model should be regarded as the valid results.

The low antibody prevalence found in the apparently healthy reindeer suggests that the gammaherpesvirus is latent or subclinical in this population. This supports the present opinion that persistent MCFV infections is common for several ruminants species in which the infection is detected, but no clinical symptoms of MCF are present (Li et al., 1996; Powers et al., 2005; Vikøren et al., 2006; Zarnke et al., 2002). It has been suggested that cervid species with the lowest seroprevalences, such as moose and roe deer (0.4% to 1%), either are suffering a fatal outcome of a MCFV infection or that these cervids are not exposed to the virus as often as red deer and reindeer (Vikøren et al., 2006; Zarnke et al., 2002).
Even though a subclinical or latent course of the infection seems to be most likely among reindeer, the appearance of different clinical forms of MCF cannot be excluded. Other studies

have described a variety of different forms of MCF among free-ranging ruminants both in Norway and other countries. Moose have been shown to suffer from either a subacute or acute form of MCF, and roe deer can suffer from a peracute or acute form of the disease (Vikøren et al., 2006). A subacute course has been described in white-tailed deer (Li et al., 2003b), whereas sika deer can have both a chronic and acute form (Crawford et al., 2002; Keel et al., 2003). No cases of MCF in wild or semi-domesticated reindeer have been reported, except in captive reindeer in contact with sheep. However, disease and even mortality to an acute form of MCF, which can take 3 days in reindeer (Kiupel et al., 2004), may pass undiscovered for free ranging animals. Species susceptible to MCF viruses that have had a history of co-evolution with these viruses may be able to better control the infection and the development of the disease through their immune response.

To the knowledge of the author this study is the first to conduct PCR on gammaherpesvirus in healthy, sub-clinically infected free-ranging reindeer. Previous studies have confirmed the presence of OvHV2 in reindeer (Kiupel et al., 2004), indicating that reindeer are susceptible to the sheep associated OvHV2, and that the virus is pathogenic in reindeer. Since OvHV2 and CpHV2 are the only known gammaherpesviruses that are recognized in free ranging and captive ruminants in Norway (Vikøren et al. 2006), these viruses were assumed to be present in the reindeer. Three alternatives were assumed to be the most likely causative virus in the reindeer population in northern Norway: 1) OvHV2, 2) CpHV2 or 3) a new, yet unrecognized reindeer gammaherpesvirus. The OvHv2 specific PCR conducted in this study failed to reveal evidence of OvHV2 in the semi-domesticated reindeer in this study, and consensus PCR did not give indications of homology to known gammaherpesviruses, indicating that a yet undiscovered gammaherpesvirus is present in the Finnmark reindeer population.

The OvHV2 specific PCR was expected to have a low sensitivity and detection rate when testing the latently or subclinical infected reindeer according to previous studies. Li et al. (2006) refers to an unpublished study revealing that only about 10% of the seropositive, clinically normal bison had detectable OvHV2 DNA in peripheral blood. This indicates that PCR detection may be limited for animals with subclinical or latent infection due to low levels of viral DNA which are under detectable levels by PCR. This indicates that an infection of OvHV2 among the reindeer in Finnmark, is difficult to detect.

A remaining question is whether a new reindeer virus belongs to the MCF virus group (Macavirus) or is another gammaherpesvirus. The phylogenetic composition (Figure 9) may indicate that the reindeer in this study, were infected with a virus closely-related to those that shares the MCFV group-specific epitope. The knowledge of gammaherpesvirus prevalence in reindeer in Finnmark is a step forward in better understanding the impact of the infection for the species and for the reindeer husbandry. The low seroprevalence of gammaherpesvirus infection in reindeer suggest that a lethal outcome of the infection is less likely, supported by a previous study (Vikøren et al., 2006). The possibility of the existence of an unknown virus in reindeer raise several question such as if the virus is specific to the reindeer, if the reindeer themselves contribute as a primary source of MCFV infection and transmission route of the virus.

Further studies are needed to answer these questions. The sequences yielded from the consensus PCR showed a closer relationship to CpHV2 than OvHV2, suggesting that a next step could be a PCR specific for CpHV2. To reveal if the causative virus is an unknown virus, a different PCR targeting a different gene region than the polymerase gene in the nested consensus PCR is relevant to conduct. Isolation and genome sequencing is a goal to identify a new reindeer virus.

# 5 Conclusions

This study revealed the presence of a gammaherpesvirus in the reindeer population of Finnmark, Norway. A serosurvey against gammaherpesvirus showed a low prevalence of 3.5%, and statistical analysis indicated that adult reindeer and high animal density on summer pastures were positively correlated with seroprevalence. A PCR specific to OvHV2 yielded sequences that did not match to the expected position (ORF 73) of the OvHV2 genome, indicating that this virus may not be present in the reindeer population in Finnmark. Alignment of nucleotide sequences from a nested consensus PCR showed little homology to sequences of other known gammaherpesviruses deposited in the GenBank. All together, this suggests that a new, as yet undiscovered gammaherpesvirus, possibly specific to reindeer, may be present in the reindeer population in Finnmark. Further analysis including genome sequencing is needed for identification of a new reindeer virus. Such information would be a valuable contribution to future research.

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# Appendix

## Protocol for Direct Competitive-Inhibition ELISA in Detection of Malignant Catarrhal Fever Virus Antibody

Components included in the kit:

- Antigen-Coated Plates, 96-well microtiter.
- Positive Control.
- Negative Control.
- Antibody-Peroxydase Conjugate.
- Substrate Solution.

Components not included in the kit:

- Serum/ Conjugate Diluting Buffer.
  1 L deonized $H_2O$ + 1 tbl PBS
- Wash Solution (1 X PBS + 0.1% Tween 20).
  5 L deonized $H_2O$ + 10 tbl PBS + 10 ml Tween.
- Stop Solution (0.18 M sulfuric acid).
  990 ml deonized $H_2O$ + 10 ml 96.04% Sulfuric Acid ($H_2SO_4$).

Preparation and dilutions for 1 X 96-well plate:

- Antibody-Peroxidase Conjugate, dilution 1 : 100
- Positive and Negative Controls, dilution 1 : 5
- Serum or Plasma samples, dilution 1 : 5

Serum or plasma samples were diluted directly into non-antigen-coated transfer plates (96-well). Positive and negative controls were diluted in test tubes before added to the transfer plate. Positive and negative controls and serum samples were diluted to a volume of 65 µl in each well on the transfer plate, and each sample were mixed with the pipettor.

Procedure:

1. Samples and controls were transferred to the corresponding antigen-coated wells (50 µl) with a multichannel micropipettor. The plates were covered with parafilm and incubated at room temperature (21-25°C) for 60 minutes.
2. An automatic plate washer was used to remove the remaining sera and controls, 12 strips X 3 washes. After last wash, the plate was striked on a clean paper towel to remove residual Wash Solution.
3. Antibody-Peroxidase Conjugate (50 µl) were added to each sample well. The plates were covered with parafilm and incubated at room temperature (21-25°C) for 60 minutes.
4. The washing procedure described in step 2 was repeated.
5. Substrate Solution (100 µl) were added to each sample well, and incubated at room temperature for 60 minutes.
6. Add Stop Solution (100 µl) to each well.
7. Immediately shake the plate for 15 seconds on the plate reader, read and record the results. Adjust the plate reader and set the Optical Density (OD) to reading wavelength for 450 nm.