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[DETECTION AND CHARACTERIZATION OF HERPES VIRUS IN WILDLIFE SPECIMENS.]

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Abstract:
A nested PCR approach was used to investigate the presence of Herpesvirusses in different species of Canadian wildlife. The PCR is based on degenerate primers targeting the DNA polymerase B gene in open reading frame 9. The polymerase B protein is a essential protein and highly conserved in the DNA polymerase B gene of Herpesvirus. In this article we describe the findings of this nested PCR on wildlife samples. A unknown Herpesvirus in the specie *Martes americana* was detected. The detected sequences are aligned in a protein alignment and shown in a pyologenetic tree. This pyologenetic tree shows the relation of the sample Herpesvirusses to Herpesvirusses that were earlier submitted to Genbank. Alpha, Gamma and possibly Beta Herpesvirusses are found with this PCR method. For further research a better overview of Herpesvirusses is desirable by looking at a larger piece of the genome to determine the relation between Herpesviruses within animal species.

Introduction/background information:
Infectious diseases of wild animals where first only considered important when agriculture (domesticated species) or human health (regarding zoonotic pathogen) have been threatened but now they are also a growing concern in endangered species conservation[1].The emergence of these diseases has increased over the past few decades and is now viewed as one of the greatest threats to biodiversity[1, 2]. Enveloped double-stranded linear DNA viruses of the family Herpesviridae are found throughout the animal kingdom, and close to 100 species have been isolated and partially characterized[3]. Herpesviruses share a common structure of double-stranded linear DNA genomes encased in an capsid what exists from a protein layer of viral mRNA/proteins and a lipid bilayer what forms a envelope. There are three sub-families alpha-, beta- and gamma-Herpesvirus each further divided in different genera(Table 1) and still viruses that are unassigned to a genera[4].

Sub-families	Genera
Alpha – Herpesvirus	*Iltovirus*
	Mardivirus
	Simplexvirus
	Varicellovirus
Beta - Herpesvirus	*Cytomegalovirus*
	Muromegalovirus
	Proboscivirus
	Roseolovirus
Gamma - Herpesvirus	*Lymphocryptovirus*
	Macavirus
	Percavirus
	Rhadinovirus

Table 1: Sub-families and genus division.

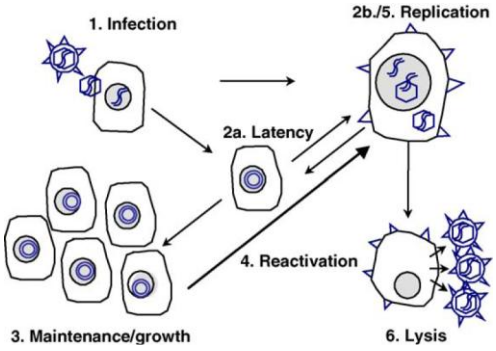

Figure 1: Cyclus of gammaherpesvirus infected cells. (1) The viral DNA is delivered to the nucleus, where it circularizes and is subject to nuclear factors, which may depend on the present cell type, activation state and differentiation state. (2) Latency may be established either without (a) or following viral replication (b). (3) Viral genes expressed throughout latency in a cell-type dependent manner will determine whether or not the infected cell will replicate, and if it does, at which rate. Signaling as well as manipulation of the immune response through release of viral cytokines and chemokines may occur in a host-dependent fashion. (4) – (6) Stress as well as other factors may induce reactivation from latency, which will eventually lead to the lysis of infected cells and to the release of new virus[18].

All Herpesviruses are nuclear-replicating. They enter the cell true receptor binding, fusion, and translocation after entry virus particles are transported along the microtubular network to the nuclear pores. The nucleocapsids associate with the nuclear pore complexes and release their DNA into the nucleus. Here the viral DNA gets transcribed to mRNA. Alpha- and beta-Herpesviruses that infect cells most likely act with lytic replication. While most gamma-Herpesvirus infected cells maintain a state of latency. In some host cells latency associated transcript (LAT) are accumulated, viral genes instead of the lytic viral genes. What result in signaling of membrane proteins (release of viral cytokines and chemokines) of the gamma Herpesvirus that protects the infected cell in the organism for

the immune system. The three sub-families have different sites where they stay latent. Alpha-Herpesviruses stay latent in the neurons, beta and gamma-Herpesviruses can reside latently in lymphocytes and macrophages[5]. If reactivations occurs due to immunosuppresion[5], for example following prolonged stress[6], transcription of viral genes transitions from LAT to viral genes that leads to enhanced replication of virus and lysis of infected cells. These latent infected animals can develop disease but mostly shed virus after the virus is reactivated but the latency period itself is usually symptom-free[7]. It is expected that many animals are infected but don't develop disease and are latent infected for life. There are different kind of Herpesviruses known that can cause disease within different animal species[7]. In wildlife Herpesviruses can be found in almost every species and in general they have neither been thoroughly identified nor characterized. Also the effect of Herpesviruses on wildlife populations is not clear. Whether the presence of various Herpesviruses in a population can lead to a restriction for the population growth, extinction or will have no consequences is unknown. It is difficult to monitor viral infections in wildlife, they will generally be noticed when animals are severely diseased or dying due to the virus infection but not in an earlier stage. Agents with a long infectious or latency period may be able to persist in small populations. It is shown for other multi-host viral pathogens (for example Canine Distemper carried by dogs on Ethiopian wolves and African wild dogs)[8, 9] that these can persists in even a relatively small group of wildlife animals[7].

As many Herpesviruses isolated from different host species are closely related, it has been suggested that the evolution of Herpesviruses occurred across host species[3]. Antibodies induced by a Herpesvirus from one animal species sometimes cross-react with a member of the family Herpesvirus from a different host[6]. Herpesvirus appear to be host specific, but the following examples show that Herpesvirusses can in some cases cross the species barrier. This is shown in malignant cattharal fever (MCF) antibodies from non wildebeest-associated MCF (NWA-MCF, OHV-2) react with the wildebeest-associated MCF (WA-MCF, AHV-1) virus what implies they are related. MCF can develop in cervids[6, 10, 11] and can cross the species barrier and infect domestic animals or the opposing way[12, 13]. Although sheep are the original host for OHV-2 and could play a role in the transmission of Herpesviruses to other species like cervids and cattle it is not proven they are a reservoir for NWA-MCF disease [6, 7, 12-17]. Domestic goats and rabbits are thought to be a reservoir, suggesting OHV-2 has a wider host range then one species[12, 13, 18]. MCF is associated with viruses from the subgroup of the ruminant rhadinoviruses only[4]. Other examples of Herpesvirusses crossing species are Pseudorabiesvirus (PRV) an alpha-Herpesvirus of porcine that can cause Aujesky's disease in a variety of species such as cattle and dogs[19, 20]. Feral swine populations could be a potential reservoir of PRV for infection of domestic swine and native wildlife for example bears[20, 21]. Cercopithecine herpesvirus-1, (CHV-1 Monkey B virus, alpha-Herpesvirus) is commonly found in Asian Macaques but can also be found by humans what is an non-natural and a dead end host. A CHV-1 infection in humans it can lead to various injuries (paralysis, dead caused by encephalomyelitis)[7]. Although several Herpesvirusses in free-ranging wildlife species were reported from different countries and continents [6, 16, 17, 22, 23] and various transmission routes (aerosols and horizontal transmission)[6] have been described, relatively little is known about the risk of interspecies transmission and infection dynamics in the wild. Especially as the knowledge on the viral dynamics between wildlife and domestic livestock is lacking, the ability to predict their emergence and to develop effective countermeasures against outbreaks is absent.

To gain a better understanding of the basic properties of emerging diseases in the wild, intensive field studies based on model systems are important[24]. Therefore a more in depth knowledge on the Herpesviruses present in wildlife species and its evolution will increase our understanding of virus exchange events between species (including livestock and humans), the fundamental knowledge of virus evolution, and the establishment of appropriate models. To study the presence and the interspecies dynamics of Herpesviruses in different species of wildlife we must be able to adequately characterize them.

In this report we describe the findings using a broad spectrum PCR method. This PCR assay targets a highly conserved region of the Herpesviral DNA polymerase gene[3, 5, 25], used for screening wildlife samples. We suspect that many of animal species are infected with at least one Herpesvirus, therefore it is expected that a substantial amount of samples from wildlife will generate a positive result. From these detections of Herpesvirus with PCR, virus isolation and characterization follow.

Materials and methods:

The tested samples are blood or various tissues. Herpesviruses can most frequently be found in blood or tissue samples[7] as these are the predispositions sites for latent infections. In blood the highest success rate to detect Herpesvirus is expected to be the peripheral blood mononuclear cell (PBMC) compartment. Beta and gamma-Herpesviruses can reside latently in lymphocytes and macrophages[5], therefore when extracted these cells EDTA whole blood derived from the field or from tissue samples, lymph nodes or spleen. It has been described that Herpesviruses can also be found in serum samples but only from a viremic animal, which is a highly unlikely event as we sample clinically healthy animals. All samples are derived during necropsy as presented to UCVM or during wildlife-captures where also blood samples were taken.

Tissue collection of necropsies:

The presented animals at the department wildlife pathology from Calgary university are from multiple sources(for example the coyotes where trapped for different research and other animals are brought to UofC as road kill, culled on suspicion disease, wildlife management). The animals can be healthy to severely diseased animals. The necropsy started with obtaining weight, sex, body length (from end of the skull to first caudal vertebrae), neck girth, body condition, absence of deciduous tooth (indicator of age). Gross post mortem examination is subsequently preformed. Samples for virological assays are taken of liver, kidney, spleen, lymph node, tonsils and lung. All material is collected in a freezer bag and stored at -80°C until further processing.

Obtained samples:

Marten material was collected by trappers and send to university of Calgary for wildlife research in general. From every organ origin in the thorax and abdominal cavity a part or in total was collected in a freezer bag as a tissue pool and stored at -80°C until further processing.
The bison samples were obtained by hunters and send to university of Calgary for wildlife research in general. Mesenteric lymph node where obtained and separately stored in a freezer bag at -80°C until further processing.

Tissue pool separation and preparation:
From the tissue pool the different organs get separated on a petri dish. From every organ 3 to 4 pieces are separated and is stored in a sterile micro-centrifuge tubes at -80 °C.

Blood sample collection of wildlife captures:
The animals are captured by a wildlife veterinarian for wildlife conservation. The animals get tagged for identification and general measurements are preformed. The animal is sedated using a dart, usually shot intramuscular (biceps femoris/quadriceps femoris) with ketamine and an alpha-2-agonist. After the animal was sedated the measurements are taken and a blood sample is taken from the *vena jugularis* or the *vena cephalica*. Serum and EDTA tubes are used for blood collection. Serum tubes are centrifuged at 4500 x g for 20 minutes. The serum is stored in a micro-centrifuged tube at -80°C.

Host order and species	**Status and origin of host**	**Specimen(s)**
Elk (*Cervus elaphus*)	Free Ranging	Blood/Serum
Bison (*Bison bison*)	Free Ranging	Lymph node
Big Hoorn Sheep (*Ovis musimon*)	Free Ranging	PBMC/ Lymph node
Marter (*Martes americana)*	Free Ranging / Trapped	Spleen/ Mesenteric lymph node
Coyote (*Canis latrans*)	Free Ranging/ Trapped	Lymph node/Spleen
Wolf (*Canis lupus*)	Free Ranging/ Trapped	Lymph node
Black Bear (*Ursus americanus*)	Free Ranging	Lymph node
Grizzly Bear (*Ursus arctos horriblis*)	Free Ranging	Spleen
Red Fox (*Vulpes vulpes*)	Free Ranging/ Trapped	Lymph node
Cougar (*Puma concolor*)	Free Ranging	Lymph node
Racoon (*Procyon lotor*)	Free Ranging	Spleen

Table 2: Used samples

DNA extraction:
For viral extraction from samples of serum or PBMC the E.Z.N.A.® Blood DNA Kit(Omega Bioservices, Norcross, America) is used. For lyses: 100 µl sample, 150µl Elution Buffer (pre-heated to 65°C), 25µl OB Protease Solution and 250 µl BLN Buffer transferred into a sterile micro-centrifuge tube. To adjust the binding conditions 260µl 100% ethanol is added. The sample is transferred to a HiBind DNA Mini Column to bind the nucleic acid to the column. In total three washings are used to remove proteins and other non-DNA material, the first is with 500 µl HB Buffer, the second and third with 700µl DNA Wash Buffer. After every wash-step the column is centrifuged at >10,000 x g for one minute. Drying the column matrix is performed by centrifugation at 10,000 x g for 2 minutes. The DNA material is eluted with 150 µl Elution Buffer(pre-heated to 65 °C) and centrifuged at >13,000 x g for one minute. The extracted DNA is stored at -80 °C.

For viral extraction from tissue samples the E.Z.N.A.® Tissue DNA kit(Omega Bioservices, Norcross, America) is used. Tissue samples are cut into small pieces and after adding 200 µL of PBS, mashed to speed up the lyses. 200 µL TL Buffer, 25 µL OB Protease Solution are added and incubated in a water bath at 55 °C for three hours so lyses can occur. The material is then centrifuged at 13,000 x g for five minutes. To adjust binding conditions the supernatant is transferred to a sterile micro-centrifuge tube where 220 µL BL Buffer is added

and incubated at 70°C for ten minutes. 220 µL of 100% ethanol is added and the sample is transferred to a HiBind DNA Mini Column. This sample is centrifuged at 10,000 x g for one minute in order to bind the DNA to the column. Washing is preformed in three times the first time with 500 µL HB Buffer and the second and third time with 700µL DNA Wash Buffer. Drying the column matrix is done by two minutes centrifuge at 10,000 x g.(This is to prevent residual ethanol interference with downstream applications). The DNA is eluted in 150 µL of preheated (70 °C) Elution Buffer and centrifuged at 13,000 x g for one minute. Extracted DNA is stored at -80 °C.

Screening for Herpes using PCR:

A pan-Herpes consensus PCR[3, 5, 25] will be used to detect the possible presence of Herpesviruses. The pan-Herpesvirus PCR is able to detect all gamma-, beta- and alpha-Herpesviruses. The area which is targeted by the nested PCR is shown in the Herpesvirus genome in figure 2. The targeted area of 215bp-315bp is in the open reading frame 9 protein(ORF) (fig. 3).

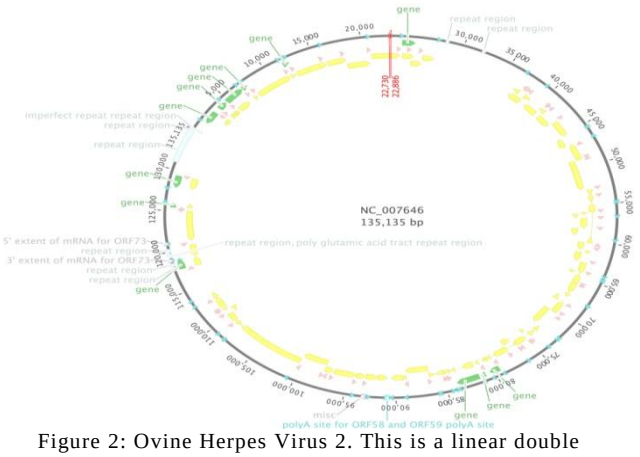

Figure 2: Ovine Herpes Virus 2. This is a linear double stranded gamma-Herpesvirus. The genome is shown as a circular genome, after entering the cell nucleus it changes from linear to circular. Between the red lines indicate the sequenced part. Left from the sequenced part TGV 5' primer is attached and right IYG 5' primer.

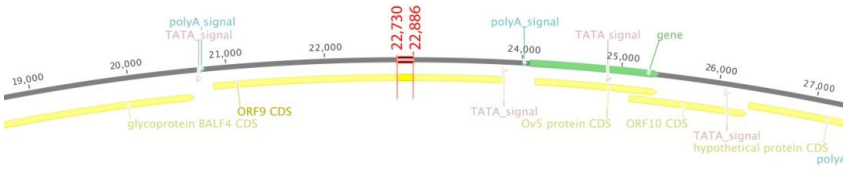

Figure 3: Zoom in on the OHV-2

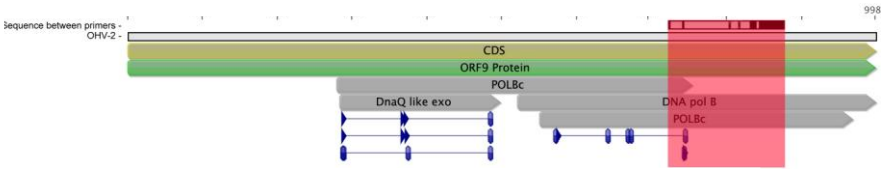

Figure 4: Zoom in on the OHV-2 (ORF9 protein) aligned with target sequence. Left from the sequenced part TGV 5' primer is attached and right IYG 5' primer

In ORF 9 the DNA polymerase B gene is found,(fig. 4) in this gene the primer binding sites are located. This is because the used primers are based on target sites of human Herpesviral DNA polymerase genes and amplify a highly conserved amino acid motif in the DNA directed DNA polymerase of the Herpesviral genome which is used to detect a part of the Herpesviruses even when there is no prior DNA sequence information is available for these viruses[3]. The primers used for this PCR are degenerate primers, dI-containing primers (fig. 5 shows where the primers attach). Degenerate bases are similar but not identical primers. This is convenient if the same gene is to be amplified from different organisms, or a primer for a protein(one amino acid can have different codons). dI is Deoxyinosine in DNA it is a base similar to Guanosine but without two amino groups. It is used as a universal base but has preference for C see table 1. The

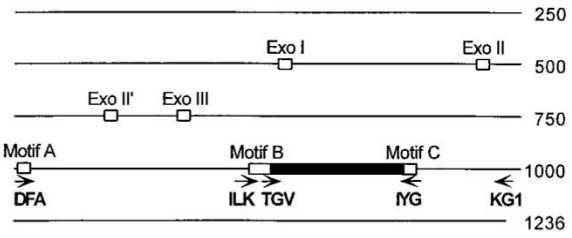

Figure 5: The positions of PCR priming sites for DFA, ILK, KG, TGV, and IYG primers within an amino acid-coding map of human Herpesvirus 1 DNA polymerase gene. The black highlight represent the region obtained with the used primers.[3]

sensitivity of this PCR is not optimal for every Herpesvirus species, because degenerate primers do not bind to related sequences with equal affinity[25].

Used **Primers:**
Three primers where used for the first-round of PCR:

Forward primers:
- DFA 5'- GAY TTY GC(N/I) AGY YT(N/I) TAY CC
-ILK 5'- TCC TGG ACA AGC AGC AR(N/I) YSG C(N/I)M T(N/I)A A

Backward primers:
-KG1 5' – GTC TTG CTC ACC AG(N/I) TC(N/I) AC(N/I) CCY TT

Two primers were used for second-round of PCR:

Forward primers:
-TGV 5' - TGT AAC TCG GTG TAY GG(N/I) TTY AC(N/I) GG(N/I) TTY AC(N/I) GG(N/I) GT

Backwards primers:
-IYG 5' – CAC AGA GTC CGT RTC (N/I)CC RTA DAT

Symbol	Description	Bases represented			
A	Adenosine	A			
C	Cytidine		C		
G	Guanosine			G	
T	Thymidine				T
I	Deoxyinosine	I-C >: I-A> I-G ~ I-T			
N	Any Base /No gab	A	C	G	T
R	Purine	A		G	
S	Strong		C	G	
Y	Pyrimidine		C		T

Table 3: IUPAC system

The nested PCR was performed in two rounds. In the first-round of PCR 3 primers (DFA, ILK and KG1) were used. In the second-round of PCR 2 primers (TGV and IYG) were used. As positive control the vaccine Novartis (Bovine Herpes Virus 1) was used. As negative control H_2O was used.

The PCR mix for the first round of PCR existed of 10 µL F or H Buffer, 1 µL of each primer (DFA/ILK/KG1), 3.8 µL H2O and 0.2 µL Taq Polymerase. 3 µL Template is added, the total 20 µL is entered in Bio Rad T100™ Thermal Cycler and undergoes the first round of PCR following the protocol 95°C (5 min.), 45 cycles: 95°C (30 sec.), 46°C (60 sec.), 72°C (90 sec.), 72°C (5 min.) and then 4°C (Forever).

The PCR mix for the second round of PCR existed of 10 µL F or H Buffer, 1 µL of the TGV and IYG Primer, 5.8 µL H2O and 0.2 µL Taq Polymerase. 2 µL Template is added, the total 20 µL is entered in Bio Rad T100™ Thermal Cycler and undergoes the second round of PCR following the protocol 95°C (5 min.), 45 cycles: 95°C (30 sec.), 46°C (30 sec.), 72°C (60 sec.), 72°C (5 min.) and then 4°C (Forever).

Amplicon Detection:
With each sample 4 µL Glycerol orange G dye is added. 15µL sample is added in each well within the 1.5% Agarose Gel (2,25 g agarose gel powder + 150 ml E.Z.N.A.® TAE buffer + 15 µL Safeview™ DNA attaching). The 1 kb plus ladder from invitrogen is added. The agarose gel is run on 110 volt for 45 minutes. Blue light gel electrophoresis is used for

detection of the positive bands. The positive bands are cut out with a scalpel stored in a sterile micro tube at -20 C°.

Gel extraction:
If a positive band was obtained with this PCR, the material was extracted from the agarose. This was done with a E.Z.N.A.® Gel Extraction Kit(Omega Bioservices, Norcross, America). 300µL of Binding Buffer (XP2) is added for incubation and melting of the gel in a 60 °C water bath for seven minutes. The sample is transferred in a HiBind DNA Mini Column and centrifuged at 10,000 x g for one minute for binding to the column. 300 µL Binding Buffer (XP2) is added and centrifuged at 13,000 x g for one minute. This step is repeated with 700 µL of SPW Wash Buffer for washing. Drying the column matrix is done by two minutes centrifuge at 13,000 x g for removal of the ethanol. To Elute the DNA 40 µL of Elution Buffer is added and centrifuging 13,000 x g for one minute. The elute DNA is stored at -80 °C to stop all enzymatic activity.

Cloning PCR gel extractions:
When positive bands from the PCR are faintly positive they are cloned in a PGEM T easy vector before the were sent for sequencing.

Ligation is achieved by 3 µL of PCR product combined with the premix for ligation (1µL vector, 1 µL ligase and 5 µL 2xbuffer) and stored at 4°C for 16 hours. Transformation is started with adding Top10 cells to the ligation product followed by a 15 min incubation on ice. Followed by heat shocking the cells for 90 seconds at 42 °C and again a 10 minutes incubation on ice. After adding 1000µL of LB at 37° C the cells were shaken at 37 °C for 60 minutes. Centrifuged at 14,000 x g for one minute. The supernatant is removed and the E.coli was resuspended in 200µL LB. 20µL and 150µL of E.coli was spread on two agar plates prepared with 40µL of xgal. These Agar plates were incubated for 16-24 hours at 37 °C. Colonies were isolated and inoculated in 30mL LB-Broth (Luria-Bertani) medium with Ampiciline (1mL Ampiciline in a liter LB-Broth) then incubated with shaking (~300 rpm) for 16 hours at 37°C. Pellet the bacterial cells by centrifugation at 4,500 x g for three minute at room temperature. Bacterial pellet is resuspended with 250 µL RNase A solution 1 (For the isolation of plasmid from E.coli the E.Z.N.A.® Plasmid Mini Kit 1 is used). For lysation Solution 2 is added. Lysis is neutralized before five minutes by adding 350 µL solution 3 and mixed immediately to prevent localized precipitation. Centrifuge at 13,000 x g for ten minutes. The supernatant is transferred to a HiBind DNA Mini Column which is prepared with a Equilibration Buffer. Centrifuge at 13,000 x g for one minute. Washing is started with 500 µL of HB Buffer and followed by 700µL of DNA Wash Buffer both after adding centrifuged at 13,000 x g for 30 seconds. The column is dried by two minutes at 13,000 x g. Column is added in 1,5 ml micro-centrifuge tube. 30 µL of Elution Buffer is added and spun down at 13,00 x g to elude the DNA. A nanodrop is used to check if the eluted DNA contains between 100-200 ng/µL plasmid DNA . Samples with a concentration between 100-200 ng/µL are ready to send for sequencing to Eurofins MWG Operon(Huntsville, USA). For higher concentrations a dilution to 100-200 ng/µL with sterile H_2O will be performed .

Characterization of Herpesvirus positive PCR material using sequencing:
The positive gel-extracted sample or the cloned sample were prepared for sequencing. The gel-extracted sample is mixed: 8µL is added to 4µL primer TGV or IYG (a 1:5 dilution of the stock solution with sterile H_2O). In total, a 12 µL sample was sent for sequencing. The cloned sample contained between 100-200 ng/µL of the plasmid with insert.

Phylogenetic analysis:
Analyses were carried out at the level of nucleic acid sequences. Sequence quality was visually inspected using Finch TV(Geospiza) and adjusted when needed. Sequences of cloning vectors or primers were removed using the Geneious program. The obtained sequence was submitted to BLAST (NCBI website) in order to determine the type of Herpesvirus the genome is most related to. Sets of bases pares for Polymerase B gene from partial gene sequences were aligned using ClustalW. Positions in alignments that had missing characters in any sequence and regions considered too divergent for justified aligning were removed before the alignments were used for phylogenetic analysis. Measures of nucleotides divergence where done in MEGA5.1.

Results:
We investigated mammals from different orders from Canada ,that were living in the wild, on the presence of Herpesvirus. Blood or tissue were collected from Elk (*Cervus elaphus*), Bison (*Bison bison*), Bighorn sheep (*Ovis musimon*), Marten (*Martes americana*), Coyotes (*Canis latrans*), Wolf (*Canis lupus*), Black Bear (*Ursus americanus***),** Grizzly Bear **(***Ursus arctos horriblis***),** Red Fox (*Vulpes vulpes*), Cougar **(***Puma concolor***),** and Racoon (*Procyon lotor).* (Table 4)

Host order and species	**Status and origin of host**	**Specimen(s)**	**Amount of samples**	**Amount Positive tested**	**Details positive tested**	**Related virus compared with NCIB(Blast)**	**Sub-Family**
Elk (*Cervus elaphus*)	Free Ranging	Bloom/Serum	40	4	E25,E29, E69, E116	97% type 2 ruminant Rhadinovirus of Elk[4]	γ
Bison (*Bison bison*)	Free Ranging	Lymph node	27	5	M10-13LN, M09-08LN, M08-07, M09-13, M09-14	American bison Herpesvirus 100%.	γ
Bighorn Sheep (*Ovis musimon*)	Free Ranging	PBMC	22	4	346BHS, 547BHS, 549BHS	99% type 2 ruminant rhadinovirus of big horn sheep and OHV-2	γ
		Lymph node	1		553BHS BHS 49-2012 PD lnn.		
Marten (*Martes americana*)	Free Ranging/ Trapped/ North West terratorium	Spleen	67	9	66 (spleen2/7/12 box: jan 16/12), 59 (spleen2/7/12 box: feb 7/12), 11(spleen2/21/12 box: feb 10), 47(spleen2/22/12 box feb:27/12), 1 (spleen2/24/12 box: feb 27/12), 35(spleen march 26/12box:march	Multiple outcome	

		Mesenteric lymph node	19	3	14), 38(spleen march28/12box:march 28), 30(spleen april 1/12box:april 1), 27(spleen april 1/12 box:april 1) FGH #3 Feb2010 MLN, Colville lake #4 Feb2010 MLN, FGH#8 Feb2010 MLN		
Coyote (*Canis latrans*)	Free Ranging/trapped	Lymph node Spleen	66 5		116/2012 nov 26 lnn., 150/2012 PD 11 dec lnn., 155/2012 PD 18dec lnn 115/2012 nov 22 lnn.	Canid Herpesvirus1 97% Multiple outcome	α
Wolf (*Canis lupus*)	Free Ranging/ Trapped	Lymph node	4	1	PD 12-46 1-10-12 lnn.	Canid Herpesvirus1 97%	α
Black Bear (*Ursus americanus*)	Free Ranging	Lymph node	5	0			
Grizzly Bear (*Ursus arctos horriblis*)	Free Ranging	Spleen	1	0			
Red Fox (*Vulpes vulpes*)	Free Ranging/ Trapped	Lymph node	3	0			
Cougar (*Puma concolor*)	Free Ranging		1	0			
Racoon (*Procyon lotor*)	Free Ranging	Spleen	1	0			

Table 4: Results

The Elk samples are 97% identical with a Rhadinovirus of Elk submitted earlier by Hong Li[4].

The positive Bison samples (Bison M10-13LN, Bison M09-08LN, M09-14) where completely identical to the sequence found in American bison and deposited in Genbank (AY057986.1) by Kleiboeker in 2001. Also found by Hong Li[4] in 13 bison.

Big horn sheep (sample 346BHS, 547BHS, 549BHS, BHS 49-2012 PD lnn.) are >96% similar to type 2 ruminant rhadinovirus of bighorn sheep but also very similar to the rhadinovirus of domestic sheep (>90%). Sample 553BHS found most (90%) comparison with ovine Herpesvirus 2. In the phylogenetic tree the Bighorn sheep samples are compared with sequences from Genbank. (BHS rhadinovirus AY237360.1 and OVH-2 HM216472.1)(Fig. 6) In the phylogenetic tree from the aligned proteins (fig. 10) also shows the group of rhadinovirus and a bigger distance to BHS553.

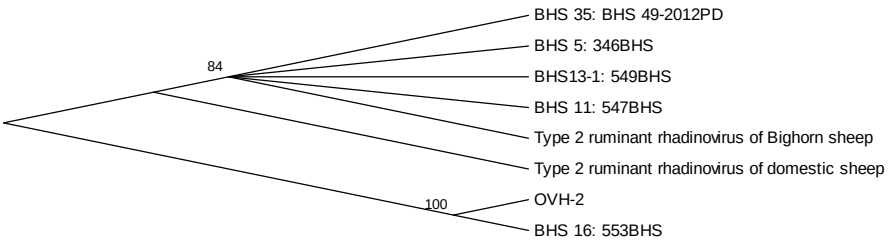

Figure 6: Comparison found Big Horn Sheep nucleotide sequences with OVH-2 and Big Horn Sheep Rhadinovirus from Blast. It can be seen here that the 553BHS has more similarity with OHV-2 then the Rhadinovirus of Bighorn Sheep or domestic sheep. Maximum likely-hood tree. Bootstrap of 2000 with cut-off value 70.

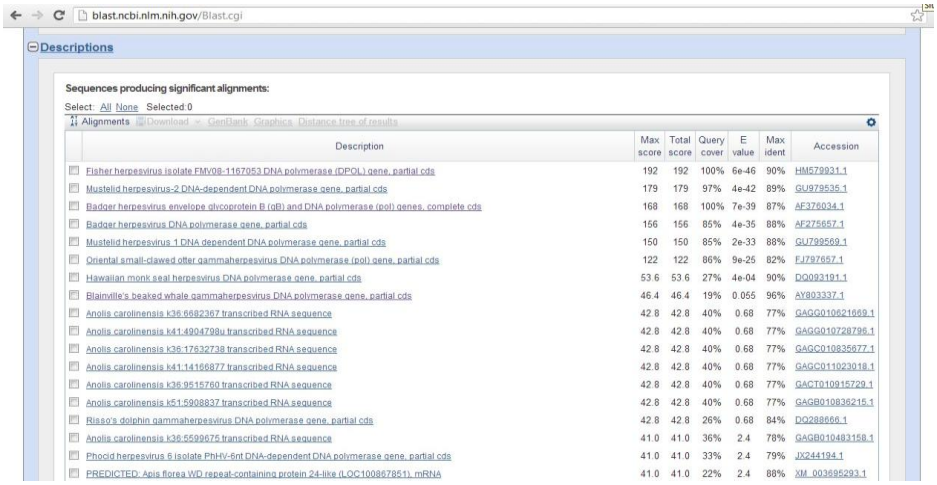

Figure 7: Blast results from Marten Colville lake #4 Feb2010 MLN.

The sequences of marten (59 spleen 2/7/12 box: feb 7/12, FGH#8 Feb2010 MLN, Colville lake #4 Feb2010 MLN, 47 spleen 2/22/12 box feb: 27/12) entered in Blast found all a similar outcome as figure 7. What can be seen is that there is no identical sequences but there are Herpesvirusses that have similarities with this sequences. In the list *mustelidae* and badger (Sub-family *Melinae, Mellivorinae,* and *Taxidiinae*) Herpesvirusses are shown. The marten belongs to the family *mustelidae* as do the badger and the otter (sub-family *Lutrinae*). After alignment these marten samples showed close relatedness. These marten samples are aligned with Mustelid Herpesvirus (GU979535.1) and Badger Herpesvirus (AF376034.1) from Genbank. This showed a distance of >88,4% and >84% with the used samples. The Herpesviruses found in these martens could be specific for the species marten. The close relation with the *mustelidae* and badger Herpesvirusses is to be expected since these animal species are closely related as well, therefore it seems logical that their Herpesvirusses will have similarities too. Both *mustelidae* and the badger Herpesvirus are gamma-Herpesvirusses. Assuming the detected marten Herpesvirus sequences are new and formally unknown, with similarities to the badger and *mustelidae* gamma-Herpesviruses, we hypothesize it could be also a gamma-Herpesvirus. In figure 10 it the relatedness of the new marten Herpesvirus to the other gamma-Herpesvirusses is shown.

The herpesvirus sequences obtained from the marten samples (66 spleen 2/7/12 box: jan 16/12, 30 spleen april 1/12 box: april 1, 27 spleen april 1/12 box:april 1,11 spleen 2/21/12 box: feb 10) searched in Blast found Mus musculus rhadinovirus 1(AY854167.1). 50 base pairs and 97% similarity. This is a rodent (house mouse) and common in Canada. The

sequences show >97,3% similarity. When aligned with the proteins the sequenced part is recognized as part of the polymerase B gene and related to human Herpesvirus 6A/B a beta-herpesvirus.

Marten samples (1 spleen 2/24/12 box: feb 27/12, 35 spleen march 26/12 box: march 14, 38 spleen march 28/12 box: march 28, FGH #3 Feb2010 MLN) are 83% similar to Equine Herpesvirus, this is only for 50 base pares, but also here Blast results showed small sequences of Herpesvirusses on different locations of the 180 base pairs sized sample. When these 4 samples are aligned the distances are >99,5%. This means the sequences are very similar to one another but don't seem to have a reference within the Genbank. In figure 10 it can be seen that at the protein level these marten Herpesviruses seem to be related to different types of Equine Herpesvirus and the Canid Herpesvirus 1. It is likely that the Herpesvirus within these martens is Alpha-herpesvirus.

Marten samples (FGH #4 Feb2010 MLN, 44 spleen 2/16/12 box: feb 10,) in these samples only short sequences (30 base pairs) of matching Herpesvirusses are discovered. Mostly Bottlenose dolphin herpesvirus DNA polymerase gene where found, this sequences has an area that is completely identical to the IYG primer. Aligning these samples with the second round primers shows similarity for the primer. This is also the case with sample Bison M09-13, 30 base pairs where found with Blast as Blainville's beaked whale gamma Herpesvirus DNA polymerase gene, this sequences also has an area that is completely identical to the IYG primer. When aligning it with second round primers it aligned 77,3% with primer TGV and 43,8% with IYG. It is likely that the sequences found is a composition of the primers overlap instead of a Herpesvirus.


blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Mus musculus rhadinovirus 1 glycoprotein B (gB) and DNA polymerase (DPOL) genes, partial cds	50.0	50.0	15%	0.007	97%	AY854167.1
Bottlenose dolphin herpesvirus DNA polymerase gene, partial cds	48.2	48.2	13%	0.024	100%	AY757301.1
Anguilla japonica contig15612 transcribed RNA sequence	46.4	46.4	15%	0.084	93%	GAGT01013935.1
Otariid herpesvirus 4 isolate OtHV-4 DNA-dependent DNA polymerase gene, partial cds	46.4	46.4	15%	0.084	93%	JX244190.1
Drosophila pseudoobscura pseudoobscura GA19253 (Dpse\GA19253), mRNA	46.4	46.4	14%	0.084	96%	XM_001352985.2
Populus trichocarpa clone POP023-C23, complete sequence	46.4	46.4	24%	0.084	83%	AC216837.1
Blainville's beaked whale gammaherpesvirus DNA polymerase gene, partial cds	46.4	46.4	18%	0.084	92%	AY803337.1
Cytomegalovirus Guenon/muscle/BM-002 polymerase gene, partial cds	44.6	44.6	16%	0.29	91%	JF828328.1
Caprine herpesvirus 2 strain 24592HIRSCH polymerase gene, partial cds	44.6	44.6	13%	0.29	96%	HM216463.1
Helicobacter pylori strain 8033 hypothetical protein gene, complete cds	44.6	44.6	12%	0.29	100%	EU553482.1
Helicobacter pylori ORF2 DNA for hypothetical protein, strain 8033	44.6	44.6	12%	0.29	100%	AM946636.1
Myodes glareolus rhadinovirus 1 glycoprotein B (gB) and DNA polymerase (DPOL) genes, partial cds	44.6	44.6	13%	0.29	96%	AY854169.2
Panulirus argus virus 1 DNA-directed DNA polymerase (pol) gene, partial cds	44.6	44.6	13%	0.29	96%	DQ465025.1
Helicobacter acinonychis str. Sheeba complete genome, strain Sheeba	44.6	44.6	12%	0.29	100%	AM260522.1
Parachlamydia-related symbiont UWE25, complete genome	44.6	44.6	20%	0.29	87%	BX908798.1
Gallus gallus prolactin receptor transcript variant 2h (PRLR) mRNA, 5' UTR	42.8	42.8	17%	1.0	89%	JX560223.1
Anolis carolinensis k26:14539052 transcribed RNA sequence	42.8	42.8	13%	1.0	96%	GACT010196834.1
Anolis carolinensis k21_20113255u transcribed RNA sequence	42.8	42.8	13%	1.0	96%	GAGA01024397.1
Anolis carolinensis k26_11116537 transcribed RNA sequence	42.8	42.8	13%	1.0	96%	GAGA01059996.1
Rivularia sp. PCC 7116, complete genome	42.8	42.8	14%	1.0	93%	CP003549.1
Mesoplodon densirostris herpesvirus isolate 51(243)K DNA polymerase gene, partial cds	42.8	42.8	16%	1.0	88%	JN863234.1

Figure 8: Blast results from marten sample 30 spleen april 1/12 box: april 1.

From the cougar and fox sequences only 30 base pairs where recognized as Herpesvirus in Blast. 89,9% was similar with the IYG primer.

4 coyote samples (129/2012 29/11/12 lnn., 86/2012 19-10-12 spleen, 128/2012 lnn. 29/11/12, 131/2012 10/12/12 lnn.) exist of 30 base pairs after removing the vector. In Genbank these match with Bottlenose dolphin Herpesvirus DNA polymerase gene, this sequences has an area that is completely identical to the IYG primer. All of these sequences align partly with primer IYG and TGV, and it is likely that the sequences found are a composition of the primers that overlap instead of a Herpesvirus.

The coyote samples (116/2012 nov 26 lnn., 150/2012 PD 11 dec lnn., 155/2012 PD 18dec lnn.,) and a wolf sample (PD 12-46 1-10-12 lnn.) where >97% similar with Canid herpesvirus 1 (EU531507.1). These are alpha-Herpesvirusses. These samples were clearly visible in the agarose gel: for example 31 in fig. 9.

Coyote (115/2012 nov 22 lnn.) Matches most with *Myodes glareolus* rhadinovirus 1. This is a rhadinovirus in a rodent group.

The phylogenetic tree in figure 10 is composed of all the positive samples found in this research, alpha and beta Herpesvirus genomes, and the sequences of herpesviruses detected by Hong Li[18] . This will provide some insight in the position within the phylogeny of Herpesviruses for the unknown marten samples.

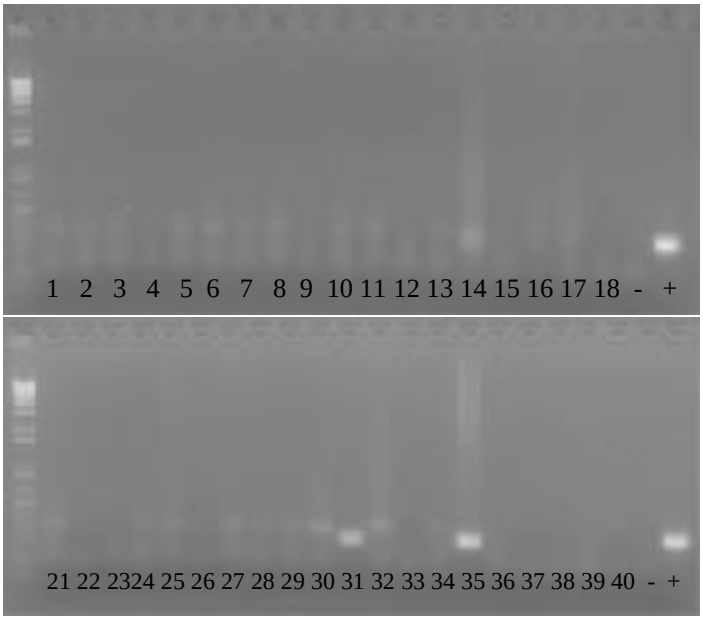

Figure 9: PCR agarose gel of Coyote samples. Sample 6(cloned), 14, 30(cloned)(131/2012 10/12/12 lnn., 142/2012 PD 11 dec lnn., 128/2012 lnn. 29/11/12) were sequenced without outcome. Bands that look similar are thought to be negative to.
31 (Wolf PD 12-46 1-10-12 lnn.) detected as Canid Herpesvirus 1.
35 (BHS 49-2012 PD lnn.) detected as ruminant rhadinovirus for Bighorn sheep type 2.

Figure 10: phylogenetic tree. Composed of the positive samples found in this research, alpha and beta Herpesvirus genomes, and results from Hong Li.[18] Protein alignment with a preformed bootstrap with value of 1000 and the consensus cut-off is 50.

Discussion:

Herpesvirusses are common in all species and most mammalian species carry at least one sort of Herpesvirus, this can be alpha, beta or gamma-Herpesvirusses. The presence of these Herpesvirusses does not mean disease occurs. The samples used are collected from animals that are offered to the UofC. This varies from healthy trapped animals to animals with severe sickness from different causes. This makes it impossible to related the presence of Herpesvirusses to any disease in these animals. Concluding from this we can say that these animals are carriers of the Herpesviruses detected, but we can't conclude that the Herpesvirus positive animals where diseased due to Herpesvirus infection. Also Herpesvirus is found in the serum of elk samples. Earlier is stated that Herpesvirus in the serum could only be found in viremic animals, so it could be that these positive elk were viremic when their blood was taken.

The sample from the BigHorn Sheep 553 that contained Herpesvirus sequences most related to Ovine Herpesvirus 2 would suggest that OVH-2 is not restricted to domestic sheep. As mentioned in the introduction it is thought that OVH-2 has a wider host range than domestic sheep only. It was known that OHV-2 could cause non wildebeest-associated MCF in cervids or domestic animals and that these could play a role in the transmission. With this finding it should be considered that Bighorn sheep can play a role in the transmission of NWA-MCF.

The Canid Herpesvirus 1 shows that Herpesvirus can cross species. It is found in coyotes and wolf. These species belong to the same family (*Canidae*) and it is likely that Canid Herpesvirus 1 can replicate in all species belonging to this order. Also the rodent Herpesvirus found in one of the coyotes that is related to a gamma Herpesvirus suggests that coyotes can be carriers of rodent gamma Herpesvirusses as well.

Within the positive marten samples three groups of sequences are recognizable. These groups represent different sub-families of Herpesvirusses. Possibly, marten can carry alpha-, beta- and gamma Herpesvirusses. If the viruses that are most related to the alpha and beta Herpesvirusses will truly fall within these groups is uncertain since they only align on protein and not on nucleotide level. As determined using Blast, the group most associated with gamma Herpesvirus has similarities with Badger Herpesvirus an animal group in the same family the *Mustelidae,* closely related to the marten. The detected gamma-Herpesvirus is identical in four different martens. It has similarities with the Mustelid Herpesvirus but it is likely that this is a gamma-Herpesvirus adapted to the marten. This Herpesvirus was previously not been described and will be tentatively called Marten gamma-Herpesvirus.

The conditions for the degenerate primers used to attach to the DNA has to be ideal and with little off set can't attach or not properly. This can cause to find more negatives than expected, as stated before it is thought most mammalian species carry at least one sort of Herpesvirus. Within this report 29 of 262 animals where tested positive and a sequence could be derived. 16 from these sequences could subsequently identified using Blastn or Blastp.

The targeted area in the polymerase B gene is a short fragment of 190-200 base pairs and is a essential protein which is highly conserved in the DNA polymerase gene of Herpesvirus. If a a longer fragment could be obtained that was less conserved, we expect that more variation may be noticed between species. Ehlers describes a PCR that targets two conserved genes (polymerase B and glycoprotein B)[25, 26].These genes are separately sequenced. Polymerase B sequences were obtained with the protocol as described in this article, plus an additional sense

primer. For glycoprotein B two sets of primers are used for the specimens amplification. When both genes are sequenced virus specific nested primers, with the sense primers located in the glycoprotein B gene and the antisense primers located in the polymerase B gene, are chosen specific for every virus. After compiling the overlapping sequences a 3.4kbp sequence is obtained. This fragment is 17 times longer and contains an area between highly conserved genes so this would be an option to get a better view of the variation of Herpesvirusses between species. This could be a direction for future research in further characterizing the viruses as described.

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Addendum:

Troubleshooting:

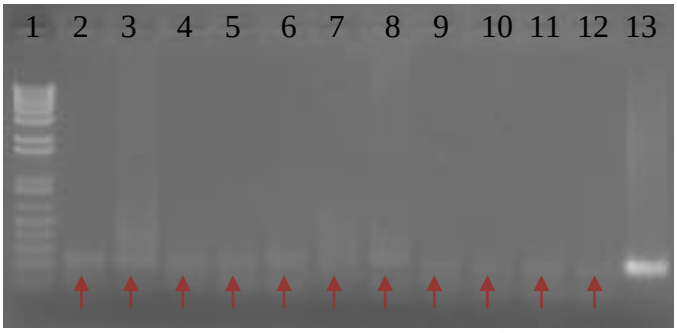


Figure 5: This agarose gel shows contamination of all samples. Negative control sample (number 12) is slightly positive. Number 2 to 11 are marten samples. Arrows point out the weak positive band.

Contamination of all samples (negative samples are also slightly positive).

Steps for cleaning:

PCR room cleaning: New PCR H_2O. Discard the used bottle.

New barrier-tips remove opened box from PCR room.

New Primer Box:
- New diluted primers
- New Taq
- New Buffer

1e Round PCR: Negative controls use PCR H_2O make in the PCR room not to be opened after first round of PCR. Positive control is added in the DNA extraction room. (Other veils are not to be opened).
For the PCR a different PCR machine is used then the previously used Bio Rad T100™ Thermal Cycler.

2e Round PCR: A different hood is used one that is close to and connected to the room with the PCR machine. This hood is cleaned with bleach (dilution 1:10) before the template from round 1 is added to the PCRmix for round 2.
The previously used barriertips are replaced by an unused box.

Amplicon detection: The first and second round of PCR are run through the agarose gel.

If this Agarose gel is negative it means PCR room is clean and the hood used to at the 2e round of PCR is free of amplicons.

For checking if the DNA extraction room is clean and the Bio Rad T100™ Thermal Cycler.
PCR with 3 samples. 1. Negative control added in the PCR room to the mastermix not to be opened in the DNA extraction room.

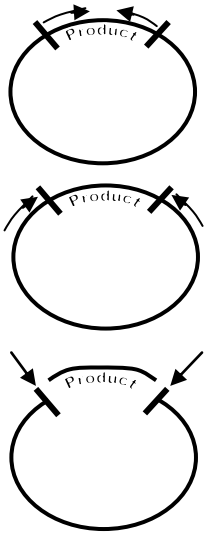
2. Negative control added in the DNA extraction room.
3. Positive control added in the DNA extraction room.

1e round of PCR is run in the Bio Rad T100™ Thermal Cycler.
2e round template is added to the PCR mastermix in the hood that is connected to the room with the PCR machine.

Both round 1 and 2 is added to the agarose gel. When the negative controls are negative it means. The DNA extraction room is clean and the Rad T100™ Thermal Cycler can be used again.

Troubleshooting 2:

After cloning concentration are significant higher than the 100-200 ng/µL. This could mean there is no insertion of the PCR product in the plasmid.
To see if there is no insertion of the PCR product before sending it for sequencing a control step is done.
Controle of PCR product in plasmid can be done with different methods.



1. The product from cloning is diluted 1:100 and 1:1000. 1 µL from the dilution is added to the master mix of the second PCR round. The second PCR round is run. The product is run true a 1,5% agarose gel. If the PCR product is present a band will appear at 200-300bp.
2. The product from cloning is rerun in a PCR with the primers SP6 and T7. These primers sequence the end of the plasmid. The product is run true a 1,5% agarose gel. If the PCR product is present a band will appear at 200-300bp.
3. The product from cloning 1µL , buffer, H_2O, EcoR1 are added together. This is put in a 37°C water bath for 1 hour. The EcoR1 enzym cuts at EcoR1 site located at 2 points. The product is run true a 1,5% agarose gel. If the PCR product is present a band will appear at 200-300bp and a band of the plasmid will be visible.

Table results and sequences:

Species	Samples	Number in PCR run	Gel Result	Sequences findings
Elk	Elk E4	1	Negative	-
Elk	Elk E6	2	Negative	-
Elk	Elk E7	3	Negative	-
Elk	Elk E8	4	Negative	-
Elk	Elk E10	5	Negative	-
Elk	Elk E11	6	Negative	-
Elk	Elk E12	7	Negative	-
Elk	Elk E16	8	Negative	-
Elk	Elk E21	9	Negative	-
Elk	Elk E24	10	Negative	-
Elk	Elk E25	11	Positive	Ruminant rhadinovirus of elk type 2 (AY237365.1)
TGCCTTAAATCGCAGAAACTGTCACACTCCAGGGTAGAACAATGCTGGAATTAACTAAAAATTTTGTTGAGAATCTTACGCTGGCAGACGTGTCTAAGATATGCCAGCATAAACTAGAGACACTCGATGAAGCGAGCAATGCCAGATTCAAAGTGATCTACGGCGACACGGACTCTG				
Elk	Elk E26	12	Negative	-
Elk	Elk E29	13	Positive	Ruminant rhadinovirus of elk type 2 (AY237365.1)
GCCTTAAATCGCAGAAACTGTCACACTCCAGGGTAGAACAATGCTGGAATTAACTAAAAATTTTGTTGAGAATCTTACGCTGGCAGACGTGTCTAAGATATGCCAGCATAAACTAGAGACACTCGATGAAGCGAGCAATGCCAGATTCAAAGTGATCTACGGCGACACGG				
Elk	Elk E32	14	Negative	-
Elk	Elk E34	15	Negative	-
Elk	Elk E36	16	Negative	-
Elk	Elk E37	17	Negative	-
Elk	Elk E38	18	Negative	-
Elk	Elk E39	19	Negative	-
Elk	Elk E41	20	Negative	-
Elk	Elk E45	21	Negative	-
Elk	Elk E54	22	Negative	-
Elk	Elk E56	23	Negative	-
Elk	Elk E57	24	Negative	-
Elk	Elk E58	25	Negative	-
Elk	Elk E59	26	Negative	-
Elk	Elk E60	27	Negative	-
Elk	Elk E63	28	Negative	-
Elk	Elk E66	29	Negative	-
Elk	Elk E67	30	Negative	-
Elk	Elk E68	31	Negative	-
Elk	Elk E69	32	Positive	Ruminant rhadinovirus of elk type 2 (AY237365.1)
TGCCTTAAATCGCAGAACTGTCACACTCCAGGGTAGAACAATGCTGGAAGTAACTAAAAATTTTGTTGAGAAT				

CTTACGCTGGCAGACATGTCTAAGATATGCCAGCATAAACTAGAGACACTCGATGAAGCGAGCAATGCCAGAT TCAAAGTGATCTACGGCGACACGGA				
Elk	Elk E74	33	Negative	-
Elk	Elk E75	34	Negative	-
Elk	Elk E78	35	Negative	-
Elk	Elk E80	36	Negative	-
Elk	Elk E81	37	Negative	-
Elk	Elk E111	38	Negative	-
Elk	Elk E112	39	Negative	-
Elk	Elk E116	40	Positive	Ruminant rhadinovirus of elk type 2 (AY237365.1)
GCTACNTGCCTTAAATCGCAGAACTGTCACACTCCAGGGTAGAACAATGCTGGAATTAACTAAAAATTTTGTTGA GAATCTTACGCTGGCAGACGTGTCTAAGATATGCCAGCATAAACTAGAGACACTCGATGAAGCGAGCAATGC CAGATTCAAAGTGATCTACGGCGACACGGACTCTG				
Bison	Bison M10-13LN	1	Positive	American bison gammagerpesvirus (AY057986.1)
CGGCGTGCCCTTTTGCGACTGCAACGGGCCTCTGGCAAATGCGCTCCAAGTCTGCGAGCTGTAAGCTCTCCA CGTAGTGCTTAGTTCTTTCCAGCATGGTCCGCCCCTGTAGAGTGACCGTTTCTGCAATTTTTAAGCAGGGAAGCA AACCGGACGCA				
Bison	Bison M10-12LN	2	Negative	-
Bison	Bison M10-11LN	3	Negative	-
Bison	Bison M10-10LN	4	Negative	-
Bison	Bison M10-09LN	5	Negative	-
Bison	Bison M09-10LN	6	Negative	-
Bison	Bison M09-09LN	7	Negative	-
Bison	Bison M09-08LN	8	Positive	American bison gammagerpesvirus (AY057986.1)
TTGCGACTGCAACGGGCCTCTGGCAAATGCGCTCCAAGTCTGCGAGCTGTAAGCTCTCCACGTAGTGCTTAGTTC TTTCCAGCATGGTCCGCCCCTGTAGAGTGACCGTTTCTGCAATTTTTAAGCAGGGAAGCAAACCGGACGCA				
Bison	Bison M09-07LN	9	Negative	-
Bison	Bison M09-04LN	10	Negative	-
Bison	Bison M09-03LN	11	Negative	-
Big Horn Sheep	339BHS	1	Negative	
Big Horn Sheep	343BHS	2	Positive	No outcome
Big Horn Sheep	344BHS	3	Negative	
Big Horn Sheep	345BHS	4	Negative	
Big Horn Sheep	346BHS	5	Positive	Ruminant rhadinovirus of bighorn sheep (AY237360.1)
GCCTTTGGCAGATTTTTTCTATATCCATGGGCTGCAAGCCTTCAACGTAGTGCTTAGTCTTTTCTAGCATAGTCCG TCCTTGCAGAGTAACAGTCTCTGCAATTTTTAAGCATGGAAGCAAGCCAGACGCAACCCCCGTAAACCC				
Big Horn Sheep	349BHS	6	Negative	
Big Horn Sheep	534BHS	7	Negative	
Big Horn Sheep	541BHS	8	Negative	
Big Horn Sheep	542BHS	9	Negative	
Big Horn Sheep	546BHS	10	Negative	
Big Horn Sheep	547BHS	11	Positive	Ruminant rhadinovirus of bighorn sheep (AY237360.1)
GCCTTTGGCAGATTTTTTCTATATCCATGGGCTGCAAGCCTTCAACGTAGTGCTTAGTCTTTTCTAGCATAGTCCG TCCTTGCAGAGTAACAGTCTCTGCAATTTTTAAGCATGGAAGCAAGCCAGACGCAACCCCCGTAGACCCGTACA CCGAGTTACAAAGATCCTCTC				

Big Horn Sheep	548BHS	12	Negative	
Big Horn Sheep	549BHS	13	Positive	Ruminant rhadinovirus of bighorn sheep (AY237360.1)
TGTAACTCGGTGTATGGGTTTACGGGGGTTGCGTCTGGCTTGCTTCCATGCTTAAAAATTGCAGAGACTGTTACTCTGCAAGGACGGACTATGCTAGAAAAGACTAAGCACTACGTTGAAGGCTTGCAGCCCATGGATATAGAAAAAATCTGCCAAAGGCCTATACCAATTTCAGAAGAACACGCTAGTTTGCGTGTAATCTATGGCG				
Big Horn Sheep	550BHS	14	Negative	
Big Horn Sheep	552BHS	15	Negative	
Big Horn Sheep	553BHS	16	Positive	Ovine Herpesvirus 2(HM216472.1)
TTAGCATCGGGGTTTGACATATTTGTTGTAGGCGCTGAACGTCCAAACTTTCCACAAATTGTTTTGTTTTCTCCAACATGGTTCGGCCCTGGAGAGTCACGGTCTCAGCTATCATGAGGCAAGGCAGCAGGCCGGAGGCTACCCCCGTAAACCCA				
Big Horn Sheep	554BHS	17	Negative	
Big Horn Sheep	555BHS	18	Negative	
Big Horn Sheep	556BHS	19	Negative	
Big Horn Sheep	558BHS	20	Negative	
Big Horn Sheep	559BHS	21	Negative	
Big Horn Sheep	957BHS	22	Negative	
Marten	65 spleen box: jan 10/12	1	Negative	
Marten	57 spleen box: jan 10/12	2	Negative	
Marten	61 spleen box: jan 10/12	3	Positive	No outcome
Marten	58 spleen box: jan 10/12	4	Negative	
Marten	02 spleen box: jan 16/12	5	Negative	
Marten	50 spleen box: jan 16/12	6	Negative	
Marten	55 spleen 2/2/12 box: jan 16/12	7	Negative	
Marten	13 spleen 2/2/12 box: jan 16/12	8	Negative	
Marten	60 spleen 2/2/12 box: jan16/12	9	Negative	
Marten	54 spleen 2/7/12 box: jan 16/12	10	Negative	
Marten	66 spleen 2/7/12 box: jan 16/12	11	Positive	Mus musculus rhadinovirus 1 (AY854167.1)
TCTGGAATTATGCCGTGTATCCAAATAGCTGCATCTATAACACATATAGGGCGCGAGATGTTAGCAACAACGTCAAGATATATATGTGATAGTTTTTCAAATGCGGATTTTATTAGAAAGTTTTTTAACAACAATGATGTTGTAAACGTGAACGATTTAATTGTAAATGTGATCTACGGCGACACGGACTCTGTG				
Marten	05 spleen 2/7/12 box: feb 7/12	12	Negative	
Marten	59 spleen 2/7/12 box: feb 7/12	13	Positive	Several Herpesvirusses top 3: HM579931.1, GU979535.1, AF376034.1
TGCTTGAAATAGCAGAAACTATAACATTTGAGGGGCGGCGCATGTTGGAGAAGTCTAAAAATTTTATAGAGAACATCACCCCCGTGGAACTCGAGAGAATTATACACAGACCAATAAGTTGTGCATATGATGCCAGCTTTAGAGTTATCTACGGCGACACGGACTCTGTGANT				
Marten	45 spleen 2/10/12 box: feb 7/12	14	Negative	
Marten	69 spleen 2/10/12 box: feb7/12	15	Negative	
Marten	48 spleen 2/10/12 box: feb 7/12	16	Negative	
Marten	46 spleen 2/10/12 box: feb7/12	17	Negative	
Marten	63 spleen 2/10/12 box: feb	18	Negative	

	7/12			
Marten	52 spleen 2/21/12 box: feb 10	19	Negative	
Marten	10 spleen 2/21/12 box: feb 10	20	Negative	
Marten	09 spleen 2/21/12 box: feb 10	21	Negative	
Marten	16 spleen 2/21/12 box: feb 10	22	Negative	
Marten	44 spleen 2/16/12 box: feb 10	23	Positive	Bottlenose dolphin herpesvirus DNA, Primer
TGTAACTCGGTGTATGGGTTTACGGGGGTAGTCCTGTTTGCACTGGGGCTTTCGACGACCGAAGTCCTCGTGTAATTCTGAGGACTGAACTTGGGCACATCGCTGGGTACTTCCTGAGGACTGCTGTTTCTTTCTACGTTTTCTTCATTCTTATGGCCAAGGAGCAACTGAAGAAGTGTTACTTTCTGGTGTGAATTTAGCTTAGAATTCCTTGAGGTATCTTGATCTTCTTTGATATCTACGGCGACACGGACTCTGTG				
Marten	11 spleen 2/21/12 box: feb 10	24	Positive	Mus musculus rhadinovirus 1 (AY854167.1)
CCGTGTATCCAATAGCTGCATCTATAACACATATAGGGCGCGAGATGTTAGCAACAACGTCAAGATATATATGTGATAGTTTTTCAAATGCGGATTTTATTAGAAAGTTTTTTAACAACAATGATGTTGTAAACGTGAACGATTTAATTGTAAATGTGATCTACGGCGACACGGACTCTGTG				
Marten	12 spleen 2/22/12 box: feb 21/12	25	Negative	
Marten	8 spleen 2/22/12 box: feb 21/12	26	Negative	
Marten	18 spleen 2/21/12 box: feb 21/12	27	Negative	
Marten	49 spleen 2/21/12 box: feb21/12	28	Negative	
Marten	17 spleen box: feb 21/12	29	Negative	
Marten	15 spleen box feb 21/12	30	Negative	
Marten	21 spleen 2/22/12 box: feb 27/12	31	Negative	
Marten	47 spleen 2/22/12 box feb: 27/12	32	Positive	Several Herpesvirusses top 3: HM579931.1, GU979535.1, AF376034.1
CACAGAGTCCGTGTCGCCGTAGATAACTCTAAAACTGGCATCATATGCACAACTTATTGGTCTGTGTATAATTCTCTCGAGTTCCACGGGGGTGATGTTTTCTATAAAATTTTTAGACTTCTCCAACATGCGCCGCCCCTCAAATGTTATAGTTTCTGCTATTTTCAAGCATGGTAGAATACCAGAGGACACCCCCGTAAACCCATACACCGAGTTACA				
Marten	1 spleen 2/24/12 box: feb 27/12	33	Positive	EHV-1 (AP012321.1)
CTCCCTGTTTGCAATTGCCGCAACTGTAACATCAATTGGGAGAGATATGTTATTGGCAACGCGAGATTATGTACATACCCGCTGGGGCACCAGGGAGCTTGTTGAGCGCGACTTCCCGATCGCTGCCACCGTATCCCTGAACAAGCCTTATGCCGTAACTGTTATCTACGGCGACACGGACTCTGTGA				
Marten	14 spleen 2/24/12 box: feb 27/12	34	Negative	-
Marten	7 spleen 2/24/12 box: feb 27/12	35	Negative	-
Marten	51 spleen 3/14/12 box: march 14	36	Negative	-
Marten	25 spleen march 26/12 box: march 14	37	Positive	Not cloned
Marten	56 spleen 2/14/12 box:march 14	38	Negative	-
Marten	67 spleen 3/14/12	39	Negative	-
Marten	4 spleen 3/14/12 box: march	40	Negative	-
Marten	35 spleen march 26/12 box: march 14	41	Positive	EHV-4 (AF030027.1)
ACTCCCTGTTTGCAATTGCCGCAACTGTAACATCAATTGGGAGAGATATGTTATTGGCAACGCGAGATTATGTACATACCCGCTGGGGCACCAGGGAGCTTGTTGAGCGCGACTTCCCGATCGCTGCCACCGTATCCCTGAACAAGCCTTATGCCGTAACTGTTATCTACGGCGACACGGACTCTGT				

Marten	53 spleen mar 29/12 box: march 14	42	Negative	-
Marten	41 spleen mar 28/12 box: march 28	43	Negative	-
Marten	3 spleen march 28/12 box march 28	44	Negative	-
Marten	22 spleen march 28/12 box: march 28	45	Negative	-
Marten	38 spleen march 28/12 box: march 28	46	Positive	EHV-1 (AP012321.1)
CTCCCTGTTTGCAATTGCCGCAACTGTAACATCAATTGGGAGAGATATGTTATTGGCAACGCGAGATTATGTACA TACCCGCTGGGGCACCAGGGAGCTTGTTGAGCGCGACTTCCCGATAGCTGCCACCGTATCCCTGAACAAGCC TTATGCCGTAACTGTTATCTACGGCGACACGGACTCTGTGA				
Marten	62 spleen mar 28/12 box: march 28	47	Negative	-
Marten	70 spleen march 28/12 box: march 28	48	Negative	-
Marten	30 spleen april 1/12 box: april 1	49	Positive	Mus musculus rhadinovirus 1 (AY854167.1)
TCTGGAATTATGCCGTGTATCCAAATAGCTGCATCTATAACACATATAGGGCGCGAGATGTTAGCAACAACGTCA AGATATATATGTGATAGTTTTTCAAATGCGGATTTTATTAGAAAGTTTTTTAACAACAATGATGTTGTAAACGTGA ACGATTTAATTGTAAATGTGATCTACGGCGACACGGACTCTGTG				
Marten	24 spleen april 2/12 box: april 1	50	Negative	-
Marten	26 spleen april 1/12 box: april 1	51	Positive	No outcome
Marten	27 spleen april 1/12 box:april 1	52	Positive	Mus musculus rhadinovirus 1 (AY854167.1)
TCTGGAATTATGCCGTGTATCCAAATAACTGCATCTATAACACATATAGGGCGCGAGATGTTAGCAACAACGTCA AGATATATATGTGGTAGTTTTTCAAATGCGGATTTTATTAGAAAGTTTTTTAACAACAATGATGTTGTAAACGTAA ACGATTTAATTGTAAATGTGATCTACGGCGATACGGACTCTGTG				
Marten	31 spleen april 2/12 box: april 2	53	Negative	-
Marten	37 spleen april 2/12 box: april 2	54	Negative	-
Marten	6 spleen april 2/12 box: april 2	55	Positive	No outcome
Marten	39 spleen april 2/12 box: april 2	56	Negative	-
Marten	33 spleen april 2/12 box: april 2 (box 2)	57	Negative	-
Marten	23 spleen box april 2 (box2)	58	Negative	-
Marten	32 spleen april 3/12 box: april 3	59	Negative	-
Marten	40 spleen apr 3/12 box: april 3	60	Negative	-
Marten	25 spleen april 3/12 box: april 3	61	Negative	-
Marten	36 spleen apr 3/13 box: april 3	62	Negative	-
Marten	42 spleen ap 3/12 box: april 3	63	Negative	-
Marten	34 spleen apr 3/12 box: april 3	64	Negative	-
Marten	29 spleen mar 26/12 box martens mar-april	65	Positive	After retesting Negative.
Marten	43 spleen box: martens mar-april	66	Negative	-
Bison	M08-01	1	Negative	
Bison	M08-03	2	Negative	

Bison	M08-04	3	Negative	
Bison	M08-05	4	Negative	
Bison	M08-07	5	Positive	No outcome
Bison	M08-08	6	Negative	
Bison	M08-09	7	Negative	
Bison	M09-03	8	Negative	
Bison	M09-05	9	Negative	
Bison	M09-07	10	Negative	
Bison	M09-09	11	Negative	
Bison	M09-10	12	Negative	
Bison	M09-11	13	Negative	
Bison	M09-13	14	Positive	Blainville's beaked whale gammaherpesvirus, Primer
TGTAACTCGGTGTACGGGTTTACGGGGGTTGGGGTGGATGTGGAAATCAGGAGTTTGACTTGGGGCATGTTGAA TGGAAATGCTGATGGCCTCAGATGAAGAGGTCAGGGAGGCACCCNGGTGTGTGN				
Bison	M09-14	15	Positive	American bison gammagerpesvirus (AY057986.1)
CACGCGCAAGCTAGGATCGGCGTGCCCTTTTGCGACTGCAACGGGCCTCTGGCAAATGCGCTCCAAGTCTGCG AGCTGTAAGCTCTCCACGTAGTGCTTAGTTCTTTCCAGCACGGTCCGCCCCTGTAGAGTGACCGTTTCTGCAAT TTTTAAGCAGGGAAGCGAACCGGACGCA				
Bison	M09-15	16	Negative	
Marten	FGH #1 feb2010 MLN	1	Negative	
Marten	FGH #2 feb2010 MLN	2	Negative	
Marten	FGH #3 Feb2010 MLN	3	Positive	EHV-1 (AP012321.1)
TACTCCCTGTTTGCAATTGCCGCAACTGTAACATCAATTGGGAGAGATATGTTATTGGCAACGCGAGATTATGTA CATACCCGCTGGGGCACCAGGGAGCTTGTTGAGCGCGACTTCCCGATCGCTGCCACCGTATCCCTGAACAAGCCT TATGCCGTAACTGTTATCTACGGCGACACGGACTCTGTG				
Marten	FGH #4 Feb2010 MLN	4	Positive	Bottlenose dolphin herpesvirus DNA, Primer
TGTAACTCGGTGTATGGGTTTACGGGGGTAGTCCTGTTTGCACTGGGGCTTTCGATGACCGAAGTCCTCGTGTAA TTCTGAGGACTGAACTTGGGCACATCGCTGGGTACTTCCTGAGGACTGCTGTTTCTTTCTACGTTTTCTTCATTCTT ATGGCCAAGGAGCAACTGAAGAAGTGTTACTTTCTGGTGTGAATTTAGCTTAGAATTCCTTGAGGTATCTTGATC TTCTTTGATATCTACGGCGACACGGACTCTGTG				
Marten	FGH#5 Feb2010 MLN	5	Negative	
Marten	FGH#6 Feb2010 MLN	6	Negative	
Marten	FGH#7 Feb2010 MLN	7	Negative	
Marten	FGH#8 Feb2010 MLN	8	Positive	Several Herpesvirusses top 3: HM579931.1, GU979535.1, AF376034.1
TGTAACTCGGTGTATGGGTTTACGGGGGTGTCCTCTGGTATTCTATCATGCTTGAAAATAGCAGAAACTATAACA TTTGAGGGGCGGCGCATGTTGGAGAAGTCTAAAAATTTTATAGAAAACATCACCCCCGTGGAACTCGAGAGAAT TATACACAGACCAATAAGTTGTGCATATGATGCCAGTTTTAGAGTTATCTACGGCGACACGGACTCTGTG				
Marten	FGH#9 Feb2010 MLN	9	Negative	
Marten	FGH#10 Feb2010 MLN	10	Negative	
Marten	Colville lake #1 Feb2010 MLN	11	Negative	
Marten	Colville lake #2 Feb2010 MLN	12	Negative	
Marten	Colville lake #3 Feb2010 MLN	13	Negative	
Marten	Colville lake #4 Feb2010 MLN	14	Positive	Several Herpesvirusses top 3: HM579931.1, GU979535.1, AF376034.1

CCTGCTTGAATAGCAGAAACTATAACATTTGAGGGGCGGCGCATGTTGGAGAAGTCTAAAAATTTTATAGAAA ACATCACCCCCGTGGAACTCGAGAGAATTATACACAGACCAATAAGTTGTGCATATGATGCCAGCTTTAGAGT TATCTACGGCGACACGGACTCTGTGA				
Marten	Colville lake #5 Feb2010 MLN	15	Negative	
Marten	Colville lake #6 Feb2010 MLN	16	Negative	
Marten	Colville lake #7 Feb2010 MLN	17	Negative	
Marten	Colville lake #8 Feb2010 MLN	18	Negative	
Marten	Colville lake #9 Feb2010 MLN	19	Negative	
Marten	2010-19 spleen	20	Negative	
Wolf	Wolf female 11-52 2010-05 07WL Axillary lnn.	21	Negative	
Coyote	106/2012 lnn	1	Negative	
Coyote	146/2012 14 dec PD lnn.	2	Negative	
Coyote	120/2012 lnn.	3	Negative	
Coyote	118/2012 lnn.	4	Negative	
Coyote	161/2012 lnn.	5	Negative	
Coyote	105/2012 lnn.	6	Negative	
Coyote	155/2012 PD 18dec lnn.	7	Positive	Canid herpesvirus 1 (EU531507.1)
TGTTTACACATAGGCTTGCGACTGTAACAACAATAGGAAGATCAATGTTAATAGCAACACAAAATTATGTGGAA AGTAGATGGGCCACACTACAATTATTGGAAAATGATTTTCCTATAACTTCAAGTATAGTTATTCCAGAAAAATCT TACAGTGTAAAAATT				
Coyote	91/2012 19-10-12 lnn.	8	Negative	
Coyote	143/2012 PD 11 dec lnn.	9	Negative	
Coyote	84/2012 lnn.	10	Negative	
Coyote	119/2012 lnn.	11	Negative	
Coyote	150/2012 PD 11 dec lnn.	12	Positive	Canid herpesvirus 1 (EU531507.1)
GTTAATGGTTTACTTCCATGTTTACACATAGCTGCGACTGTAACAACAATAGGAAGAACAATGTTAATAGCAAC ACAAAATTATGTGGAAAGTAGATGGGCCACACTACAATTATTGGAAAATGATTTTCCTATAACTTCAAGTATAG TTATTCCAGAAAAATCTCACAGTGTAAAAATT				
Coyote	145/2012 PD 14 dec lnn.	13	Negative	
Coyote	114/2012 nov 22 lnn.	14	Negative	
Coyote	110/2012 nov 22 lnn.	15	Negative	
Coyote	112/2012 nov 22 lnn.	16	Negative	
Coyote	115/2012 nov 22 lnn.	17	Positive	Myodes glareolus rhadinovirus 1 (AY854169.2)
GTTACCCTGCCTACCAATAGCTGAACCATAACTTTTCAGGGGCGAAGAATGTTGGACCTTTCCAAAAACTATATA GAGTCTTTAACACCTGAGAGAATTTCAGAATTAACAGGTGTCAGGACATCGCCAGAAGGCTCTTTAAGAGTAATCTA CGGCGACACGGACTCTGTGA				
Coyote	123/2012 lnn.	18	Negative	
Coyote	92/2012 19-10-12 lnn.	21	Negative	
Coyote	80/2012 lnn.	22	Negative	
Coyote	135/2012 PD 11 dec lnn.	23	Negative	
Coyote	139/2012 PD 11 dec lnn.	24	Negative	
Coyote	160/2012 PD 18 dec lnn.	25	Negative	
Coyote	153/2012 PD 18 dec lnn.	26	Negative	

Coyote	116/2012 nov 26 lnn.	27	Positive	Canid herpesvirus 1 (EU531507.1)
GTTAATGGTTTACTTCCATGTTTACACATAGCTGCGGCTGTAACAACAATAGGAAGATCAATGTTAATAGCAACACA AAATTATGTGGAAAGTAGATGGGCCACACTACAATTATTGGAAAATGATTTTCCTATAACTTCAAGTATAGTTATTCC AGAAAAATCTTACAGTGTAAAAATT				
Coyote	152/2012 PD 18 dec lnn.	28	Negative	
Coyote	156/2012 PD 18 Dec lnn.	29	Negative	
Coyote	107/2012 lnn.	30	Negative	
Coyote	90/2012 19-10-12 lnn.	31	Negative	
Coyote	151/2012 PD 18 dec lnn.	32	Positive	clonen
Coyote	83/2012 lnn.	33	Negative	
Coyote	158/2012 PD 18 dec lnn.	34	Negative	
Coyote	109/2012 lnn.	35	Negative	
Coyote	148/2012 17 dec 2012 lnn.	36	Negative	
Coyote	127/2012 29/11/12 lnn.	37	Positive	No outcome
Coyote	140/2012 PD 11 dec lnn.	38	Negative	
Coyote	#133 dec 10, 2012 lnn.	39	Negative	
Coyote	149/2012 dec 17,2012 lnn.	40	Negative	
Coyote	147/2012 PD 14 dec lnn.	1	Negative	
Coyote	121/2012 lnn.	2	Negative	
Coyote	117/2012 lnn.	3	Cut out	
Coyote	100/2012 lnn.	4	Negative	
Coyote	154/2012 18 dec 12 lnn.	5	Cut out	
Coyote	131/2012 10/12/12 lnn.	6	Cut out	Only 30 bp, primer
CACCCCCGTAAACCCATACACCGAGTTACA				
Coyote	134/2012 10/12/12 lnn.	7	Negative	
Coyote	137/2012 PD 11 dec lnn.	8	Cut out	
Coyote	125/2012 29/11/12 lnn.	9	Negative	
Coyote	84/2012 lnn.	10	Negative	
Coyote	159/2012 PD 18 dec lnn.	11	Cut out	
Coyote	141/2012 PD 11 dec lnn.	12	Negative	
Coyote	138/2012 PD 11 dec lnn.	13	Negative	
Coyote	142/2012 PD 11 dec lnn.	14	Positive	No outcome
Coyote	104/2012 lnn.	15	Negative	
Coyote	122/2012 lnn.	16	Negative	
Coyote	75/2012 lnn.	17	Negative	
Coyote	144/2012 PD 11 dec 2012 lnn.	18	Negative	
Coyote	129/2012 29/11/12 lnn.	21	Cut out	Only 30 bp, primer
CACCCCCGTGAACCCGTACACCGAGTTACA				
Coyote	79/2012 31 aug 12 lnn.	22	Negative	
Coyote	77/2012 31 aug 12 lnn.	23	Negative	
Coyote	111/2012 nov 22 lnn.	24	Negative	
Coyote	113/2012 lnn.	25	Cut out	
Coyote	124/2012 lnn.	26	Negative	
Coyote	136/2012 PD 11 dec lnn.	27	Cut out	
Coyote	151/2012 PD 17 dec lnn.	28	Negative	

Coyote	108/2012 lnn.	29	Cut out	
Coyote	128/2012 lnn. 29/11/12	30	Cut out	Only 30 bp, primer
CACCCCCGTAAACCCGTACACCGAGTTACA				
Wolf	PD 12-46 1-10-12 lnn.	31	Positive	Canid herpesvirus 1 (EU531507.1)
GTTTACNCATAGCTGCGACTGTAACAACAATAGGAAGATCAATGTTAATAGCAACACAAAATTATGTAGAAAGTAG ATGGGCCACACTACAATTATTGGAAAATGATTTTCCTATAACTTCAAGTATAGTTATTCCAGAAAAATCTTACAGCG TAAAAATT				
Black Bear	PD 12-34 sept 11, 2012 lnn.	32	Cut out	clonen
Wolf	PD 2012-24 july 24 lnn.	33	Negative	
Black Bear	64-2012 PD lnn.	34	Negative	
Bighorn sheep	49-2012 PD lnn.	35	Positive	Ruminant rhadinovirus of bighorn sheep (AY237360.1)
GGNTTGCTTCNTGCTTAAAATTGCAGAGACTGTTACTCTGCAAGGACGGACTATGCTAGAAAAGACTAAGCACTAC GTTGAAGGCTTGCAGCCCATGGATATAGAAAAAATCTGCCAAAGGCCTATACCAATTTCAGAAGAACACGCTAGTT TGCGTGTAATCTACGGCG				
Wolf	53/2012 PD nov 12/2012 lnn.	36	Negative	
Black Bear	PD 12-32 lnn.	37	Negative	
Red Fox	AB/VIC/3.4C/#15 lnn.	38	Negative	
Fox	10/2012 nov 22 lnn.	39	Negative	
Black Bear	PD 12-33 lnn.	40	Negative	
Fox	9/2012 22 nov lnn.	1	Cut out	Only 30 bp found similar to Herpesvirus, primer.
TGTAACTCGGTGTACGGGTTTACGGGGGTCGGTAGCGGTAAGTCGAGAATGGCACGCAGACGCAGCTCAAACTGG CTGATTGATGCGCCATTTTGTGTCCAATGCCCACTGTTATGCACTCGCGGAGCCAGTTCGTTAATCAACAGGCTATC ATCGACAATGAAACATTCCATGGCCATGACGCCCACGTAATTAAGCGCGTTCATGATCGCGCTGAGCATCTGCTCT GCTTGCTGCTGCAACTGTGGATTAGGCTGTGGCAATGCAACGCTGATACGTAAGATACCCTCTTCATGCAGGTTAT GTGTCAGAGGATAAAATACGCAGCGACCATCGCGAGCGCGAGCGCCAATCAGAGAAACCTCTCCAGAGAATTTGG TGTCTTGCTCACCAGGTCGACGCCTTATTCATCGCGACCTGCTGCTTGTCCAGGA				
Coyote	126-2012 spleen	2	Negative	
Grizzly bear	66-2012 5 dec 2012 spleen	3	Negative	
Racoon	19-10-12 spleen	4	Negative	
Coyote	78/2012 31 aug 2012 spleen	5	Negative	
Cougar	65-2012 PD 5 dec 2012 lnn.	6	Cut out	Only 30 bp found similar to Herpesvirus, primer
CACAGAGTCCGTGTCGCCATAGATGGAGATGGTTACTAAGGGCACCCCTGGGGAATCAGTGGGGGTGGGGACACA AAGTCAGGAGAGATGGGAATGATGAGTGCGGGGAGAGGGGCTTCCCGAAGGTGGGGCCTGGCCTGGCCTTGCAAC ACCAGACGGGATTGGACAGAAAGGCCCCTCCTCAGGGCCCTGCTCCTCTCACCGGTCACCACATGCCTCAGTGGGG TGAGTGAAATGCCATCAAGGCCCGGCGACAAAGTAGCAGGAAGCTTCGTGATGGAGAAGAATGGTAAATTAATTTC AGAAGTAGAGCTGGCCTGAAAGTAAAGAAGAGAGGTTTGGACTTAAGTTTTTCTTTGTTTCATTTTTATTATGGAAA ATTCCAAACTTAAAAAATAGACCCATGTAATGAACCCCCAAATGCCCCTTACCCAGCTTTCAACAATCACCGATATT GTGTCTACACTGCTTCACCTCTCCTTCCCATTCCCAGCCTCACCCCTACACCCCCGTAAACCCATACACCGAGTT				
Black Bear	PD 12-36 sept 11/2012 lnn.	7	Negative	
Coyote	39/2012 19-10-12 spleen	8	Negative	
Coyote	86/2012 19-10-12 spleen	9	Positive	Only 30 bp, primer
CACCCCCGTAAACCCATACACCGAGTTAC				
Coyote	88/2012 19-10-12 spleen	10	Negative	