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Virus Research 259 (2019) 46–53

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Identification of gammaherpesvirus infection in free-ranging black bears (*Ursus americanus*)

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ARTICLE INFO

Keywords:
UrHV-1
Black bears
Virus isolation
PCR
Degenerative PCR
DNA sequencing

ABSTRACT

Herpesvirus infection was investigated in black bears (*Ursus americanus*) with neurological signs and brain lesions of nonsuppurative encephalitis of unknown cause. Visible cytopathic effects (CPE) could only be observed on days 3–5 post-infection in HrT-18G cell line inoculated with bear tissue extracts. The observed CPE in HrT-18G cells included syncytia, intranuclear inclusions, and cell detachments seen in herpesvirus infection *in vitro*. Herpesvirus-like particles were observed in viral culture supernatant under the electron microscope, however, capsids ranging from 60 nm to 100 nm in size were often observed in viral cultures within the first two passages of propagation. Herpesvirus infection in the bear tissues and tissue cultures were detected by PCR using degenerate primers specific to the DNA polymerase gene (DPOL) and glycoprotein B gene (gB). DNA sequencing of the amplicon revealed that the detected herpesvirus has 94–95% identity to Ursid gammaherpesvirus 1 (UrHV-1) DNA sequences of DPOL. Phylogenetic analysis of DPOL sequences indicates that black bear herpesviruses and UrHV-1 are closely related and have small distances to members of *Rhadinovirus*. Interestingly, black bear herpesvirus infections were also found in bears without neurological signs. The DPOL DNA sequence of black bear herpesviruses detected in neurological bears were similar to the those detected in the non-neurological bears. However, the gB DNA sequence detected from the neurological bear is different from non-neurological bear and has only 64.5%–70% identity to each other. It is possible that at least two different types of gammaherpesviruses are present in the *U. americanus* population or several gammaherpesviruses exist in ursine species.

1. Introduction

Gammaherpesviruses are common infections in a wide variety of mammalian species. The subfamily of *Gammaherpesvirinae* includes four characterized genera: *Lymphocryptovirus*, *Macavirus*, *Pervavirus*, *Rhadinovirus* and unassigned genus. Lymphocryptoviruses are often associated with lymphoproliferative diseases, and have been identified in at least 9 different species, such as human, macacine, cercopithecine, leporid and ovine. Human herpesvirus 4 (HHV4) is one of the prominent lymphocryptoviruses associated with a number of human cancers (Grywalska et al., 2013; Petersson, 2015). HHV4, also known as Epstein-Barr virus (EBV), prevalent in 90 to 95% of the human population, is also clinically associated with various neurological diseases such as primary central nervous system lymphoma, multiple sclerosis, Alzheimer's disease, cerebellar ataxia, and encephalitis (Ali and Lawthom, 2013; Corssmit et al., 1997; Davies et al., 2016; Fallo et al., 2005). Human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), and Murine gammaherpesvirus-68 (MHV-68) are most studied rhadinoviruses. HHV8 or KHSV is mainly linked to the development of Kaposi's sarcoma and a rare B cell lymphoma, primary effusion lymphoma in human (Moore et al., 1996; Wong and Damania, 2005). However, both HHV-4 and HHV8 are

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https://doi.org/10.1016/j.virusres.2018.10.016
Received 19 June 2018; Received in revised form 12 October 2018; Accepted 19 October 2018
Available online 30 October 2018
0168-1702/

neurotropic and can infect neuronal cell lines, Sh-Sy5y and Ntera2 cells, as well as human primary neurons (Cho and Song, 2014). MHV-68, also called murid gammaherpesvirus 4, was originally isolated from a bank vole by passage through mouse brain (Terry et al., 2000). MHV-68 is known to persist in the brain after cerebral infection and can causes cerebral infection with inflammation in 50% of the infected animals (Hausler et al., 2005). Rhesus macaque rhadinovirus, another member of the *Rhadinovirus* genus, has been shown to cause encephalomyelitis and diseases similar to multiple sclerosis in human (Axthelm et al., 2011). Members of *Percavirus*, such as equine gammaherpesvirus 2 and mustelid gammaherpesvirus 1, are not linked to a specific disease, except equine gammaherpesvirus 5 (EHV-5), which is linked to equine multinodular pulmonary fibrosis (Williams et al., 2007). Members of the *Macavirus* can be either non-pathogenic, such as BHV-6, or pathogenic, such as malignant catarrhal fever virus (MCFV) and caprine herpesvirus 2 (CHV-2). BHV-6 is ubiquitous in healthy cattle populations (Koptopoulos et al., 1988), but MCFV and CHV-2 can cause fatal multisystemic disease in sheep and buffalo (Brenner et al., 2002; Li et al., 2005, 1999; Loken et al., 2009).

Herpesvirus infection in bears has rarely been documented. Recovery of viral sequences corresponding to the alphaherpesviruses, such as equine herpesvirus 1 (EHV1) and equine herpesvirus 9 (EHV9), have been published in case reports involving captive ursid mortalities (Dombrowski et al., 2016). An investigation of squamous cell carcinomas revealed the presence of a gammaherpesvirus in multiple captive sun bears (*Heloarctos malayanus*) (Lam et al., 2013). However, successful culture of a gammaherpesvirus of ursine origin has not been demonstrated. This report documents the detection and partial characterization of gammaherpesvirus from multiple black bears (*Ursus americanus*) with and without neurological diseases from Nevada, California and Oregon, USA.

2. Materials and methods

2.1. Bears

Bear 1(B1) and Bear 2 (B2) were two of four black bears showing neurological diseases that had been euthanized by the Nevada Department of Fish and Wildlife. B1, an approximately 2 year old male, was unable to rise, but could drag itself by his front legs for a short distance. B2, a 1 year old female, was not afraid of people and exhibited slight unsteadiness or tremor. Bears NV17a-17c were bears that either died from vehicular trauma or were urbanized animals euthanized as a result of their habituation to humans. Bears Z1 and Z3 were urbanized bears around 1–1.5 years old euthanized by California Department of Fish & wildlife. Bear O1 was juvenile, male American black bear and was euthanized by Oregon Department of Fish & Wildlife due to unsuitability for rehabilitation and release. Following necropsy examination of these animals, tissues were submitted to the Oregon Veterinary Diagnostic Laboratory for diagnostic testing.

2.2. Cell culture and virus isolation

The human rectal tumor (HrT-18G) cells, Marbin Darby Canine Kidney (MDCK), Vero Maru (Vero), Crandell Feline Kidney (CRFK cells), Porcine Kidney 15 (PK-15) and Rabbit Kidney-13B (RK-13B) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Seradigm), penicillin (100U/ml) and streptomycin (100 µg/ml) (Sigma-Aldrich, Inc.) at 37 °C with 5% CO2 in a humidified incubator. The viral isolate was propagated in HrT-18G cells in F-15 media supplemented with 2.5 µg trypsin and 2.5 µg/ml pancreatin (1XTPC) (GIBCO).

The pooled tissue preparation from each bear was inoculated to HrT-18G cells, absorbed for 1 h on a rocker, washed three times with serum free F-15 media containing 1XTPC following absorption, and cultured in serum free F-15 media containing penicillin (100U/ml) and streptomycin (100 µg/ml) (Sigma-Aldrich, Inc.) at 37 °C with 5% CO2 in a humidified incubator.

2.3. Electron microscopy negative staining

Virus particles purified by ultracentrifugation of infected cell supernatants at 29 K rpm, were adsorbed to formvar-coated carbon-stabilized copper grids by floating grids on a drop of 10 µl sample mixed with 10 µl of 2% phosphotungstic acid (PTA) (pH 6.9) in water on the parafilm. The grids were then blotted dry with Whatman filter paper by side-blotting and allowed to air dry before viewing under the electron microscope. Images were obtained with TEI Titan 80–200 transmission electron microscope.

2.4. Total tissue DNA isolation

Total tissue DNA was isolated from approximately 100 mg of each tissue using EZNA Tissue DNA extraction kit (Omega Bio-tek). For each tissue sample, about 0.5 µg of total DNA was used as templates in either degenerate PCR or PCR with UrHV-1 specific primers. Viral DNA from tissue culture was isolated from cell pellet of 5 ml total tissue lysates using EZNA Tissue DNA extraction kit (Omega Bio-tek).

2.5. Primers and PCR amplification

Nested degenerate (or panherpesvirus) PCR Primers, POLdegF1 (GAYTTYSMIAGYYTITAYCC) and POLdegR1 (TTICKIACSARITCIACICCYTT), POLdegF2 (ATIATIMWRGCICAYAAYYTITG) and POLdegR2 (AAIAIISWRTCIGTRTCICCRTA) targeting conserved regions of the catalytic subunit of the DNA polymerase of herpesviruses were used to detect herpesvirus as previously described (Pozo et al., 2016). The nested panherpesvirus PCR was optimized with purified viral DNA of human herpesvirus type 1 (HHV-1), leporid herpesvirus type 4 (LHV-4) and koi herpesvirus (KHV) (Supplementary Fig. 1). The primers UG341F (5′-ACCTACACAGGCACCCTCAC-3′) and UG341R (5′-AATGA AGGCCCTTGACCTCT-3′) specific to Ursid herpesvirus 1 were selected based on the DNA sequence deposited in Genbank (Accession No. JX220982.1). In addition, another sets of nested herpesvirus degenerate (or consensus) primers, DFA (5′-GAYT TYGCNAGYYTNTAYCC-3′), ILK (5′-TCCTGGACAAGCAGCARNYS GCNMTNAA-3′), and KG1 (5′-GTCTT GCTCACC AGNTCNACNCCYTT-3′); TGV (5′-TGTAACTCGGTGTAYGGNTTYACNGGNGT-3′) and IYG (5′-CACAGAGTCCGTRTCNCCRTADAT-3′) targeting the DNA polymerase gene, were also used to detect herpesvirus as previously described (VanDevanter et al., 1996). In addition, genus-specific glycoprotein B gene (gB) primers were used to detect gB DNA as reported previously (Ehlers et al., 2008).

PCR with the panherpesvirus oligomers POLdegF1 and POLdegR1 was performed in a 50-µl solution consisting of 5.0 µl 10 × PCR amplification buffer (GBiosciences), 5.0 µl 360 GC Enhancer (Applied Biosystems) or 5 µl of 50% DMSO (Invitrogen), 1.0 µl dNTPs at 10mMeach, 0.8 µM primers of POLdegF1and POLdegR1, 1.25 U Taq DNA polymerase (GBiosciences), and 0.2-0.5 µg total tissue DNA. The mixture was subjected to 94 °C for 2 min, and 40 cycles of 94 °C for 30 s, 40 °C for 45 s, and 72 °C for 45 s, and followed by a 5-min elongation reaction at 72 °C after the final cycle. The products of the first-round amplification with primers POLdegF1 and POLdegR1are then subject to a second-round of PCR with primers POLdegF2 and POLdegR2. The second-round of PCR or the nested PCR was run at the same conditions as the first round of PCR amplification. PCR with the herpesvirus consensus primers DFA, ILK, KG1, TGV, and IYG were performed as described previously (VanDevanter et al., 1996). PCR with genus-specific glycoprotein B gene (gB) primers were performed as reported previously (Ehlers et al., 2008).

PCR with UrHV-1 specific primers, UG341F and UG341R, was performed as follows: a 50-µl solution consisting of 5.0 µl 10× amplification buffer (GBiosciences), 1.0 µl dNTPs at 10 mM each, 0.4 µM

primers (UG314F and UG341R), 1.0 U Taq DNA polymerase (GBiosciences), and 0.25–0.5 µg total tissue DNA, was subjected to 94 °C for 2 min, and 30 cycles of 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 45 s, followed by a 5-min elongation reaction at 72 °C after the final cycle.

2.6. DNA sequencing

Sequences of PCR products were determined by direct sequencing of PCR products purified by a ChargeSwitch PCR Clean-Up kit (Invitrogen) or QIAquick Gel Extraction Kit (QIAGEN). Sequencing was performed by the Center for Gene Research and Biocomputing (CGRB) at Oregon State University and by the Research Technology Support Facility Genomics Core at Michigan State University. The CGRB used an ABI Prism®3730 Genetic Analyzer with a BigDye® Terminator v. 3.1 Cycle Sequencing Kit, employing ABI Prism®3730 Data Collection Software v. 3.0 and ABI Prism®DNA Sequencing Analysis Software v. 5.2. The nucleotide sequences were analyzed with the Geneious software.

3. Results

3.1. Virus isolation

To determine whether infectious viruses were present in the tissues collected from Nevada (NV) black bears B1 and B2, tissue preparations from each organ were inoculated to MDCK, Vero, CRFK, PK-15 and RK-13B cells. No visible cytopathic effect (CPE) was observed in any of those cells 2 weeks post-infection. However, inoculation of HrT-18G cell with the pooled tissue preparations from B1 brain, kidney, spleen and lymph node, yielded visible CPEs, such as syncytia, intranuclear inclusions and cell detachments, at 3–5 day post-infection (dpi) (Fig. 1C and D), although fusion (black arrows) (Fig. 1A and B) was also observed in uninfected controls; this was attributed to trypsin and pancreatin treatment and was different from syncytia (white arrow) seen in the infected cells (Fig. 1C and D). To determine if similar CPE could be produced from tissues collected from black bears without any clinical neurological disease, tissue preparations from Oregon (OR) bear O1 were also investigated by virus isolation. Similar CPEs were observed in HrT-18G cells following infection of pooled bear tissue preparations from O1 (Fig. 1E and F).

3.2. Electron microscopy

The virions of herpesviruses are enveloped particles and have icosahedral capsids at 100–120 nm in size. To determine if CPE observed in the infected HrT-18G was caused by herpesvirus infection, virions isolated from tissue culture supernatant were subjected to negative staining and examined by transmission electron microscopy. Herpesvirus like capsids were seen in the cells infected with tissue preparations from NV bear B1, which had CNS clinical signs (Fig. 2A). Herpesvirus like particles were also seen in cells infected from tissue preparation from OR bear O1, which had no CNS clinical signs (Fig. 2B). In addition, varied sizes of capsids and virions were observed in tissue cultures infected with the fecal samples of CA bear Z3 and OR bear 1 (Fig. 2C-D), where coronavirus-like particles (open arrow) and capsids over 50 nm (solid arrow) were also observed in the infected tissue cultures. Coronaviruses are commonly observed in the intestine tract of animals, such as cervids and cattle. It is possible that coronaviruses are common in ursine species.

3.3. PCR detection with panherpesvirus primers

There are conserved amino acid sequences within the DNA polymerase within the herpesvirus family, which can be used to design degenerative primers for detection of herpesviruses. To determine whether the observed viral particles are herpesviruses, tissues collected from the two NV black bears B1 and B2 were examined by nested PCR using panherpesvirus primers (Pozo et al., 2016) and herpesvirus consensus primers (VanDevanter et al., 1996). To determine if herpesviral genome is present in NV bear B1 and B2, total DNA from brain, kidney, liver and lymph nodes was analyzed by the nested panherpesvirus PCR. As shown in Fig. 3, amplicons within the predicted size were detected in all tissue DNA samples from both NV bears B1 and B2, when 360 GC Enhancer was included in the PCR reactions. DMSO is also an enhancer for GC rich samples and when DMSO was included in PCR reaction, only correct amplicons were detected in total DNA of all B1 tissues, but only one of B2 tissues. Therefore, 360 GC Enhancer was used in the rest of the panherpesvirus PCR tests. In addition, correct PCR products were also obtained when herpesvirus consensus primers were used (data not shown here). To determine if the herpesviral genome is present in black bears without clinical neurological signs, another four bears (NV17a, NV17b, NV17c and NV14a) from Nevada and one bear (Z3) from

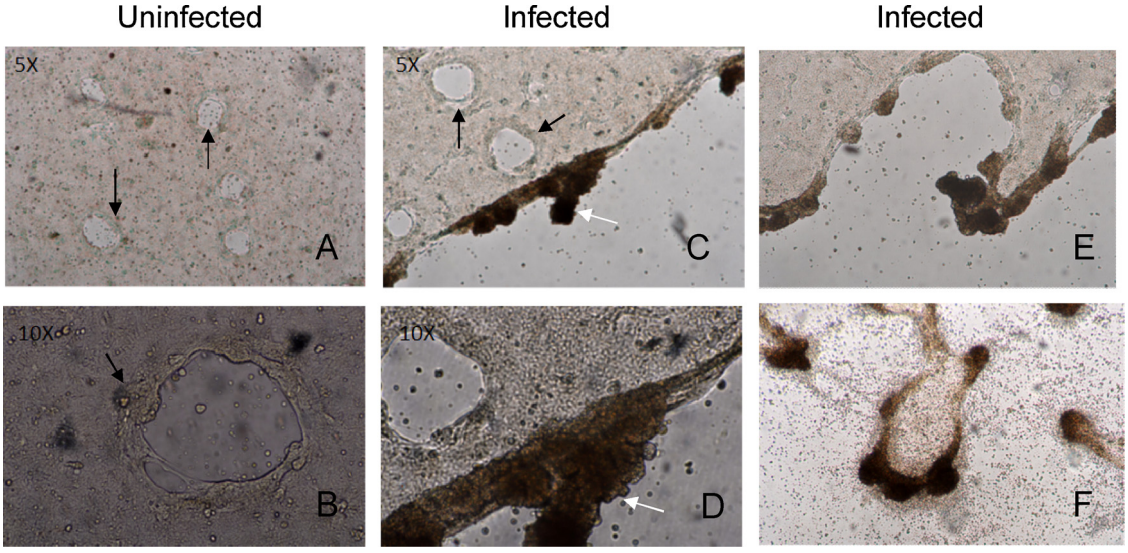

Fig. 1. Light microscope images of HrT18G cells with or without exposure to bear tissue extracts. A (5X) & B (10X): mock infected HrT-18G cells at day 4 post-infection. C (5X) &D (10X): HrT-18G cells infected with B1 bear tissue extracts at day 4 post-infection. E &F: HrT-18G cells exposed to O1 tissue extract at day 4 post-infection (E), and at day 8 post-infection (F).

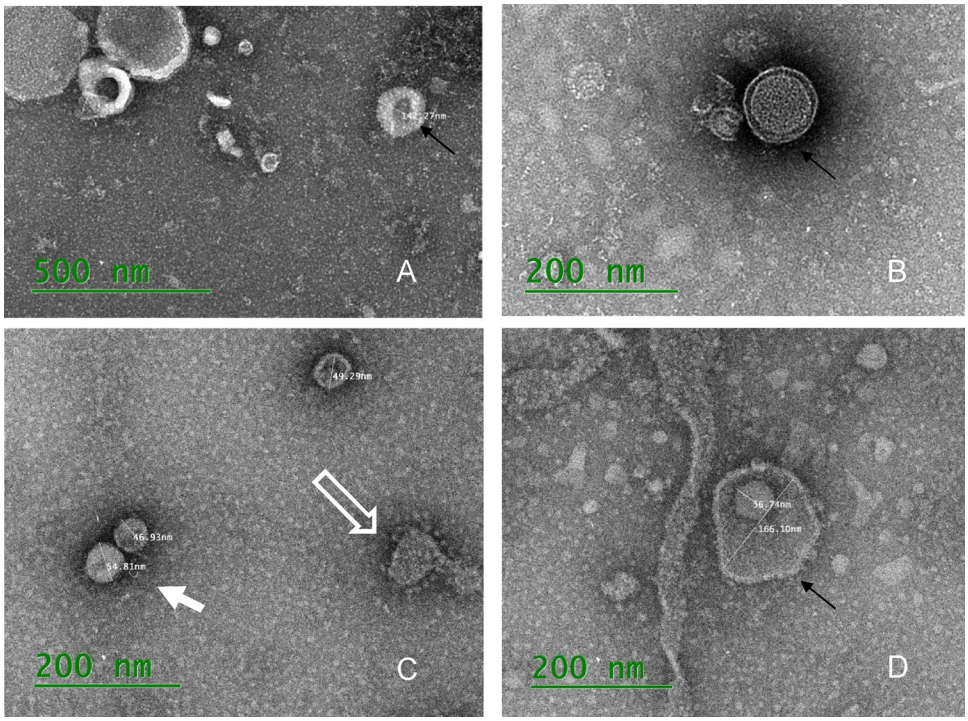

Fig. 2. Electron micrograph images of virions purified from infected HrT-18G cell culture supernatant. A: Purified virions from tissue isolation with B1 bear showing neurological signs; B: Negative staining of purified virions from tissue culture infected with pooled tissues from O1 bear (B), without neurological signs. C and D: Negative staining of purified virions from tissue culture infected with fecal samples of CA bear Z3 and OR bear 1, without neurological signs. The black arrow indicates the herpesvirus like virion. The open arrow indicates coronavirus like virion. The solid white arrow indicates capsids with sizes between 40-60nm: Scale bar = 0.2 µm or 0.5 µm.

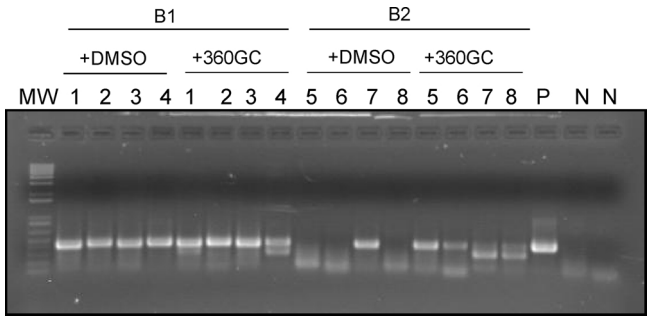

Fig. 3. Panherpesvirus PCR analysis of tissue total DNA isolated from B1 and B2 bears diagnosed with neurological diseases. The PCR reaction was performed similarly as described in Supplemental Fig. 1 with either DMSO or 360 GC Enhancer. The predicted product is > 400 bp in size. Template DNA was as follows: 1&5: brain, 2&6: kidney; 3&7: liver; 4&8: lymph node; P: HHV-1; MW: 1 kb-plus DNA ladder (Invitrogen).

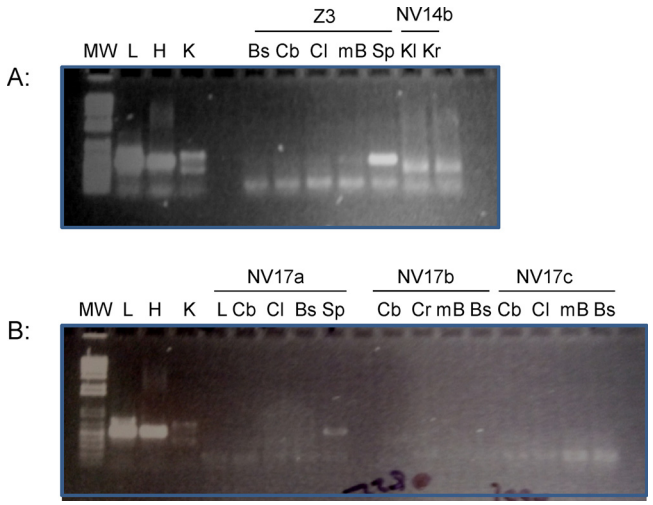

Fig. 4. Panherpesvirus PCR analysis of tissue total DNA isolated from bears without clinical signs. The reaction was performed as described in Supplemental Fig. 1 with 360 GC Enhancer included in the PCR. A: Z3 is bear from California, NV14b: bear collected in 2015 from Nevada. B: NV17a-c: bears collected in 2017 from Nevada. The abbreviations of tissues are as follows: Bs: Brain stem, Cb: cerebrum, Cl: cerebellum, mB: mid-brain, Sp: spleen, Kl: left kidney, Kr: right kidney, L: liver; Pos: L: LHV-4, H: HHV-1, K: KHV; MW: 1 kb-plus DNA ladder.

California were tested by nested panherpesvirus PCR primers, which were more sensitive than the herpesvirus consensus primers (unpublished data). As shown in Fig. 4, a product within the predicted size was amplified from total DNA of spleen from CA bear Z3 and NV bear17a. However, the positive amplicon was not detected in all the tested tissues and tested bears (Fig. 4B, NV17b and NV17c). To ensure the panherpesvirus PCR reactions worked properly, HHV-1 (H), LHV-4 (L) and KHV (K) genomic DNA were always included as controls when bear tissue DNA were analyzed. Their products were also sequenced to confirm the reactions worked correctly.

3.4. DNA sequence analysis

To confirm the panherpesvirus PCR amplicons are indeed herpesviral DNA, amplicons of lymph node total DNA from NV bear B1, spleen total DNA from CA bear (Z3) and NV bear NV17a were sequenced and analyzed by Geneious software. As shown in Fig. 5, the DNA sequence alignments of amplicons from B1, NV17a, and Z3 total DNA are almost identical except the reads near the end, and they have > 94% identity to Ursid gammaherpesvirus 1 (UrHV-1). It suggests the herpesviral DNA detected from NV bear and CA bear are very similar. DNA sequencing of the PCR product amplified by the herpesvirus consensus primer of VanDevanter et al. (VanDevanter et al., 1996) also confirmed the detection of herpesviruses sequences from bears B1 and B2. BLAST (Altschul et al., 1990) analysis of these sequences revealed nucleotide similarities ranging from 90% to 96% to corresponding DNA polymerase sequences of gammaherpesviruses from diverse species of seals (data not shown). To investigate phylogenetic relatedness, the panherpesvirus PCR amplicon DNA sequences from bears B1, Z3, and N17a were compared to DNA sequences from representative members of the *Gammaherpesvirinae* using Jukes Cantor, neighbor-joining analysis. As

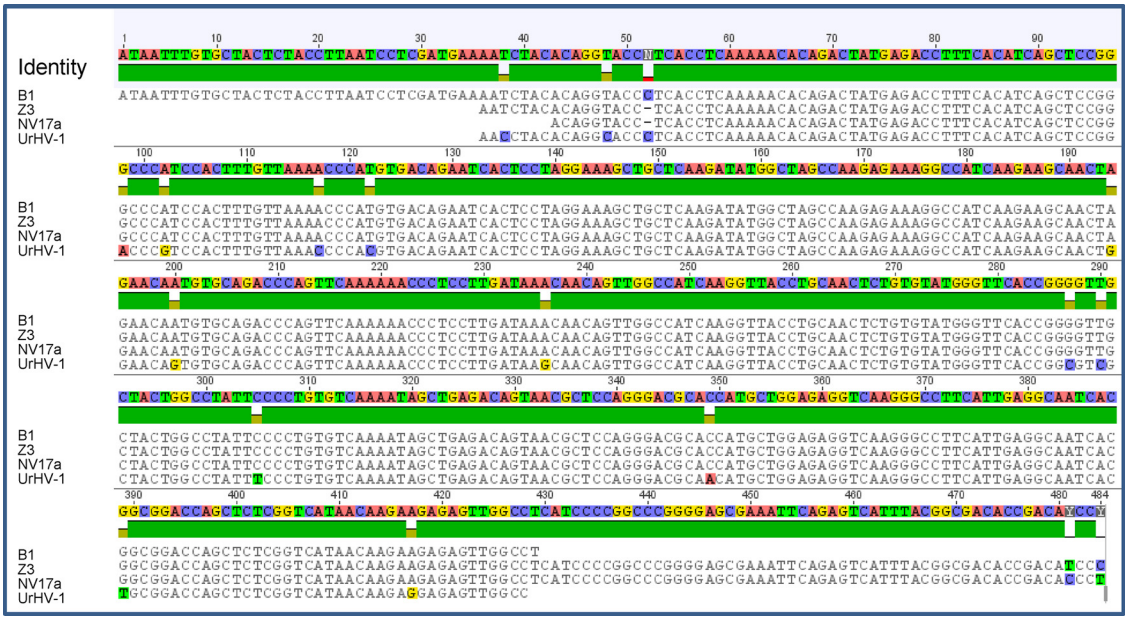

Fig. 5. DNA sequence alignments of amplicons from panherpesvirus PCR analysis with UrHV-1 DNA sequence available in the Genbank. PCR amplicons are produced by panherpesvirus PCR using primers POLdegF1 and POLdegR1 in the first round of reaction, POLdegF2 and POLdegR2 in second round of reaction. B1, Z3, NV17a: PCR products amplified from total DNA from lymph node of bear B1, spleen of bear Z3, spleen od bear NV17a, respectively. UrHV-1: DNA sequence detected in the sun bear (accession number JX220982). Green bar stands for identical sequences.

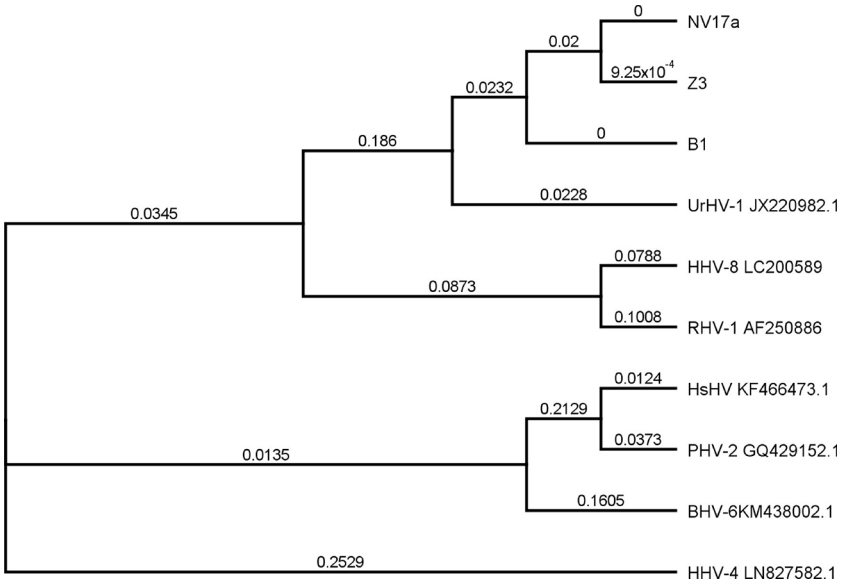

Fig. 6. Phylogenetic analysis of the amplicons from panherpesvirus PCR. Phylogenetic tree of the DNA sequences of the DNA polymerase subunit. The scale bar represents genetic distance (nucleotide substitutions per site), and branch lengths are given above the branches. The abbreviations in the virus names are as follows: HHV-4: human herpesvirus type; BHV-6: bovine gammaherpesvirus type 6; CPV-2: Caprine herpesvirus 2; HsHV: Harp seal herpesvirus isolate; PHV-2: Phocid herpesvirus 2; EHV-2: Equid gammaherpesvirus 2; HHV-8: human herpesvirus type 8; RHV-1: Gorilla rhadinovirus 1 isolate; UrHV-1: Ursid gammaherpesvirus 1; B1: amplicon from Nevada bear B1lymph node; NV17a: amplicon from Nevada bear NV17a spleen; Z3: amplicon from California bear Z3 spleen. The accession numbers used for DNA polymerase coding sequence analysis were given following each virus name.

shown in Fig. 6, the amplicons from bear tissues and UrHV-1 branch out together and have smaller distance to members of *Rhadinvirus*, such as HHV-8 and Rhadinovirus 1 (RHV-1). The sequences of amplicons by the herpesvirus consensus primers are more close to Phocid herpesvirus 2 (PHV-2) and harp seal herpesvirus isolate (HsHV), which have not been assigned genus (data not shown here). Both PHV-2 and HsHV branched together with member of *Macavirus*, such as BHV-6, which used to be member of *Rhadinavirus* in older classifications. These data suggest that UrHV-1 and the bear herpesviruses under investigation are phylogenetically close to each other and have DNA sequences similarity to members of *Rhadinavirus* within the subfamily of *Gammaherpesvirinae*.

The glycoprotein B gene (gB) encodes a structural protein on the surface of the envelope, which plays an important role in viral entry to specific host cells{Petrovic, 2017 #376}. It does not have high homology between different groups of herpesviruses. Genus-specific (GS) glycoprotein B gene (gB) primers have been used successfully in characterization of several novel gammaherpesviruses {Ehlers, 2008 #374; Lozano, 2015 #375}. Here, using the GS gB primers, gB like DNA sequence were amplified from DNA samples from both neurological bear B1 and non-neurological bears. Interestingly, gB DNA sequences from neurological bear B1 had only 64.5% identity to the gB DNA amplified from non-neurological bear O1. However, the gB DNA sequence amplified from B1 DNA has higher identity (92.4%) to gB of Harp seal herpesvirus isolate FMV04-1493874, while the gB DNA sequences from bear O1 had 68.3% identity to gB DNA sequence of Sea otter herpesvirus clone herpes_23 (KX024493.1). It is possible that different types of gammaherpesviruses exist in the black bear population. Phylogenetic analysis revealed that both gB DNA from neurological bear B1 and non-neurological bear O1 branch out together with Harp seal herpesvirus and Sea otter herpesvirus (Fig. 7), and are distant to gB DNA of Ovine herpesvirus type 2 (OHV-2) of *Macavirus*, Equine herpesvirus 2 (EHV-2) from *Percavirus*.

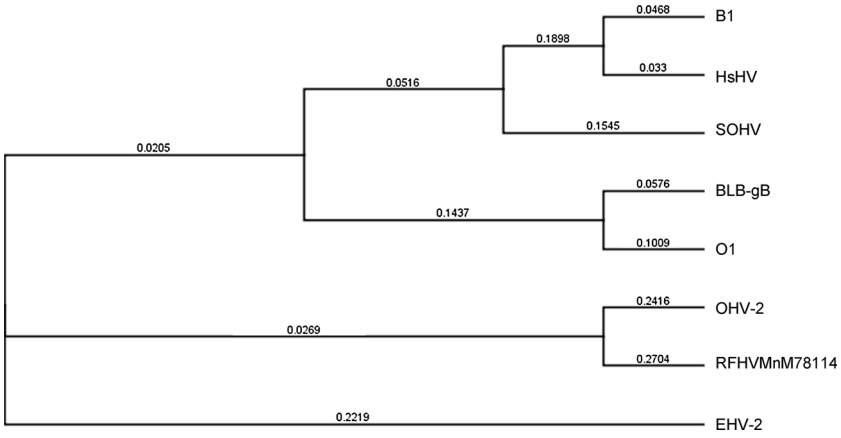

Fig. 7. Phylogenetic tree of the gB DNA sequences. The scale bar represents genetic distance (nucleotide substitutions per site), and branch lengths are given above the branches. The abbreviations in the virus names are as follows: HsHV: Harp seal herpesvirus isolate FMV04-1493874 (KP136799); SOHV: Sea otter herpesvirus (KX024493.1); OHV-2: Ovine herpesvirus 2 (AF385442.1); RFHVMnM78114: the Macaque Homolog of Kaposi's Sarcoma (KS)-Associated Herpesvirus (KF703446); EHV-2: Equid herpesvirus 2 (HQ247756.1); BLB-gB (MK089801) : gB DNA amplified from non-neurological black bear. B1: gB amplicon from Nevada bear B1 lymph node; O1: gB amplicon from Oregon bear spleen.

3.5. PCR detection with UrHV-1 specific primers

To confirm that the herpesvirus like particles observed under EM were indeed UrHV-1 like herpesvirus virions, both HrT-18G cell line passages 1 and 2 from bears B1 and O1 were tested by PCR using UrHV-1 specific primers, UG341F and UG341R. When the total DNA from tissue cultures passage 1 (P1) and passage 2 (P2) were tested directly by UrHV-1 specific PCR, no amplicons were observed (data not shown). However, when the total DNA of P1 and P2 were tested by hybrid nested PCR, with the first round of PCR reaction using panherpesvirus primers POLdegF1 and POLdegR1, and the second round of PCR reaction using UrHV-1 specific primers UG341 and UG341R, visible amplicons were observed in UrHV-1 specific PCR reactions (Fig. 8). Uninfected cell cultures yielded no amplicons. DNA sequence alignment shows amplicons of total DNA from P1 and P2 have > 90% identity to UrHV-1 (Fig. 9). To determine how common this UrHV-1 like virus is in black bear population, various tissues from 15 bears collected from Nevada, California, and Oregon were analyzed by this hybrid nested PCR with panherpesvirus primers in primary reaction and UrHV-1specific primers in the secondary reactions. As shown in Table 1, 7 out of 10 bears from Nevada, 3 out of 4 from California, and the sole bear from Oregon tested positive by this technique. This suggests that black bear herpesviruses have DPOL DNA similar to UrHV-1.

4. Discussion

An investigation of a cluster of cases of black bears in the Reno, Nevada area exhibiting neurologic clinical signs was undertaken in the winter and spring of 2014. A nonsuppurative encephalitis was identified in these animals via histopathology, but at this time, the causative agent remains unclear. However, during this investigation, herpesviruses were considered a potential cause, as ursine encephalitis cases have been linked to alphaherpesviruses (Dombrowski et al., 2016). Investigations using degenerate PCR primers, both at the OVDL and at Michigan State University VDL, resulted in amplification of DPOL DNA sequences with homology to gammaherpesviruses in a variety of tissues and in bears with and without evidence of neurologic disease. However, investigations using genus-specific glycoprotein B gene (gB) primers revealed that the gB DNA sequence obtained from neurological bear B1 is different from those amplified from bears without neurologic disease. It is possible that a specific type of gammaherpesvirus is associated with bear neurologic disease. However, if several gammaherpesviruses with similar DPOL DNA sequences exist in black bears, it will require further study to connect a specific herpesvirus with neurological diseases in bears.

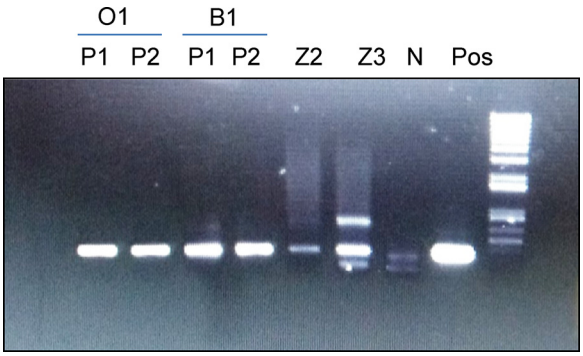

Fig. 8. Detection of UrHV-1 like DNA using UrHV-1 specific primers. Total DNA samples were analyzed by PCR using UrHV-1 specific primers UG341F and UG341R. The abbreviations of tissues are as follows: P: total DNA of lymph node from bear B1; N: total DNA of HrT-18G cells; Z2: total DNA of spleen from Z2; Z3: total DNA of spleen from Z3; P1: total DNA of viral culture passage 1, P2: total DNA of viral culture passage 2. O1: Oregon bear without neurological signs, B1: Nevada bear with neurological signs. MW, 1 kb-plus DNA ladder.

Tissue culture of an ursid herpesvirus has not been reported before. Our initial virus isolation attempts were in cell lines that are commonly used for animal virus isolations. Vero, CRFK, RK-15, and MDBK are all susceptible to alphaherpesvirus infections, such as bovine herpesvirus type 1, equine herpesvirus type 1, pseudorabies virus, feline herpesvirus type 1, and canine herpesvirus type 1. Infections of those alphaherpesviruses produce CPE, such as syncytia, intranuclear inclusions, and cell detachments. No CPE was observed in those cells following inoculation of the bear tissue preparation within the 2 week observation period. HrT-18G cells are normally used for animal coronavirus isolations in the veterinary diagnostic lab. The fecal sample from Z3 was suspected for coronavirus and was inoculated to HrT-18G cells. Interestingly, cell fusion and cell detachment were observed at 3–5 day post-infection, which was different from the CPE seen in coronavirus infections. CPEs from coronavirus infections are characterized with cell death and cell detachment. It is interesting that human rectal epithelial cells are susceptible to this bear herpesvirus infection. Under EM examination, herpesvirus-like capsids and particles were observed in infected HrT-18 cells. However, the observation of complete herpesvirus virions is rare in infected HrT-18G cells. In addition, capsids of variable size were also observed in tissue culture infected with bear fecal samples, and many of them have morphology similar to rotavirus and coronavirus (Fig. 2C and D). Since HrT-18G cells are susceptible to coronavirus infection, it is not surprising to see coronavirus in cultures infected with fecal samples. It is possible coronaviruses are common in black bears. Another interesting finding in this study is that the production of UrHV-1like virus in vitro is low. The viral genome can only be detected in direct cell lysates by nested UrHV-1 specific PCR. Again, it is possible that the black bear herpesvirus replicates poorly in vitro. This correlates with the few CPE plaques observed in infected Hrt-18G cells.

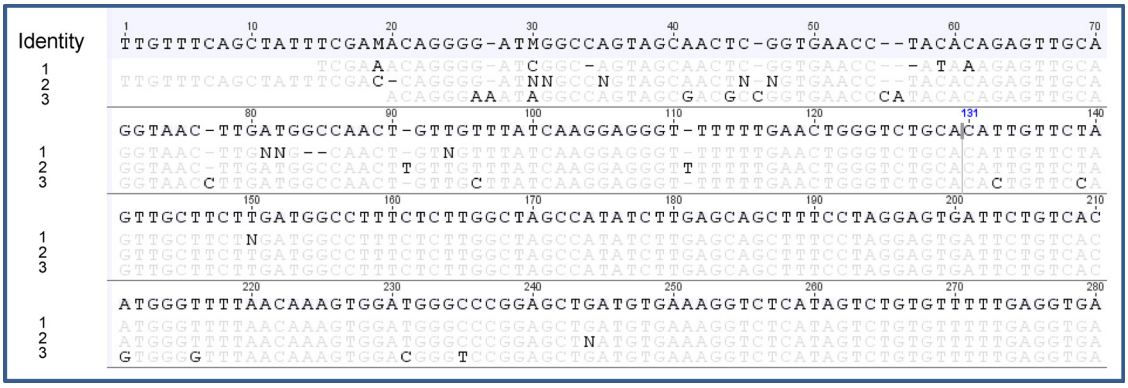

Fig. 9. DNA sequence alignments of UG341 PCR product with UrHV-1 sequence detected in the sun bear (accession number JX220982). 1: amplicon of P2 from Nevada bear B1 virus isolation shown in Fig. 8. 2: amplicon of P2 from Oregon bear O1 virus isolation shown in Fig. 8. 3: JX220982, UrHV-1 detected in sun bear.

Table 1
Results of Ursid gammaherpesvirus 1 isolation and PCR using degenerate PCR primers and UG341specific primers.

States	No. of bears		Virus Isolation	Degenerate PCR	UG341
	Total	CNS signs			
Nevada	12	2	pos	7/10	7/10
California	4		pos	3/4	3/4
Oregon	1		pos	1/1	1/1

Panherpesvirus PCR was developed by Pozo F. et al to detect bat herpesviruses (Pozo et al., 2016). There were two sets of nested degenerate PCR primers selected to detect the conserved herpesvirus genes, DNA polymerase and terminase in the bat herpesvirus study. The selection of those primers was based on HHV-5 reference genome (Accession No. NC_006273). Here, we find we can use panDPOL primer sets, which target the HHV-5 DNA polymerase, genome position between 79774 and 78991, 79750-79201, respectively. The anticipated PCR product with POLdeg1F/POLdeg1R and POLdeg2F/POLdeg2R are 713–992 bp and 482-758 bp, respectively. The conditions reported by Pozo F. et al can amplify the DNA polymerase sequence of alphaherpesviruses, such as HHV-1 and LHV-4, but not alloherpesviruses, such koi herpesvirus (KHV). However, inclusion of 360 enhancer in the PCR was able to amplify DNA polymerase coding sequence of KHV DNA. Another interesting observation in this study is that positive amplification occurred in all the tested tissues from bears B1 and B2 with neurological signs, however, fewer tissues tested positive in bears NV17a-c without clinical signs, and some instances, no tissue tested positive in bears without clinical signs. The lymphoplasmacytic nature of the encephalitis may account for the positive result in brain tissues from neurologic bears. The tissues which tested positive in non-neurological bears were often spleen and lymph nodes (Fig. 4). It is possible that this black bear herpesvirus persists in these lymphoid rich tissues, like many other gammaherpesviruses and this hypothesis was pursued using whole blood EDTA samples collected from three other bears that had no neurologic signs. Positive amplification with UrHV-1 specific primers was observed in total DNA of white blood cells (WBC) from two black bears from Nevada and one from Oregon. DNA sequence of the amplicon from the WBC total DNA is similar to sequences amplified from bear spleen samples from CA Z3 and NV17a and lymph node sample of B1 (Fig. 10). These findings are in agreement with the common feature of B cell latency of gammaherpesviruses.

DNA sequence alignment analysis shows that PCR amplicons of DPOL from the black bear tissues have the highest homology to UrHV-1 (Fig. 5). UrHV-1 is a novel herpesvirus detected by degenerate PCR primers in sun bears (*Helarctos malayanus*) with oral squamous cell carcinoma (Lam et al., 2013). Phylogenetic analysis shows that UrHV-1

Fig. 10. DNA sequence alignments of UG341 PCR product amplified from white blood cell (WBC) total DNA. 1: amplicon of lymph node total DNA from bear B1; 2: amplicon of spleen total DNA from bear NV17a. 3: amplicon of WBC total DNA from a new Oregon bear submitted to OVDL in 2018. 4: amplicon of WBC total DNA from a new Nevada bear submitted to OVDL in 2018. 5: amplicon of spleen total DNA from bear Z3.

is closest to members of *Rhadinovirus* (Fig. 6). The old classification of *Gammaherpesvirinae* consists of 2 genera, *Lymphocryptovirus* and *Rhadinovirus,* the later includes *the Macavirus* and *Percavirus*. The new classification separates the *Macavirus* and *Percavirus* from *Rhadinovirus,* which consists of viruses appearing to be paraphyletic. It is possible that this black bear herpesvirus is paraphyletic under certain conditions. It is unknown if UrHV-1 is oncogenic. We speculate that the virus may merely have been present in lymphocytes associated with the carcinomas. The phylogenetic analysis shown in Fig. 6, suggests that this black bear virus and UrHV-1 are close to members of *Rhadinovirus* and *Macavirus*. However, when genus-specific glycoprotein B (gB) gene primers (Ehlers et al., 2008) were used, gB DNA sequences from neurological bear had lower identity to gB DNA sequence amplified from non-neurological bear. Since they have less than 70% identity in gB gene, we propose that at least two different black bear herpesviruses exist in free-ranging black bears (*Ursus americanus*) and they may be close related to UrHV-1. More DNA sequences will be needed for further classification.

In summary, new black bear herpesviruses that are phylogenetically close to UrHV-1 and Harp seal herpesvirus isolate FMV04-1493874 have been detected in free-ranging black bears (*Ursus americanus*) with and without neurological disease. These black bear herpesviruses may have limited replication in HrT-18G cells. Further study will be needed to determine if a specific black bear herpesvirus is associated with neurological disease in black bears (*Ursus americanus*).

Acknowledgements

We thank Carlson College of Veterinary Medicine at Oregon State University (OSU) and Biomedical Undergraduate Summer Research (KW) for funding this study. We thank the supports from Oregon Department of Fish and Wildlife and California Department of Fish and Wildlife.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2018.10.016.

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