NRC Research Press

ARTICLE

Alphaherpesvirus: isolation, identification, partial characterisation, associated pathologic findings, and epidemiology in beluga whales (*Delphinapterus leucas*) in Alaska and Arctic Canada[1]

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Abstract: Live, dead stranded, and harvested belugas (*Delphinapterus leucas*) in Alaska and the western Canadian Arctic were screened for viruses utilizing a primary beluga cell line. Samples consisted of swabs from blowhole, anus, and genital tract. Virus cytopathic effect was seen after the incubation of 6–30 days post infection, and virus-like particles consistent with herpesvirus were observed upon electron microscopy. DNA extraction, cetacean-specific polymerase chain reaction (PCR) amplification, and sequencing of the DNA-dependent DNA polymerase gene fragments of approximately 700 nucleotides revealed the presence of a new species of alphaherpesvirus. Culture positive isolates were recovered from all swab types, from 2001 to 2016. PCR testing of swab and skin lesions from Bristol Bay, Alaska belugas revealed that the herpesvirus was present in the blowholes of a high proportion of the animals. Results suggest that belugas from Canadian and Alaskan locations are infected with alphaherpesvirus. Eight culture-positive belugas were identified from Alaska, all but one were adults and all had evidence of skin disease. No Canadian belugas showed signs of skin disease. Virus was isolated from three separate populations indicating it is likely enzootic in belugas. This is the first report of an alphaherpesvirus isolated and propagated from a monodontid species.

Key words: alphaherpesvirus, beluga, epidemiology, polymerase chain reaction, virus isolation.

Résumé : Des bélugas (*Delphinapterus leucas*) vivants, morts échoués et chassés en Alaska et dans l'ouest de l'Arctique canadien ont fait l'objet de dépistage de virus en utilisant une lignée cellulaire primaire de béluga. Les échantillons consistaient en prélèvements de l'évent, de l'anus et du tractus génital. L'effet cytopathologique du virus a été vu après 6–30 jours d'incubation suivant l'infection et des particules semblables à un virus correspondant à l'herpèsvirus ont été observées par microscopie électronique. L'extraction d'ADN, l'amplification de réaction en chaîne de polymérase (RCP) spécifique aux cétacés

Received 29 August 2017. Accepted 20 November 2017.

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[1]This article is part of a Special issue entitled "The beluga summit: knowledge sharing of the eastern Beaufort Sea beluga whale".
Lisa L. Loseto currently serves as Co-Editor; peer review and editorial decisions regarding this manuscript were handled by John Iacozza and Greg Henry.

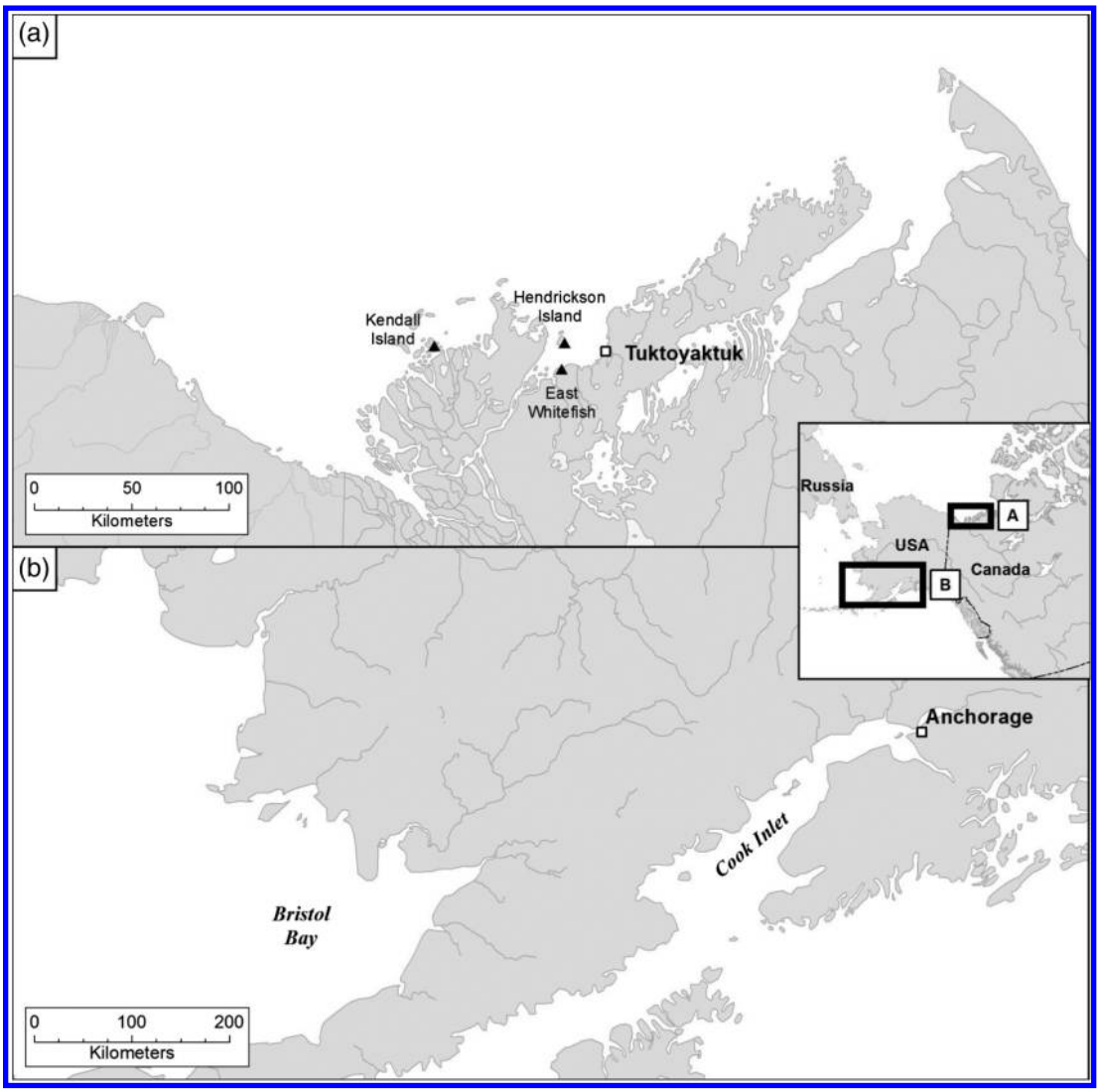
et le séquençage des fragments de gène de polymérase d'ADN dépendants de l'ADN d'environ 700 nucléotides ont révélé la présence d'une nouvelle espèce d'alphaherpèsvirus. Des isolats positifs ont été récupérés depuis tous les types de prélèvements de 2001 à 2016. Les tests RCP des prélèvements et des lésions de peau des bélugas de la baie de Bristol, en Alaska ont révélé que l'herpèsvirus était présent dans les évents d'une grande proportion des animaux. Les résultats suggèrent que les bélugas des régions canadiennes et de l'Alaska sont infectés par l'alphaherpèsvirus. Huit bélugas révélant une culture positive ont été identifiés en Alaska, tous sauf un étaient adultes et tous avaient des signes de maladie de la peau. Aucun des bélugas canadiens n'a montré des signes de maladie de peau. Le virus a été isolé de trois populations séparées indiquant l'enzootie probable chez les bélugas. Ceci est le premier signalement de l'alphaherpèsvirus isolé et propagé par une espèce monodontidé. [Traduit par la Rédaction]

Mots-clés : alphaherpèsvirus, béluga, épidémiologie, réaction en chaîne de polymérase, isolation d'un virus.

Introduction

The beluga whale (*Delphinapterus leucas*) is an Arctic and sub-Arctic toothed cetacean. Beluga whales are also called white whales because they lose the pigment in their skin as they age, with adults becoming almost pure white. They have a circumpolar distribution and are hunted for subsistence use by Inuit and other indigenous peoples in Canada, Alaska, Russia, and Greenland (McGhee 1988). Threats to sustainable beluga populations world-wide include over hunting, human interaction, pollution, and loss of ice-associated habitat due to climate change (Huntington 2009). Little is known about the effects that infectious diseases may have on this species. There are five genetically distinct beluga populations which use Alaskan waters and one of these summers in Canadian waters (O'Corry-Crowe et al. 1997). The five populations include from northeast to south: The Eastern Beaufort Sea (EBS), the Eastern Chukchi Sea, the Eastern Bering Sea (primarily Norton Sound), the Bristol Bay (BB), and the Cook Inlet (CI). The range for the EBS population is trans-boundary with belugas migrating between Canadian and US waters in summer and into Russian waters in fall. The most recent population estimates for the EBS beluga population numbered 19 629 belugas and was, therefore, not considered to be in danger of overexploitation and remains a healthy robust population (DFO 2000). Samples obtained during summer subsistence hunt in the Canadian Inuvialuit Settlement Region (ISR) confirm that these belugas are genetically distinct from the other stocks of belugas found in Alaskan waters (O'Corry-Crowe et al. 1997). EBS belugas congregate in the Mackenzie estuary in early summer, disperse eastward towards Amundsen Gulf and Viscount Melville Sound in August and migrate westward along the Alaska coast and far off shore under the polar pack ice in autumn and are thought to over winter in the Bering Sea and Chukchi Sea (Fig. 1). The BB beluga population is estimated to be 2467 and this population size appears to be increasing and is, therefore, not designated as "depleted" under the Marine Mammal Protection Act or listed as "threatened" or "endangered" under the Endangered Species Act. Though genetically distinct from EBS, both populations overwinter in the Bering Sea but may utilize separate locations (Allen and Angliss 2015; Citta et al. 2017). The Alaska CI beluga population is considered sedentary and geographically isolated with belugas permanently residing within the inlet. This population declined significantly in the 1990s and despite limits on the subsistence harvest, the population has not grown as anticipated. In 2008, the abundance estimate was only 375 whales and the US government subsequently listed the population as "endangered" (Shelden et al. 2015). The reasons for the population's failure to thrive are unknown, though, predation by killer whales, disease, fisheries interactions, pollution, and anthropogenic disturbances have all

Fig. 1. Map of the sampling locations in Canada and Alaska.



been proposed as having a role in limiting the growth of this population (Hobbs et al. 2008; Norman et al. 2015).

Health is a major concern in all three populations of belugas and regular monitoring takes place opportunistically, as in the case of BB and CI, where stranded, captured, and tagged belugas are examined, sampled, and released by veterinary professionals, native community members, and aquarium crews. The subsistence hunt that takes place in a number of whaling camps in the ISR has gone on for hundreds of years, and has been monitored by industry, scientists, and community members since the 1950s; a monitoring program was formalized in the mid-1980s (Harwood et al. 2002). Hunters make available their harvested belugas where a range of measurements and samples are collected and tested, not only for diseases (Nielsen et al. 2000, 2001*a*, 2001*b*) but also for changes in body condition, stock discrimination genetic markers, diet assessments, hormone, and contaminant levels (Loseto et al. 2009, 2015; Harwood et al. 2014). Hunters also provide valuable information regarding

historical changes in behaviour, migration patterns as well as ice conditions that are incorporated into reports and scientific advice for consideration by wildlife management and government regulatory bodies.

The Herpesviridae family encompasses a large group of DNA viruses that primarily infect vertebrates, which they have done for millions of years, making them one of the most successful of all the viruses. Although there are a wide variety of different herpesviruses with different biological characteristics, they have in common basic properties such as morphology and size of the virus particles, a highly regulated gene transcription system and the ability to establish latency (Roizman and Sears 1996). Alphaherpesvirus latency occurs when viral DNA remains, as an episome within the nuclei of nerve cells mostly in the trigeminal or sacral ganglia, or in lymphoid cells, without causing clinical signs. Reactivation of latency, production of infectious virus, and virus shedding may occur at any time, sometimes years later, ensuring the long-term survival of the virus and its spread to uninfected individuals. One well-known example is infection with chicken pox caused by human herpesvirus 3 (HHV-3) or varicella zoster virus (VZV), which usually occurs in childhood with subsequent development of "shingles" decades later (Grinde 2013). The Herpesviridae are subdivided into three major subfamilies: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae. Alphaherpesviruses cause a variety of significant diseases in humans, domestic animals, livestock, and wildlife. Alphaherpesviruses are capable of causing a range of diseases, from inconsequential mouth sores to severe encephalitis in newborns and immunocompromised individuals, as in the case of human herpes simplex virus (HHV-1) infection (Roizman and Sears 1996). Given the widespread nature of herpesviruses, it is not surprising that both cetaceans and phocids are known to be infected with alpha and gammaherpesviruses (Van Bressem et al. 1999; Smolarek Benson et al. 2006; Maness et al. 2011; Bellehumeur et al. 2016). To date, the occurrence of betaherpesviruses has not been reported in cetaceans.

Skin lesions associated with an alphaherpesvirus have been described in a captive beluga originally from Hudson Bay, as confirmed by transmission electron microscopy (Barr et al. 1989). Skin lesions consistent with herpes infection also have been observed in beluga from the St. Lawrence River Estuary (Martineau et al. 1988). The described skin lesions were circular to elliptical ulcers surrounded by paler than normal skin with characteristic intranuclear inclusion bodies in epithelial cells. One young beluga in Cook Inlet died of a systemic herpesvirus infection associated with vasculitis in multiple organs and intranuclear inclusion bodies (Burek-Huntington et al. 2015). Virus isolation and propagation in cell cultures of herpesviruses from cetaceans have not been reported, probably due to the lack of available permissive cell lines. Other pathologies associated with herpesvirus infection in cetaceans include proliferative dermatitis in an Atlantic bottlenose dolphin (*Tursiops truncatus*) (Manire et al. 2006), penile lesions in a dead stranded beluga, from the St. Lawrence River Estuary (Bellehumeur et al. 2015), encephalitis in a harbour porpoise (*Phocoena phocoena*) (Kennedy et al. 1992), interstitial nephritis in a beaked whale (*Mesoplodon densirostris*) (Arbelo et al. 2012), and lymphoid necrosis, also in a beaked whale (Arbelo et al. 2010). A pan-herpesvirus nested PCR assay performed on a frozen tissue lesion from the beluga from the St. Lawrence River was positive, and sequencing of the amplified DNA fragment revealed the identity to be an alphaherpesvirus tentatively named beluga whale herpes virus (BWHV). Virus isolation attempts utilizing a primary beluga whale kidney (BWK) cell line were negative, probably due to the advanced state of decomposition of the carcass (Bellehumeur et al. 2015). Subsequent evaluation of archived material from other dead stranded beluga suggested that BWHV is endemic in the St. Lawrence Estuary beluga population (Bellehumeur et al. 2015).

This study addresses the concern that BWHV maybe circulating in Arctic populations of belugas and describes attempts to isolate and characterize recovered viruses from hunter-harvested and stranded/dead belugas across three populations that vary in population size and health status.

Material and methods

Animals and sampling

Alaska, USA

Information on the individual belugas sampled from BB (approximately 59°1′58″N, 158°21′45″W) and CI (approximately 60°28′36″N, 151°50′24″W) from which virus isolates were recovered and characterized from blowholes are detailed in Table 1. Age was estimated by body length using charts developed in other studies comparing body length to dentine growth layer groups (GLGs) (Vos 2003; Suydam 2009). Samples of skin lesions and healed scar tissue were taken using a 6 mm diameter biopsy punch from live captured animals and larger samples from dead stranded animals. These were fixed in 10% neutral buffered formalin for histological examination and frozen at −80 °C for virus isolation. Twenty-seven blowhole swabs were collected in viral transport media (VTM) (Zhou et al. 1998) and immediately frozen on dry ice and held at −80 °C prior to shipping to Winnipeg for virus isolation attempts. Similarly, a total of 47 samples corresponding to blowhole, urogenital, and anal/fecal swabs as well as skin lesions and peripheral blood buffy coats were frozen in dry ice and shipped to the University of Florida in Gainesville, for viral testing by PCR (Table 2).

ISR, Canada

Hunter-harvested belugas were sampled soon after death from three closely located hunting camps in the Northwest Territories, Canada; namely Hendrickson Island (HI) (69°29′55″N, 133°34′59″W), Kendall Island (KI) (69°29′23″N, 135°16′60″W), and East Whitefish (68°53′49″N, 136°55′14″W) (EWF) (Fig. 1). Information such as the location, years harvested, along with tissue samples was collected from each beluga by hunt monitors and is detailed in Table 3. Ages were determined by counting growth layer groups in the dentine of tooth sections (Stewart et al. 2006). Blowhole, rectal, and urogenital swabs were collected in ampoules containing VTM and immediately frozen in a dry shipper containing liquid nitrogen prior to shipping to Winnipeg for virus isolation attempts. Samples from 2001 were archived samples also collected from the beluga hunt and stored from a previous study and held at −80 °C. None of the hunter-harvested belugas had any active skin lesions upon a visual examination of the carcasses.

Histopathology

Biopsies of lesions from live captured belugas in Bristol Bay and dead stranded beluga in Cook Inlet were preserved in 10% neutral buffered formalin, routinely processed at Histology Consulting Services, Everson, Washington, USA, embedded in paraffin, sectioned at 5 mm, stained with hematoxylin and eosin, and reviewed by Alaska Veterinary Pathology Services.

Virus isolation

Virus isolation was done by standardized testing methodologies employed by most veterinary diagnostic laboratories modified for beluga virus isolation by using primary beluga whale kidney (BWK) cells, which would be more likely to be susceptible to beluga-specific viruses. Briefly, frozen ampoules were rapidly thawed and 350 μL aliquot samples were aseptically inoculated onto 25 cm^2 tissue culture flasks (Corning Inc., Corning, New York, USA) containing 80% confluent cultures of BWK cells grown in Dulbecco's Minimal Essential Medium/Ham's F-12 (1:1) containing HEPES buffer (to control the pH),

Table 1. Information on Alaska and ISR belugas from which virus isolations were made from blowhole swabs.

Isolate ID	GenBank accession number	Location	Year	Sex	Age/years	Clinical assessment	Gross and histologic findings
DLBBN-AK_18-08	KF679805	BB-AK	September 2008	M	Sub adult	Unhealthy	Numerous healed and active skin lesions; spongiosis, ballooning degeneration, and inclusion bodies in skin lesion
DLBB-AK_12-01	KJ191537	BB-AK	September 2012	M	Adult	Healthy	Shallow lesions — possibly molt
DLBB-AK_12-04	KJ191539	BB-AK	August 2012	M	Adult	Healthy	Skin lesions
DLBB-AK_12-08	KJ191540	BB-AK	2012	F	Adult	Healthy	Erosive and vesicular dermatitis
DL-AK_12-01	KJ191536	CI-AK	2012	M	Sub adult	Unhealthy	Skin lesions — not specific for herpes (Found dead — Drowned/Systemic illness related to parasites)
DLBB-AK_13-07	KU522246	BB-AK	August 2013	M	Adult/15	Healthy	Small almost healed skin lesion on flank
DLBB-AK_14-06	KU311703	BB-AK	August 2014	F	Adult/14	Unhealthy	Proliferative lesion in blowhole; Skin — indications of viral infection — spongiosis/vesiculation
DLEWF-NWT_14-10_	KU311705	EWF-NWT	2014	M	Adult/10	Healthy	—
DLHI-NWT_14-09	KU323796	HI-NWT	2014	M	Adult/46	Healthy	—
DLKI-NWT_01-12	KU311704	KI-NWT	2012	M	Adult/29	Healthy	—

Note: BB-AK: Bristol Bay, Alaska, USA; CI-AK: Cook Inlet, Alaska, USA; EWF-NWT: East Whitefish, North West Territories, Canada; HI-NWT: Hendrickson Island, NWT. Canada; and KI-NWT: Kendall Island, NWT, Canada.

Table 2. Detection of cetacean herpesvirus DNA in tissues and lesions of beluga whales from Bristol Bay, Alaska using a direct polymerase chain reaction targeting the DNA polymerase gene.

Beluga ID	Blowhole swab	Skin lesion	Urogenital swab	Rectal swab	Buffy coat	Age/years	Sex	Animal/skin
DLBB12-01	+	NT	NT	NT	NT	Adult	M	Healthy — minor skin lesions
DLBB12-02	–	NT	NT	NT	NT	Adult	F	Healthy
DLBB12-03	+	+	NT	NT	–	Juvenile	F	Healthy — vesicular dermatitis; spongiosis; small atypical inclusions
DLBB12-04	+	NT	NT	NT	NT	Adult	M	Healthy, minor resolving skin lesion
DLBB12-05	+	NT	NT	–	–	Adult/8–10	F	Healthy, minor skin lesion
DLBB12-06	+	NT	NT	NT	–	Adult	F	Healthy, minor skin lesion
DLBB12-07	–	NT	NT	NT	–	Sub adult	M	Healthy; no skin lesion
DLBB-AK_12-08	+	NT	NT	NT	–	Adult	F	Healthy, minor skin lesion
DLBB12-09	+	#1 POS #2 NEG	NT	NT	–	Adult	F	Healthy, multiple erosive lesion
DLBB13-01	+	–	NT	–	–	Adult	F	Healthy, rare lesion
DLBB13-02	—	—	—	—	–	Adult >25	F	Healthy, no skin lesions
DLBB13-03	+	NT	+	–	NT	Sub adult	F	Grey, wrinkled skin with many erosive changes; ballooning degeneration and spongiosis
DLBB13-04	NT	NT	NT	–	–	Adult	F	Healthy
DLBB13-05	+	–	NT	NT	–	Adult	M	Healthy, minor skin lesion; very mild erosive, lymphocytic erosive dermatitis. No inclusions
DLBB13-06	–	–	NT	NT	–	Adult/10	M	Healthy
DLBB13-07	+	–	+	+	–	Adult	F	Healthy. Tiny lesion
DLBB13-08	+	–	NT	NT	–	Aged adult	M	Healthy. Several minor ulcerative/erosive lesions with spongiosis
DLBB13-09	–	NT	NT	NT	NT	Aged adult	M	Strong, Healthy. 2 small lesions
DLBB13-10	+	NT	+	NT	–	Sub adult	F	Extensive skin lesions including a large chronic ulcer. Ulcerative dermatitis with intranuclear inclusion bodies and secondary bacterial infection
BBN01-08	+	–	NT	NT	NT	Sub adult	—	Animal in molt, very mild nonspecific dermatitis
Number positive/ number tested	14/18	2/7	3/3	1/5	0/14	—	—	—

Note: NT, not tested.

Table 3. Virus isolations from Canadian beluga swab samples from 2001 to 2015, and Alaskan belugas from 2008 to 2016.

Location	Year	Number of belugas	Blowhole	Urogenital	Rectal	Skin/skin lesions	#Positive/total
HI-NWT	2001	11	0/11	ND	ND	ND	0/11
KI-NWT	2001	16	4/16	ND	ND	ND	4/16
HI-NWT	2014	14	10/14	ND	ND	ND	10/14
EWF-NWT	2014	1	1/1	ND	ND	ND	1/1
HI-NWT	2015	18	7/18	3/17	2/17	ND	8/18
EWF-NWT	2015	6	3/6	3/5	1/6	ND	5/6
BB-AK	2008–2014	16	10/16	ND	0/4	0/11	10/33
CI-AK	2009–2016	11	4/11	0/2	1/6	1/3	5/11

Note: BB-AK: Bristol Bay, Alaska, USA; CI-AK: Cook Inlet, Alaska, USA; EWF-NWT: East Whitefish, North West Territories, Canada; HI-NWT: Hendrickson Island, NWT, Canada; KI-NWT: Kendall Island, NWT, Canada. ND, not determined.

supplemented with 10% foetal bovine serum (HyClone Laboratories Inc., Logan, Utah, USA) containing antibiotics (penicillin 200 units mL^{-1}, streptomycin 200 μg mL^{-1}, and gentamycin 50 μg mL^{-1}) as previously described (Tuomi et al. 2014). After adsorption for 1 h at 37 °C, the inoculum was removed and 5.0 mL of fresh media were added to each flask. A mock-infected flask inoculated with sterile media always served as the negative controls. Flasks were incubated at 37 °C and examined daily using an inverted microscope for signs of virus infection as visualized by the development of cytopathic effect (CPE). Cells were passaged (1:2) at weekly intervals into fresh media and observed for up to one month. Media from flasks showing CPE were passed through a 0.45 μm filter diluted (1/100) and passaged onto fresh cells. Only if the CPE could be successfully passaged were the presumptive virus isolates included for further study.

DNA extraction, PCR, and sequencing

A sub-set of isolates from the ISR and Alaska belugas was tested targeting the DNA polymerase gene of known cetacean herpesviruses. Nucleic acids were extracted from infected and uninfected BWK cell cultures using the QIAamp DSP Spin Kit (QIAGEN) following the manufacturer's protocol. All PCRs were performed in a MJ Research, Inc. thermal cycler in 50 μL volumes. PCR assays targeting the DNA polymerase gene of cetacean herpesviruses contained 200 μmol L^{-1} dNTPs, 2.0 mmol L^{-1} $MgSO_4$, 1 μL of Enzyme Mix Elongase (Invitrogen), and 0.4 μmol L^{-1} of each oligonucleotide primer (FP-Dpol_632: 5′-GAC TTY GCY AGY YTV TAC CCH AGC AT-3′ and (RP-Dpol_633: 5′-TTG CKC ACS AGG TCS ACS CCY TTC AT-3′). These primers were designed after performing multiple sequence alignments (MegAlign, Lasergene, and DNASTAR) of the complete open reading frames (ORFs) of the DNA polymerase genes of alpha and gammaherpesviruses of bovine, ovine, and caprine species, available in the GenBank database of the National Centre for Biotechnology Information (NCBI), and selection of highly conserved nucleotide sequences. Over the years, these primers have been modified to their present composition, as homologous cetacean herpesvirus sequences became available (GenBank accession numbers: AY757301, AY949830, AY949828, AY952778, KJ406184, MF678601, KX424961, and KX424962). The primers drive the amplification of DNA fragments that range in length from 728 to 733 nucleotides, including the primer sequences.

Cycling conditions consisted of an initial denaturation at 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 56 °C × 45 s, and 68 °C × 1 min. Under these conditions, a herpesvirus DNA polymerase gene fragment of the expected 733 nucleotides was amplified, including the primer sequences. Two cloned and eight uncloned DNA fragments were sequenced by standard Sanger sequencing procedures using the BigDye Terminator

Sequencing Kit 3.1 (Applied Biosystems), and their chromatograms were manually reviewed for potential misreading using the Chromas 2.5.1 software (Technelysium Pty Ltd), and exported to the SeqEd function of the Lasergene software (DNASTAR). The BLAST Nucleotide and BLAST X functions from the NCBI website were used to identify herpesvirus sequences most closely related to those derived from the beluga whales. All 10 sequences obtained from the DNA polymerase gene of the beluga whale alphaherpesviruses have been deposited in the GenBank database of the NCBI and their accession numbers are provided in Table 1.

Total DNA was also extracted from blowhole, urogenital, and fecal swabs, skin lesions, and buffy coat samples obtained from beluga whales from the Bristol Bay stock, Alaska (Table 2), using the QIAamp DSP Spin Kit (QIAGEN) as described above, following the manufacturer's protocol, and tested directly by PCR the same way as for the nucleic acids extracted from the inoculated BWK cell cultures.

Transmission electron microscopy (TEM)

BWK cells propagated in a 75 cm^2 flask previously infected with the BBN18-08 beluga isolate were incubated at 37 °C until approximately 75% destruction of the cell monolayer was observed. The remaining attached cells were fixed at room temperature by first removing the supernatant media and replacing it with 2.5% glutaraldehyde in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.4) for 2 h. The fixative was removed and the fixed cells washed twice with 5.0 mL of cacodylate buffer. Cells were detached using a cell scraper in 1.0 mL cacodylate buffer and transferred to a vial. Buffered 4% low melting point agarose-embedded cells were re-fixed with Karnovsky's fixative, washed with 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.4), post fixed with buffered 1% OsO_4 for 1 h at room temperature, dehydrated with increasing concentrations of ethanol, then embedded in Spurr's epoxy resin using a Pelco (Ted Pella, Redding, CA, USA) microwave. Ultrathin sections were observed using Hitachi H7600 TEM (University of British Columbia Bioimaging Facility) and FEI Tecnai G2 Spirit TEM (Department of Cellular and Physiological Sciences, University of British Columbia).

The images were treated with Adobe Photoshop® CS3 to enhance brightness and contrast.

Results

Gross findings and histopathology

Unless otherwise described here, all animals live captured and harvested were considered to be normal, healthy individuals. Samples were submitted for virus isolation and PCR from both healthy individuals and animals with skin lesions. In a few of the Alaskan cases, there were some cases with pathology possibly related to herpesvirus infection.

A male beluga (DLBBN18-08) (58°52′40″N, 158°46′16″W) was sampled alive at Snake River, Bristol Bay (Fig. 2) on the 24th of September 2008. It did not behave normally and had very worn teeth for a small animal with grey skin (presumably a sub adult). It had numerous healed and active skin lesions characterized as an oval, depressed, white with sharp, distinct edges (Fig. 2*a*). On histopathology, this animal had erosive lesions in which the tips of the rete pegs were heavily infiltrated with neutrophils, lymphocytes as well as some macrophages. The remaining epithelial cells often had clearing of the cytoplasm and marginalization of the chromatin, and a central basophilic to amphophilic Cowdry type A intranuclear inclusion body (Fig. 2*b*). This skin lesion was not sub sampled, submitted for virus isolation or screened by PCR for herpesvirus, but the blowhole swab sample was positive by culture and PCR.

Several other animals had many serpiginous to circular erosive, sharp-edged lesions. Both DLBB12-03 and DLBB12-09 were PCR positive for herpesvirus in the skin lesion

Fig. 2. (*a*) Gross lesion in the skin of beluga BBN18-08 from Bristol Bay in 2008. This lesion was 5 cm × 3.5 cm and was one of several on this animal. (*b*) Histopathology of BBN18-08 skin lesion presenting intranuclear inclusion bodies (arrows) typical of herpesvirus with marginated chromatin and amphophilic inclusion bodies (100×).

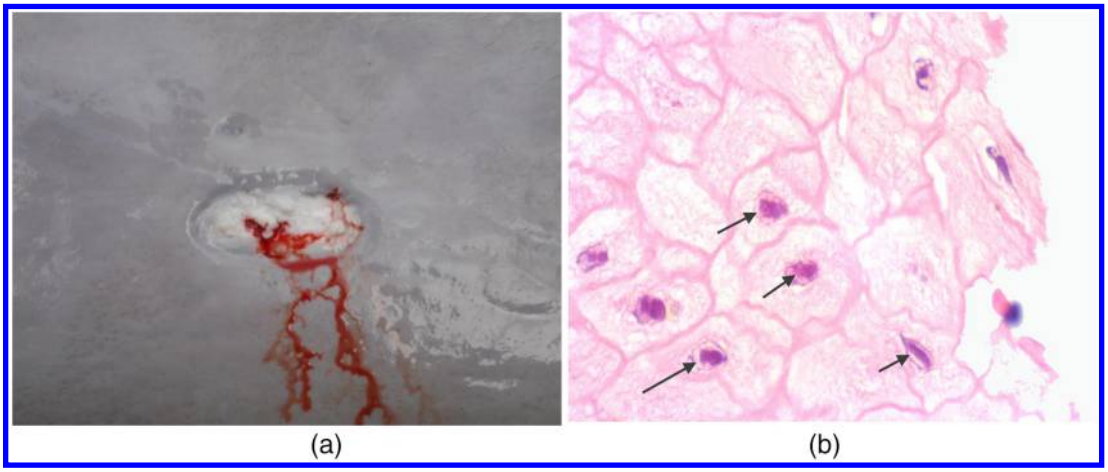
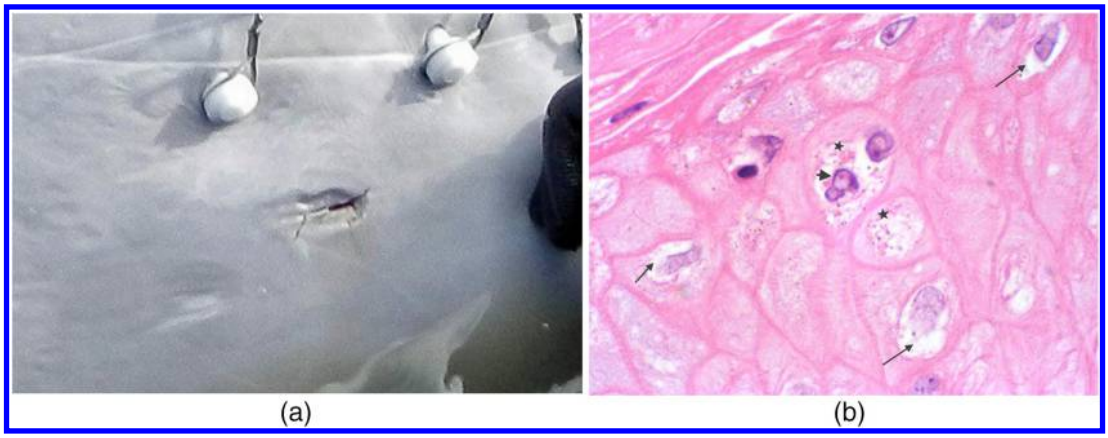


Fig. 3. (*a*) DLBB12-03 was herpes-positive by PCR in blowhole and skin lesion. The skin lesion was a very minor erosive dermatitis. (*b*) Histopathology of demonstrated spongiosis, ballooning (small arrows) degeneration with small eosinophilic intranuclear (arrowhead), and intracytoplasmic inclusions (star).

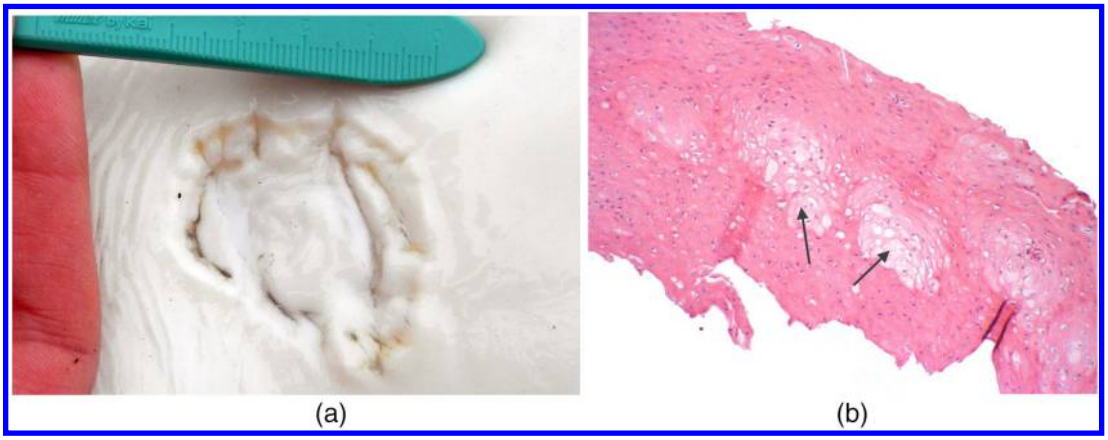


(Fig. 3*a*). Histopathology was slightly different in these two animals, and was characterized by spongiosis, ballooning degeneration with small eosinophilic intranuclear and intracytoplasmic inclusions in DLBB12-03 (Fig. 3*b*). DLBB12-09 had a very minor skin lesion (Fig. 4*a*) and patches of spongiosis and ballooning degeneration in the superficial epithelium with no inclusion bodies (Fig. 4*b*). This finding was very common in most of the cases. Other notes on gross and histologic findings are presented in Table 2.

Cook Inlet case

A juvenile male beluga (DL-AK_12-01) was found drowned as a result of net entanglement in the Kenai River, Cook Inlet (60°32′38″N, 151°16′43″W) (Fig. 1) on the 7th of May 2012. Cause of death was drowning, but it also had severe multisystemic illness related to parasites and multifocal healed traumatic skin lesions. Other notes on gross and histologic findings are presented in Table 1.

Fig. 4. (*a*) Gross lesion on DLBB12-09: Multifocal an irregularly shaped erosive lesion with sharp edges scattered along the lateral and ventral surface. (*b*) Histologically, there were patches of spongiosis and ballooning degeneration (arrow) in the superficial epithelium (10×).



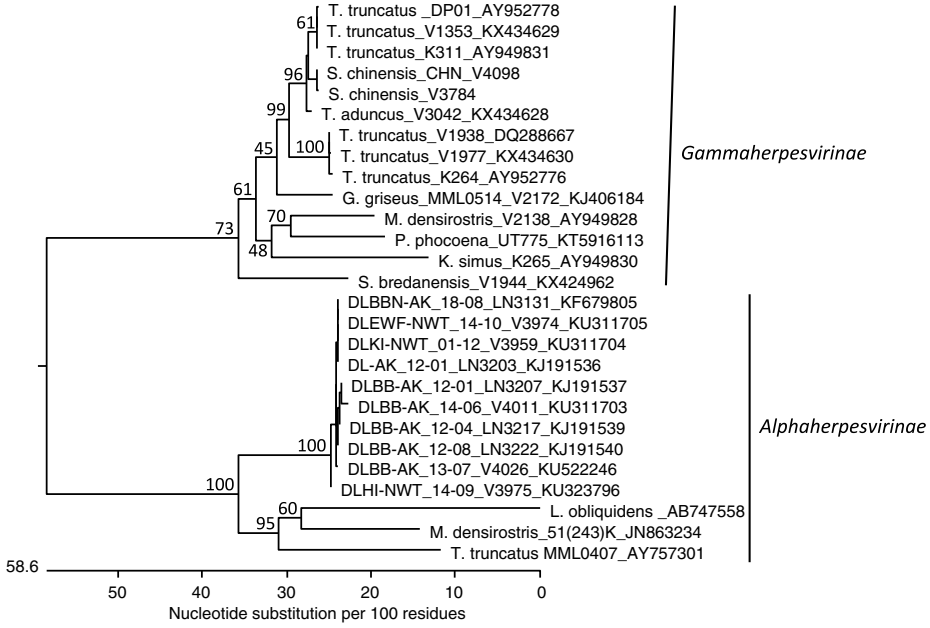
Virus isolation

Following inoculation and incubation of swab material, the cells in some of the flasks became multinucleated, swollen and syncytia developed after 6–30 days post infection. This CPE progressed for an additional week until all cells showed signs of infection, death, and detached into the medium. This progressive CPE could be transmitted to fresh cell cultures inoculated with CPE positive cell lysates and appeared to be identical in all cases. Cell control flasks remained unaffected throughout the course of these experiments. Positive virus isolation results were obtained from all locations in the ISR and both populations of Alaska belugas with prevalence of 28/66 (42.4%) for ISR, 10/33 (30.3%) in Bristol Bay, and 5/11 (45.5%) in Cook Inlet (Table 3). The ISR and all but two of the BB belugas were presumably healthy, whereas the CI beluga was dead stranded. Virus isolations were successful from blowhole, fecal, and urogenital swabs, with some belugas being positive in multiple swab types. Blowhole swabs gave the most virus isolations with 25/66 (37.9%) giving positive results. Virus isolations were made from samples as far back as 2001 and in samples tested from all years (2008, 2012, 2013, 2014, and 2015) (Table 3).

DNA extraction, PCR, and sequencing

PCR of total DNA extracted from infected BWK cell cultures showing characteristic herpesvirus CPE, including the presence of multinucleated giant cells, yielded DNA fragments of 733-nucleotides long, including the primer sequences, as determined by sequencing of two amplified and cloned fragments. The sequences obtained from the remaining eight uncloned fragments varied in size from 658 to 681 nucleotides. These DNA polymerase gene fragments shared a nucleotide identity of 99.7%–100% among themselves and had highest identity with their homologue fragment from an alphaherpesvirus from a Blainville's beaked whale (*M. densirostris*) (74.0%–75.0%) from the Canary Islands, Spain, an Atlantic bottlenose dolphin (*T. truncatus*) (72.0%–73.0%) from Florida, USA, and a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) (63.5%–64.4%) from Japan (Fig. 5). The direct PCR performed on total DNA extracted from swabs and lesions revealed that the herpesvirus could be detected at high frequency in the blowhole samples (14/18 or 77.8%), in the generally healthy belugas (Table 2). All urogenital samples 3/3 and one fecal sample out of five also yielded fragments of the herpesvirus DNA polymerase gene of the expected size

Fig. 5. Neighbour-joining phylogenetic tree of a 733-nucleotide fragment of the DNA polymerase gene of cetacean herpesviruses, including the newly described beluga whale (DL) alphaherpesviruses. Shown also are cetacean herpesviruses of the Alphaherpesvirinae and Gammaherpesvirinae subfamilies of the Herpesviridae. Nucleotide sequences were aligned using the Clustal W slow and accurate function of the MegAlign suite of the Lasergene version 14 (DNASTAR). The bootstrap analysis was set at 1000 replicates. Sequences from cetacean herpesviruses retrieved from the GenBank database were only used if they were about 700 nucleotides in length or more. The GenBank accession numbers of all sequences are indicated at the end of the cetacean herpesvirus name. *Tursiops truncatus* (Atlantic bottlenose dolphin), *Tursiops aduncus* (Indo-Pacific bottlenose dolphin), *Grampus griseus* (Risso's dolphin), *Mesoplodon densirostris* (Blainville's beaked whale), *Phocoena phocoena* (Harbour porpoise), *Kogia simus* (Dwarf sperm whale), *Delphinapterus leucas* as in DLBB-AK (beluga whale from Bristol Bay, Alaska), DLEWF-NWT (beluga whale from East White Fish, Northwest Territories, Canada), DLKI-NWT (beluga whale from Kendall Island), DLCI-AK (beluga whale from Cook Inlet, Alaska), DLHI-NWT (beluga from Hendrickson Island), *Lagenorhynchus obliquidens* (Pacific white-sided dolphin).



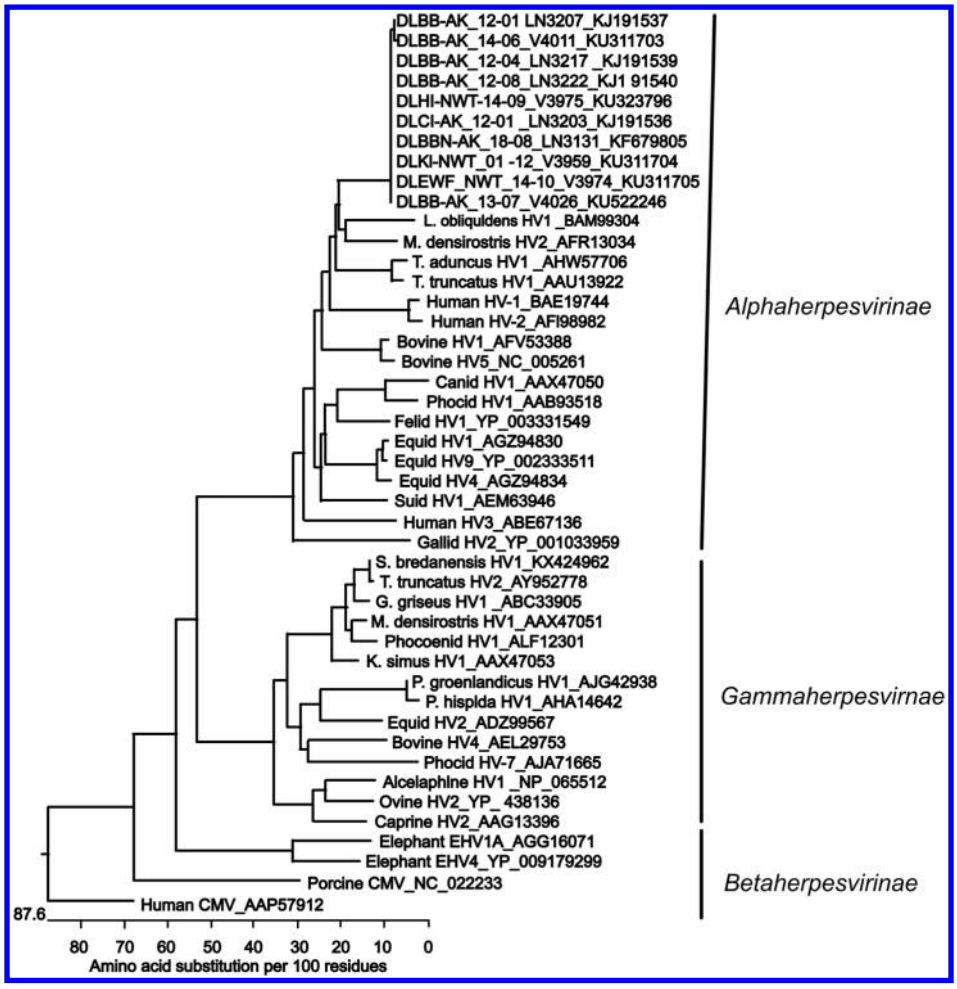
of approximately 700 nucleotides. Only one of these DNA polymerase fragments was sequenced and confirmed to correspond to the targeted alphaherpesvirus gene.

Phylogenetic analysis of the deduced amino acid sequences from the DNA polymerase gene fragments of all 10 beluga whale virus isolates, using homologous sequences from selected members of all three subfamilies of the Herpesviridae family, showed that the beluga whale isolates shared identities of 99.6%–100% among themselves, with the highest percent identity to alphaherpesviruses from cetacean species such as *M. densirostris* (77.6%–78.9%), *T. truncatus* (76.6%–76.9%), *Tursiops aduncus* (74.7%–76.0%), and *L. obliquidens* (75.3%–75.9%). The phylogenetic grouping of the beluga whale herpesviruses with thoroughly researched alphaherpesviruses of terrestrial mammals such as HHV-1, bovine HV-1 (BoHV-1), equine HV-1 (EHV-1), and suid HV-1 or pseudorabies virus (PRV) amongst others, further supports their inclusion in the Alphaherpesvirinae subfamily of the Herpesviridae (Fig. 6).

Transmission electron microscopy

Virus particles were imaged from transmission electron microscopy examination of fixed infected BWK cells displaying CPE following inoculation with a blowhole isolate from

Fig. 6. Neighbour-joining phylogenetic tree constructed with the deduced amino acid sequences (between 223 and 235 amino acids) of the DNA polymerase gene fragments of all 10 beluga whale alphaherpesviruses and their homologous sequences of selected members of the Herpesviridae retrieved from the GenBank database of the NCBI. Sequences were subjected to a multiple sequence alignment using the Clustal W function of the MegAlign suite (Lasergene Version 14, DNASTAR). The branch lengths are proportional to evolutionary distance. The three herpesvirus subfamilies are indicated. Cetacean virus names are provided in Fig. 5. GenBank accession numbers are indicated at the end of the sequence names.



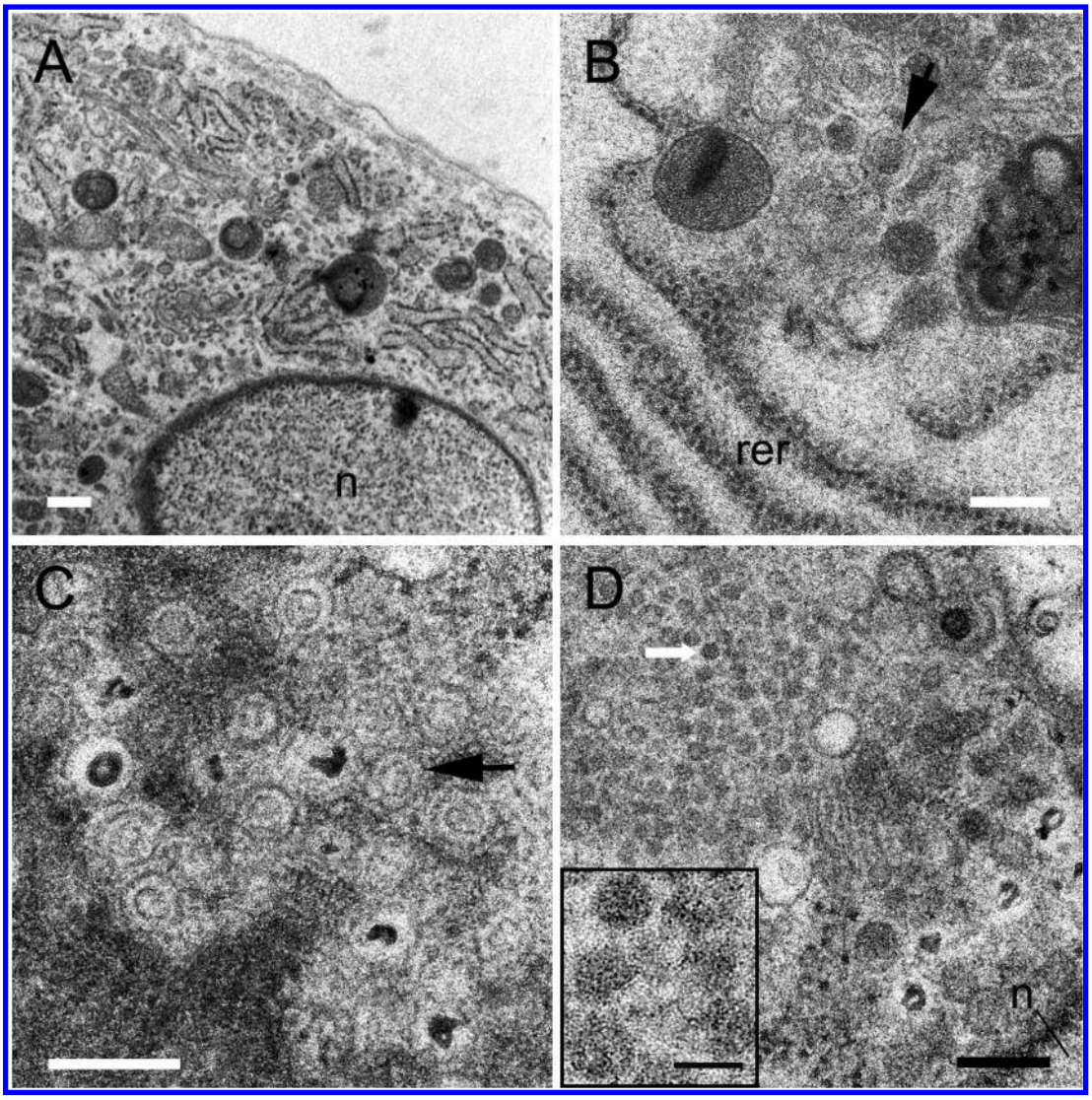
DLBBN18-08, described in Table 1 and Figs. 2*a* and 2*b*. Virus-like particles consistent with the size (approximately 100 nm in diameter) and icosahedral symmetry consistent with herpesvirus morphology could be seen within the cytoplasm of the infected cells but not in control cells (Fig. 7). Immature virus particles (without envelope Fig. 7*d*) and complete viruses including their envelopes could be seen in the cytoplasm (Figs. 7*b* and 7*c*).

Discussion

Virus isolation, identification, and relationship to other alphaherpesviruses

The present study reports on the first isolation and in vitro propagation of an alphaherpesvirus infecting a cetacean species utilizing beluga primary cell culture (Nielsen et al. 1989). The technique of virus isolation is still considered the "gold standard" in

Fig. 7. Transmission electron microscopy images of an uninfected control cell (A) and infected cells (B–D). In B and C, herpesviruses presented their virus core and envelope (highlighted with black arrows). In D, they presented only the capsids (one of them marked with a white arrow). Scale bars: (A) 500 nm, (B, C, and D) 200 nm. Insert in (D) Higher magnification of the area around the white arrow. Scale bar: 50 nm. rer: rough endoplasmic reticulum, n: nucleus.



establishing unequivocally the identity of viruses causing disease in affected animals (Leland and Ginocchio 2007). This finding has been confirmed by PCR amplification and sequencing of a 733-nucleotide fragment of the DNA polymerase gene as well as by TEM. Virus particles consistent with the size and morphology of herpesvirus could be seen in the cytoplasm of experimentally infected cultures from which virus isolations had originally been made.

Our finding also confirms the findings of Bellehumeur et al. (2015), who concluded that BWHV was commonly found in dead stranded belugas from the St. Lawrence River Estuary population. Using a molecular approach, they tested a herpes suspect penile lesion

utilizing a pan-herpesvirus nested PCR technique (Van Devanter et al. 1996) with the subsequent sequencing of the positive PCR product. This revealed a partial herpesvirus DNA polymerase (DPOL) gene fragment of 218 nucleotides. Its nearest nucleotide identity was with the partial DPOL gene of an alphaherpesvirus, confirming the identity of the virus. In this manuscript, we showed that a larger fragment (681–733-nucleotides) of the DNA polymerase gene of all 10 beluga whale herpesviruses analyzed, had the highest level of identity with the homologous gene fragment of alphaherpesviruses from a Blainesville's beaked whale, Atlantic- and Indo-Pacific bottlenose dolphins and Pacific white-sided dolphin (Fig. 5).

Furthermore, phylogenetic analysis of the deduced amino acid sequences of the beluga whale herpesviruses also showed that these viruses grouped together with important alphaherpesviruses of humans and livestock such as, HHV-1, HHV-2, BoHV-1, BoHV-5, EHV-1, and PRV, amongst others (Fig. 6). Direct PCR performed on total DNA extracted from blowhole, urogenital and in one case, with rectal swabs obtained from the Bristol Bay stock of beluga whales showed that this alphaherpesvirus can be readily detected in the blowhole, even in the absence of apparent lesions (Table 2). Testing of archived lesions from other dead belugas from the St. Lawrence River also gave positive PCR results indicating that BWHV infection of beluga whales was endemic in this beluga stock (Bellehumeur et al. 2015).

BWHV is likely enzootic in beluga populations

We have also shown that BWHV is widespread among Arctic and subarctic beluga around North America, with virus isolations being made from cases representing two separate beluga populations in Alaska (BB and CI) as well as from belugas harvested in the Canadian subsistence hunt. It is likely that BWHV is enzootic in beluga populations world-wide given that alphaherpesviruses are known to persistently infect their hosts and co-diverge with them over millions of years as is the case with the human herpes simplex viruses HV-1 and HV-2 (Wertheim et al. 2014). Beluga populations have a circumpolar distribution and there is a likelihood of contact between individuals from different populations during migration to allow for the spread of the virus. The St. Lawrence River Estuary beluga population is isolated from other arctic populations has already been shown to maintain BWHV enzootically (Bellehumeur et al. 2015). Further testing of belugas from all the representative populations would be needed to confirm the hypothesis that BWHV is enzootic in all belugas.

Epizootiology of BWHV

EBS virus isolations were made from samples harvested as far back as 2001 and was subsequently made in 2014 and 2015 with an overall frequency of virus isolation of 42.4%. Blowhole swabs gave the most positive virus isolations regardless whether the belugas had skin lesions or not (Table 3). All except two of the Alaskan belugas were adults, and all save one had evidence of some active skin disease. Some of the BB belugas had both healed and active skin lesions that might indicate recrudescence of latent infection leading to active infection and then reverting to latency periodically. The CI beluga (DL-AK_12-01) died of cardiac collapse as a result of drowning entrapment was severely parasitized, and had skin lesions that did not appear to be consistent with herpesvirus infection yet an alphaherpesvirus isolate was made from the blowhole (Table 1). The three belugas from the ISR from which virus was recovered and characterised were also adult males over 10 years of age. It should be noted that the subsistence hunt in the ISR is biased towards the harvesting of healthy males and typically avoid females, since most are present with calves. Thus, it would be premature to conclude that only adults in this population are

infected with the alphaherpesvirus. Immunity to herpes and other viruses is known to wane in old age as a result of immunosenescence, a process whereby the immune system gradually deteriorates over time (Caruso et al. 2009). This phenomenon would in part explain the observation that older BB and EBS belugas were frequently virus-isolation positive. Virus isolations were made from all types of swabs that were collected and tested; including blowhole, fecal, and urogenital swabs, suggesting that rapidly dividing epithelial cells at these sites provide a suitable source of susceptible cells for the replication and shedding of infectious virus; perhaps continuously. Live virus shed by the host may be transferred to other susceptible belugas by direct sexual contact, since virus is present in the urogenital tracts of both sexes, as well as by dispersal of virus in aerosols produced by breathing, since virus is present in the blowhole of a large number of infected belugas (37.9%). Belugas are highly social animals travelling together in family groups, where they are often in close physical contact; with ample opportunity to exchange virus containing aerosols between infected and susceptible individuals. These behaviours would explain the high prevalence of infection seen in the ISR.

Latency

Alphaherpesviruses possess the ability to latently infect their host. Primary infection usually occurs first in the epithelial cells of the oral or genital mucosa followed by virus replication until the virus reaches local nerve ends and axons to then spread centripetally towards the neural ganglia that innervates the affected area. It is at these ganglia that the virus usually undergoes latency and remains dormant for the most variable periods of time, until it is reactivated, mainly due to immunosuppression and stress, travelling this time centrifugally along the nerves and eventually being shed as infectious virus through the original site of infection (Roizman 1996). Latency and reactivation of herpesviruses have not yet been demonstrated to occur in any species of cetaceans, and it would be important to determine if these hallmarks of terrestrial herpesvirus infection follow the same pattern in aquatic mammals.

Pathogenicity

The herein described novel alphaherpesvirus of beluga whales has some biological properties, similarities, and differences when compared with other related alphaherpesviruses from terrestrial mammals. It infects mucous membranes in the oral, respiratory, and urogenital tissues in a similar fashion to HHV-1 and HHV-2, but differs in that infectious BWHV can always be recovered from a high proportion of belugas in the absence of lesions. BWHV causes skin lesions in some infected belugas as is seen in infections with BoHV-2 (Iman 2012) and PRV (Yang et al. 2016). Both these viruses may cause significant morbidity and mortality in their natural hosts, especially in the young. Cervid herpesvirus 2 (CvHV-2) is highly prevalent in reindeer (*Rangifer tarandus tarandus*) populations (Evans et al. 2012), also causing high mortality in young animals due to respiratory infection, as well as infection of the female reproductive tract (das Neves et al. 2009).

BWHV has been isolated from the urogenital tract and penile swabs from the ISR belugas. Whether young beluga can be infected via the vertical route (mother to offspring) and what effect the infection may have on the reproduction cycle, fetal exposure, infection, fetal development or survival of newborn belugas is unknown. In the case of one alphaherpesvirus isolation positive case from BB Alaska, herpesvirus suspect lesions were seen on the skin and inclusion bodies consistent with herpesvirus infection were seen in the histologic preparations of the skin biopsy samples. Two other cases had more active skin lesions, and these were PCR positive for herpesvirus; however, histopathology was not typical of herpesvirus infection. Further work, including electron microscopy needs to be

done to determine whether this is a different histologic manifestation of herpesvirus infection, or perhaps a consequence of a dual infection with a different pathogen. A systemic herpesvirus infection in a young beluga from Cook Inlet has been reported, raising questions on the potential pathogenicity of these viruses (Burek-Huntington et al. 2015).

Host range

The host range of alphaherpesviruses can also vary widely among different species. HHV-1 has a wide host range with infection and disease being reported in mice, rabbits, guinea pigs, and even zebrafish. HHV-1 can also infect numerous human cell lines derived from various organs and tissues other than skin and neural tissues (Karasneh and Shukla 2011). Suid HV-1 is the causative agent of Aujeszky's disease in swine and can also "jump" species barriers and establishes lethal infections in sheep, dogs, cattle, panthers, and mink. Infections of species other than swine invariably result in severe neurological symptoms and death of the affected animals (Tischer and Osterrieder 2010). In this context, the host range of BWHV has not yet been determined. Belugas are consumed for food; therefore, it would be a logical next step to determine whether BWHV has zoonotic potential. As a first step, screening of a number of human cell lines for their ability to support productive replication in vitro would provide a crude yet beneficial preliminary assessment of the zoonotic potential of BWHV.

Conclusions

Results from this study show that it is possible to isolate and propagate BWHV from swab and tissue samples from both healthy and unhealthy belugas from three different beluga populations. Identity as an alphaherpesvirus was confirmed by two methods; first, visualisation of virus particles having the size and morphological characteristics of herpesvirus by TEM and second, by DNA sequencing of amplified and cloned fragments representing the DNA polymerase gene found in infected cell cultures, swab, and tissue samples from infected belugas. Sequenced DNA products had a high degree of nucleotide identity with other cetacean alphaherpesviruses, thereby confirming the identity of these new isolates as a new species of alphaherpesvirus. BWHV was identified in samples and swabs from non-healthy Alaskan belugas and histopathological evidence of an association between the development of skin lesions and BWHV infection could be shown in some of the BB belugas. However, the multisystemic illness seen in the CI case could not be directly linked to BWHV infection alone and other unknown pathogens may have been involved in the development of the observed pathologies. Further work needs to be done to establish what pathologies other than skin lesions are associated with BWHV infection.

Northerners continue to utilize belugas as a food source and food safety concerns regarding harvested belugas are a major concern for hunters and their families. No human cases of disease as a result of consuming or butchering harvested belugas have been reported in either Canada or Alaska to the best of our knowledge. However, the continuation of beluga health assessments provides an effective means of detecting new pathogens that maybe emerging as threats to the animals themselves or to the people who use them for food.

Acknowledgements

Beluga samples from Alaska were collected under permit with the Marine Mammal Health and Stranding Response Network 932-1905-00/MA-009526 and MMHSRP 932-1905-00/MA-009526 for Cook Inlet and permit with National Marine Mammal Laboratory permit 782-1719-07 and 14245 for Bristol Bay. All live capture work was in accordance with approval from the Marine Mammal Laboratory of the Alaska Fisheries Science Center (MML/AFSC) IACUC protocols (ID No. AFSC-NWFSC2012-1) and funded through the Georgia Aquarium

and MML/AFSC and with the support of the Bristol Bay Native Association, Bristol Bay Marine Mammal Council, Alaska SeaLife Center, Alaska Department of Fish and Game, Mystic Aquarium, Shedd Aquarium, and National Institute of Standards and Technology. Cook Inlet sample collections were supported by Prescott grant NA12NMF4390162. Beluga samples from Canada were collected under DFO permits and in partnership with the Fisheries Joint Management Committee (FJMC) beluga harvest monitoring program. The authors would like to thank the beluga harvesters and Hunters and Trappers Committee at Tuktoyaktuk and Inuvik, Northwest Territories for supporting the beluga monitoring program and our research program. We thank the beluga monitors and research assistance for sample collections; Frank Pokiak, Verna Pokiak, Danny Swainson, Lawrence Angasuk, Bertha Joe, Steve Cockney, Paden Lennie, and Andrew Gordon. We thank Sonja Ostertag, Department of Fisheries and Oceans Canada, for coordinating the collection of the samples and Mark Ouellette, Department of Fisheries and Oceans Canada, for providing the map.

We thank the Fisheries Joint Management Committee established under the Inuvialuit Final Agreement, for funding some of the field work and laboratory analyses. We thank Canadian Natural Sciences and Engineering Research Council Discovery and Accelerator Grants for funding the electron microscopy studies.

In addition, we would like to thank Derrick Horne (UBC Bioimaging Facility) and Wayne Vogl (Department of Cellular and Physiological Sciences, UBC) for their technical support.

We thank Henry P. Huntington for help in editing and formatting the final manuscript.

The authors have no conflicts of interest of any nature.

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