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September 2021

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National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Alaska Fisheries Science Center

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False-Positive Polyomavirus Infection in a Stranded, Pregnant Cook Inlet Beluga Whale (*Delphinapterus leucas*)

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ABSTRACT

Polyomaviruses affect a wide range of wildlife including marine mammals with varying levels clinical significance. On 26 May 2014, a dead, adult female Cook Inlet beluga whale was reported stranded at Kincaid Park in Anchorage, Alaska. A necropsy was performed approximately 14 hours later on 27 May 2014. Gross examination revealed a nearly full term-pregnancy, petechial hemorrhage on the bladder, heavy *Crassicauda* in the kidneys, and no obvious cause of death. Histopathological examination found mild inflammation in the brain and moderate lymphoplasmacytic mastitis and eosinophilic and clear intranuclear inclusion bodies in epithelial cells. Tissue from the mammary gland taken at the time of necropsy were cultured for virus and possible signs of cytopathic effect were observed but were not definitive. Cellular/viral supernatant was tested using polymerase chain reaction (PCR) for herpesvirus and polyomavirus and was initially negative for herpesvirus and suspect positive for polyomavirus. Sequencing of the PCR product, however, did not confirm the presence of polyomavirus. A polyomavirus was also initially identified on paraffin-embedded blocks of mammary tissue via electron microscopy (EM); however, a second examination determined no virus to be present. This finding indicates that polyomavirus is not considered to be a possible factor in the health status or death of this beluga whale. While polyomavirus are generally subclinical, they can sometimes cause disease and thus it was important to investigate this finding given the critically endangered status of this population.

CONTENTS

INTRODUCTION

Beluga whales (*Delphinapterus leucas*) inhabit northern hemisphere oceans, seas, and bays, and, in Alaska, their range extends from Yakutat Bay to the Beaufort Sea (Hazard 1988). Five stocks are managed under the U.S. Marine Mammal Protection Act: Cook Inlet, Bristol Bay, eastern Bering Sea, eastern Chukchi Sea, and Beaufort Sea, the most isolated is the Cook Inlet stock (hereafter CIB) (Muto et al. 2020). Numbering fewer than 300 whales (Wade et al. 2019), their geographic and genetic isolation from other stocks (O'Corry-Crowe et al. 1997, Laidre et al. 2000), and site fidelity (Rugh et al. 2010, Shelden et al. 2018, McGuire et al. 2020) increases their vulnerability to natural and anthropogenic sources of mortality and morbidity (Norman et al. 2015, Castellote et al. 2018). CIB are listed as endangered under the U.S. Endangered Species Act (73 Fed. Reg. 62919, October 2008) and in 2015, the National Oceanic and Atmospheric Administration (NOAA) recognized this population as a Species in the Spotlight. This report describes the laboratory investigation of a possible polyomavirus detected post-necropsy in a stranded pregnant CIB. Given their critically endangered status, a full investigation was deemed worthwhile to pursue.

Polyoma viruses are 40–45 nm, icosahedral, nonenveloped, circular, double-stranded DNA viruses which can cause a wide variety of primarily subclinical and clinical infections and tumor induction in a variety of avian species and immunocompetent mammals (Jiang et al. 2009). The family Polyomaviridae has recently been split into three genera: two genera utilize mammalian hosts, Orthopolyomavirus and Wukipolyomavirus, and one genus is found in birds, Avipolyomavirus. In mammals, polyomaviruses typically cause minimal clinical disease in their immunocompetent endemic hosts, followed by latent infection. However, exceptions exist, as murine pneumotropic polyomavirus (MPtV) causes significant mortalities in newborn mice. An association between brain tumors and a recently discovered raccoon polyomavirus has also been identified (Cortez-Hinojosa et al. 2016).

Polyomaviruses have been found in a variety of wildlife species including mammals, fish (López-Bueno et al. 2016), and birds. These viruses have specifically been documented in marine mammals including the common dolphin (*Delphinus delphis*), California sea lion (*Zalophus californianus*), sea otter (*Enhydra lutris*), northern fur seal (*Callorhinus ursinus*),

Weddell seal (*Leptonychotes weddellii*), and Hawaiian monk seal (*Monachus schauinslandi*) (Colgrove et al. 2010, Anthony et al. 2013, Duncan et al. 2013, Cortes-Hinojosa et al. 2016, Siqueira et al. 2017, Varsani et al. 2017). The significance of these viruses was generally not clear in the marine mammals but may have caused ulcerative tracheobronchitis in common dolphins (Anthony et al. 2013), was found present in the placenta of a northern fur seal (Duncan et al. 2013), and was associated with an oral mass found in a California sea lion (Colgrove et al. 2010).

Several polyomaviruses are known to cause clinical disease in immunosuppressed humans and laboratory animals. Some of these include BK virus causing hemorrhagic cystitis and nephropathy (Li et al. 2013, Hu et al. 2018), Merkel cell polyomavirus causing Merkel Cell carcinoma (Jaeger et al. 2012) in individuals with HIV or organ transplants, and JC virus causing progressive multifocal leukoencephalopathy (PML) in individuals on immunosuppressive medication (Tan and Koralnik 2010, Shishido-Hara et al. 2014).

MATERIALS AND METHODS

Necropsy and Histopathological Examination

External exam, photographs, morphometrics, and subsampling were performed at the site of the stranding on the beach at Kincaid Park, Anchorage, Alaska (61.15 N, 150.04 W) approximately 14 hours after this animal was reported on 26 May 2014 (Fig. 1). Due to extreme incoming tides, large samples of tissues (pluck, major organs, and head) were removed, refrigerated at 4°C overnight, and subsampled the following day (5/28/2014). Experienced marine mammal veterinarians assisted by technicians and volunteers (MMPA permit 932-1905/MA-009526-00, 01) performed all gross examinations.

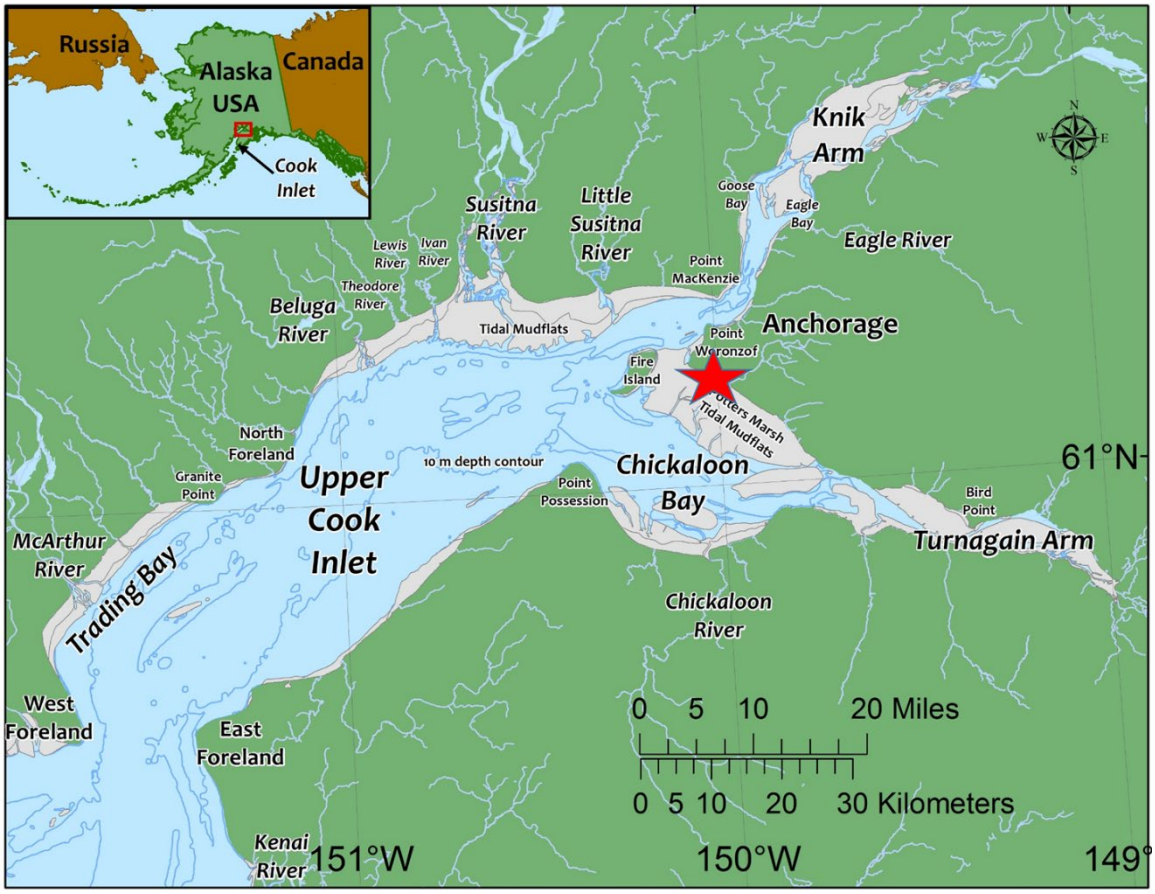

Figure 1. -- Map of Cook Inlet and location of the stranded beluga whale (star symbol).

The animal was in right lateral recumbency and had dark red fluid (postmortem seepage) draining from the eye (Fig. 2). The necropsy was performed using standard procedures per Geraci and Lounsbury (2005). Twenty-seven samples were taken for a combination of life history, toxicological, bacterial, and viral analysis and 45 tissues were sampled for histopathological analysis, all using standard necropsy protocols. Viral samples consisted of duplicate samples of approximately 1 g of whole tissue placed in cryovials (2 mL or 5 mL containing a) no media; b) viral transport media (Remel, Lenexa, KS), and c) RNAlater (Applied Biosystems, Waltham, MA). Samples for histopathological analysis were placed in 10% neutral buffered formalin (changed after 24 hours, then allowed to fix for 10 days), and sent to Histopathological Consulting Services (Everson, WA) for slide preparation. All slides were interpreted by a board-certified veterinary pathologist (K. Burek-Huntington).

Figure 2. -- Ventrolateral view of the intact beluga whale carcass in right lateral recumbency.

Viral Cultures

Viral culture was performed at the Department of Fisheries and Oceans (Winnipeg, Manitoba). Samples were received on dry ice and stored at -80°C. 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 (Nielsen et al., 1989), which would be more likely to be susceptible to beluga-specific viruses and Vero.DogSLAMtag cells, a cell line which has been shown useful for the isolation of cetacean viruses (Rodrigues et al. 2020).

Samples were rapidly thawed and diluted in Hanks balanced salt solution (HBSS) plus penicillin 200 IU/mL, streptomycin 200 µg/ml and gentamicin 50 µg/mL) to give approximately a 10% w/v ratio of tissue suspension and then clarified by low-speed centrifugation for 10 min (2,060 × g). Aliquots of 0.5 mL of the supernatant were used to inoculate 25 cm^2 tissue culture flasks (Corning Inc., Corning, New York, USA) containing 80% confluent cell cultures of primary BWK cells and Vero.DogSLAMtag cells. Flasks were incubated at 37° C for one hour, then the inoculum was removed and 10 mL of Dulbecco's Modified Eagle's

Medium/Ham's F-12 (DMEM/ F-12) containing 10% fetal calf serum (FCS) was added to the BWK flasks and media with 2% FCS to the Vero.DogSLAMtag flasks. Mock-infected flasks inoculated with sterile medium served as negative controls.

Flasks were incubated at 37° C and examined daily for cytopathic effect (CPE). Cells were blind passaged weekly for four weeks, at which time cells from flasks were harvested and submitted for polymerase chain reaction (PCR) testing for polyomavirus.

Transmission Electron Microscopy

Paraffin embedded blocks of formalin fixed tissues with suspected viral inclusions on histopathology (mammary gland and brain) were sent to California Animal Health Food Safety Laboratory (CAHFS; Davis, California) for thin section transmission electron microscopy (TEM) (completed October 2016). Blocks were retained at CAHFS and re-sectioned for second examination in May 2021.

PCR for Polyomavirus and Viral Sequencing

Tissue culture cell pellets of Vero.DogSLAMtag and BWK were tested for polyomavirus by PCR at the University of Florida Romero Laboratory in October 2018. Total DNA was extracted using standard technique and a commercial DNA kit. Primers were designed using sequences from dolphin and bovine polyomavirus and targeted a 600 base pair region of the polyomavirus VP1 gene (Johne et al. 2005, Anthony et al. 2013). Tissue culture fluid and mammary tissue were also sequenced via high throughput screening (HTS) and screened for viral sequence at the Waltzek Laboratory (University of Florida) and the Varsani Laboratory at Arizona State University, respectively (Varsani et al. 2017).

RESULTS

Gross Examination

Upon gross examination, cause of death was presumed to be secondary to live stranding due to glacial silt found in the airways. Externally there was partial scarring and shrinkage of the left eye and enlarged mammary glands leaking amber-colored fluid (Fig. 3). The 386 cm (standard length) female was pregnant with a full-term male fetus (150 cm length: Shelden et al. 2020a, b). Internal exam determined the carcass to be fresh dead (code 2). Body condition was average. Nematode parasites were in the kidneys, gastrointestinal tract, and subcutaneous layer (Fig. 4). These parasites are commonly found in Cook Inlet beluga whales (Burek-Huntington et al. 2015). The nematodes in the kidney were consistent with *Crassicauda giliakiana*. Petechiae were present on the mucosa of the bladder and cervix (Fig. 5) and hilar and mesenteric lymph nodes were mildly reactive.

Figure 3. -- Mammary glands were active.

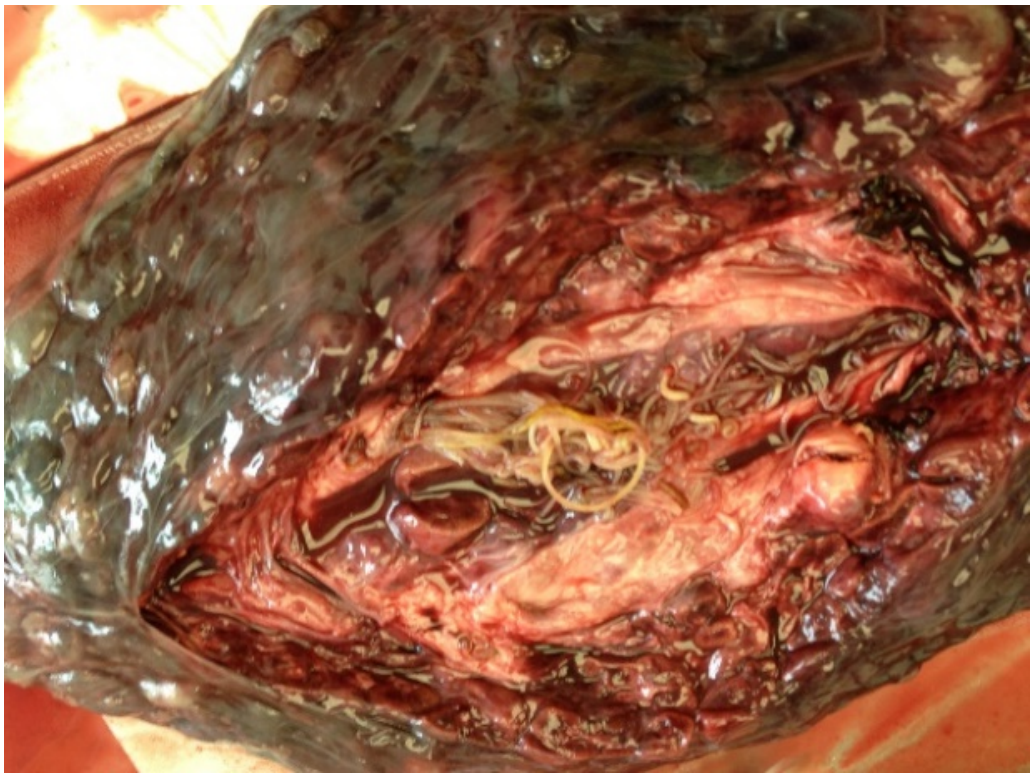

Figure 4. -- Large numbers of nematode parasites were present in the kidney.

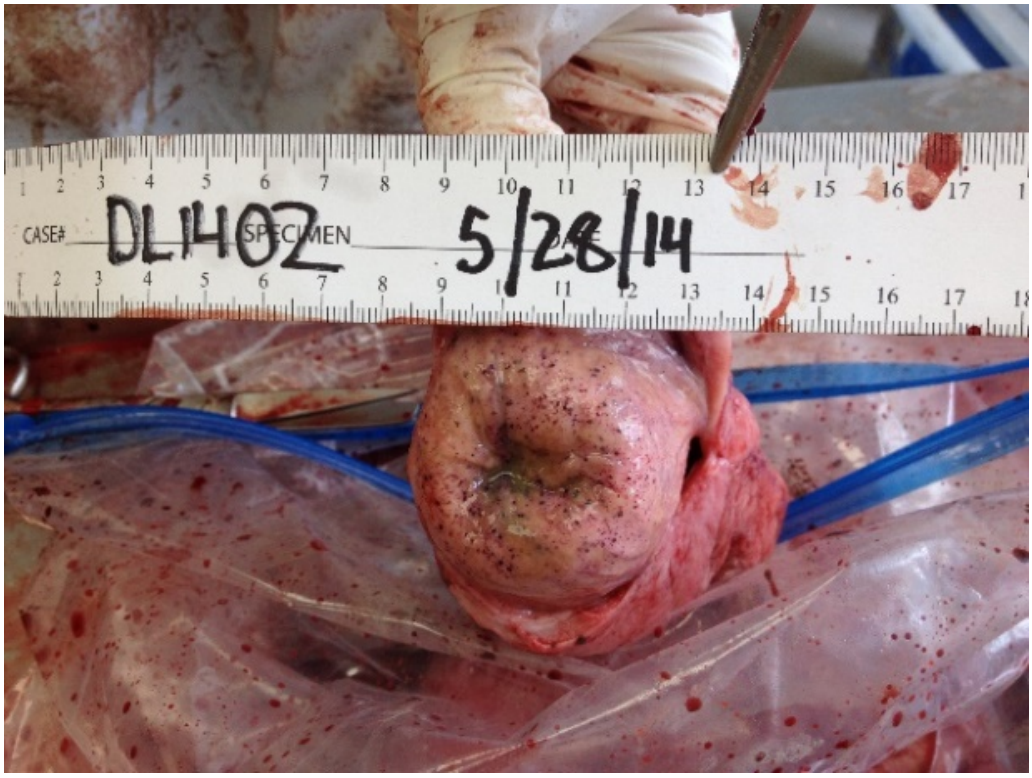

Figure 5. -- Petechial hemorrhages were present on the cervix.

Histopathological Examination of Non-mammary Tissues

Histopathological examination demonstrated a wide variety of findings in multiple organs. There was terminal aspiration of stomach contents indicated by skeletal muscle fibers in the airway. Marked generalized organ congestion and marked pulmonary edema, peracute degenerative myopathy of the cervical muscle and acute drainage of hemorrhage in the tonsil were likely consequences of the live stranding and terminal event. Mild neutrophilic tracheitis was likely related to the aspiration.

Various lesions associated with parasitism included eosinophilic pleuritis, mixed cell chronic interstitial pneumonia with marked fibrosis, eosinophilic drainage reaction in the hilar, moderate eosinophilic and lymphoplasmacytic enterocolitis, mild portal eosinophilic and lymphocytic hepatitis, mild to moderate chronic granulomatous steatitis, and a subcutaneous parasitic granuloma (probably *Crassicauda* sp*.* cyst). These were interpreted to be due to parasitism due to the strong eosinophilic component of the inflammation.

Degenerative changes which are likely age-related, as age obtained from a tooth indicated the whale was at least 41 years old (Vos et al. 2020, Shelden et al. 2020b), included mild to moderate myocardial fibrosis and degeneration. Phthisis bulbi was also present and is consistent with an old traumatic incident to the eye.

Histopathological Examination Mammary Tissues

The mammary gland was mildly active and had a moderate lymphoplasmacytic mastitis. There were aggregates of epithelial cells which had nuclei with chromatin margination and central clearing (clear inclusion bodies) (Fig. 6A). Duct cells were affected most often. Rarely these cells also had small brightly eosinophilic bodies. The nipple tissue had intense infiltration of lymphoplasmacytic inflammation subjacent to the epithelium of the ducts and surrounding skin. Frequent mitotic figures were present in the stratum basale, with piling up of the basal cells, as well as dyskaryotic cells throughout the epithelium. There were scattered foci of lymphocytic exocytosis. Many of the superficial

epithelial cells had cleared cytoplasm, chromatin margination, and rare distinct intranuclear eosinophilic bodies (see arrows in Fig. 6B).

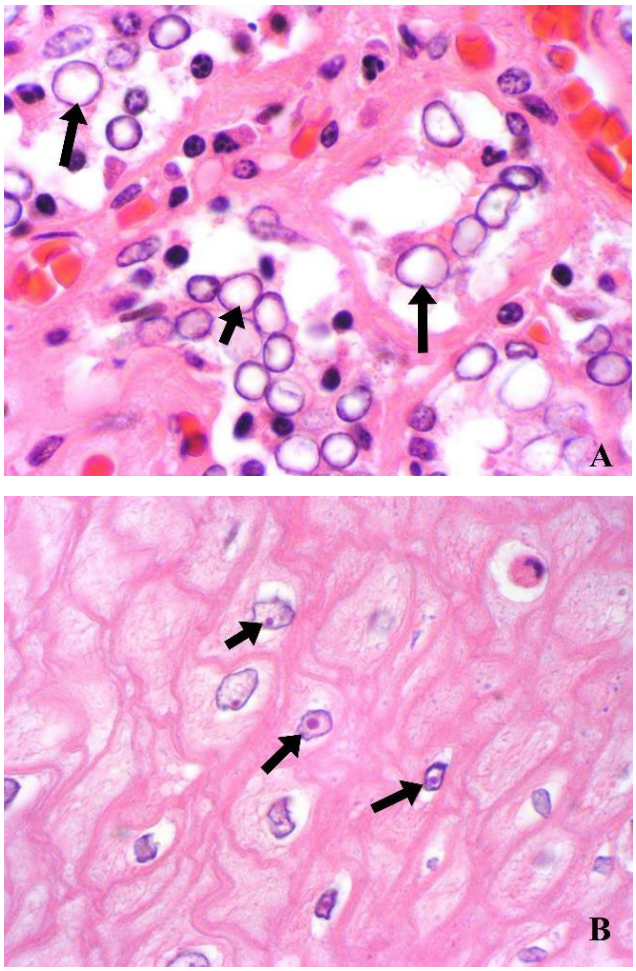

Figure 6. -- Distinct clear inclusion bodies (arrows) were present primarily in the duct cells. (A). Small brightly eosinophilic intranuclear inclusion bodies (arrows) were present in the nipple epithelial cells (B). Magnification 100×.

Histopathological Examination Brain

There were foci of intensive perivascular edema, lymphocytes, and larger mononuclear cells packed full of deeply eosinophilic material consistent with Gitter cells (Fig. 7A). There were scattered areas of mild perivascular lymphocytic inflammation

present (Fig. 7B). There was one lightly eosinophilic intracytoplasmic possible inclusion body (Fig. 7C-D).

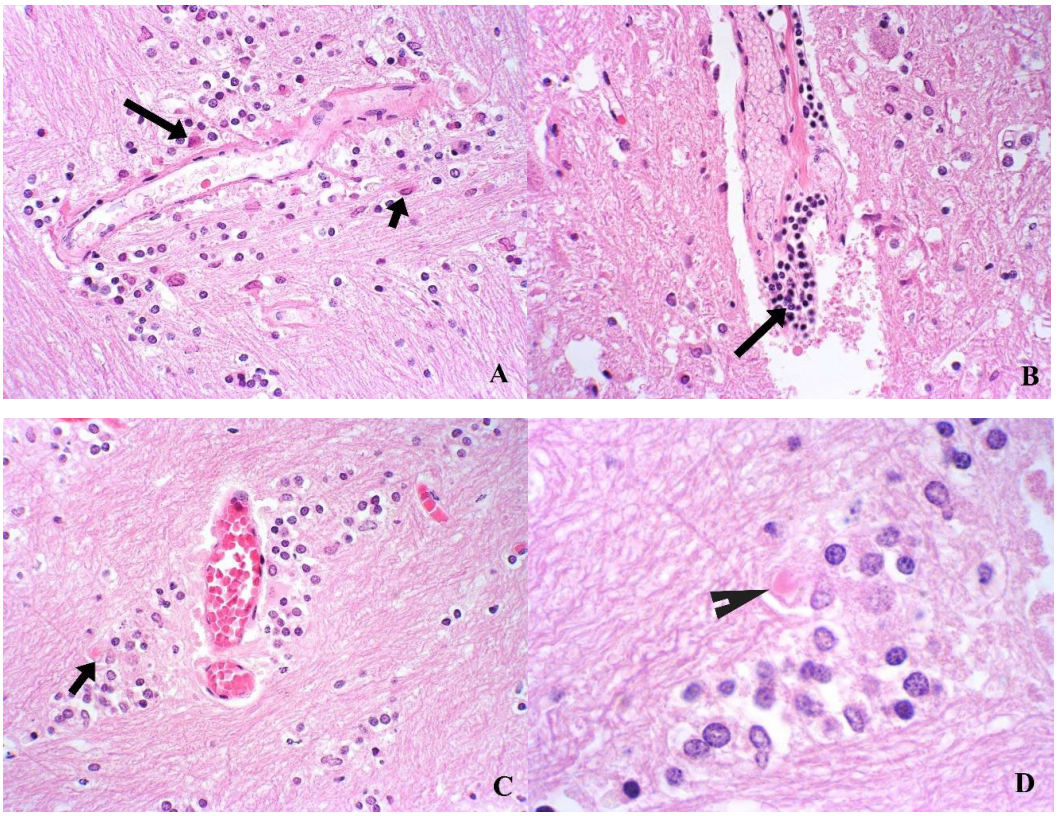

Figure 7. -- Foci of intensive perivascular edema, lymphocytes and larger mononuclear cells packed full of deeply eosinophilic material consistent with Gitter cells (arrows) magnification 40× (A). Scattered areas of mild perivascular lymphocytic inflammation (arrow) magnification 40× (B). One lightly eosinophilic intracytoplasmic possible inclusion body (arrow) magnification 40× (C). Magnification 100x of possible inclusion body (arrow) (D).

Transmission Electron Microscopy (TEM) from Paraffin-embedded Blocks

CAHFS reported that virus was not observed on brain tissue. For mammary tissue, it was initially reported (10/31/2016) that virus was observed on the thin-section (Fig. 8), and that the structure was consistent with polyomavirus (Appendix 1). Because the quality of the photomicrographs was poor and viral sequence could not be obtained via molecular

means, recuts with better quality photomicrographs were requested in May of 2021. No virus was found on recuts of this tissue indicating no viral presence (Appendix 2).

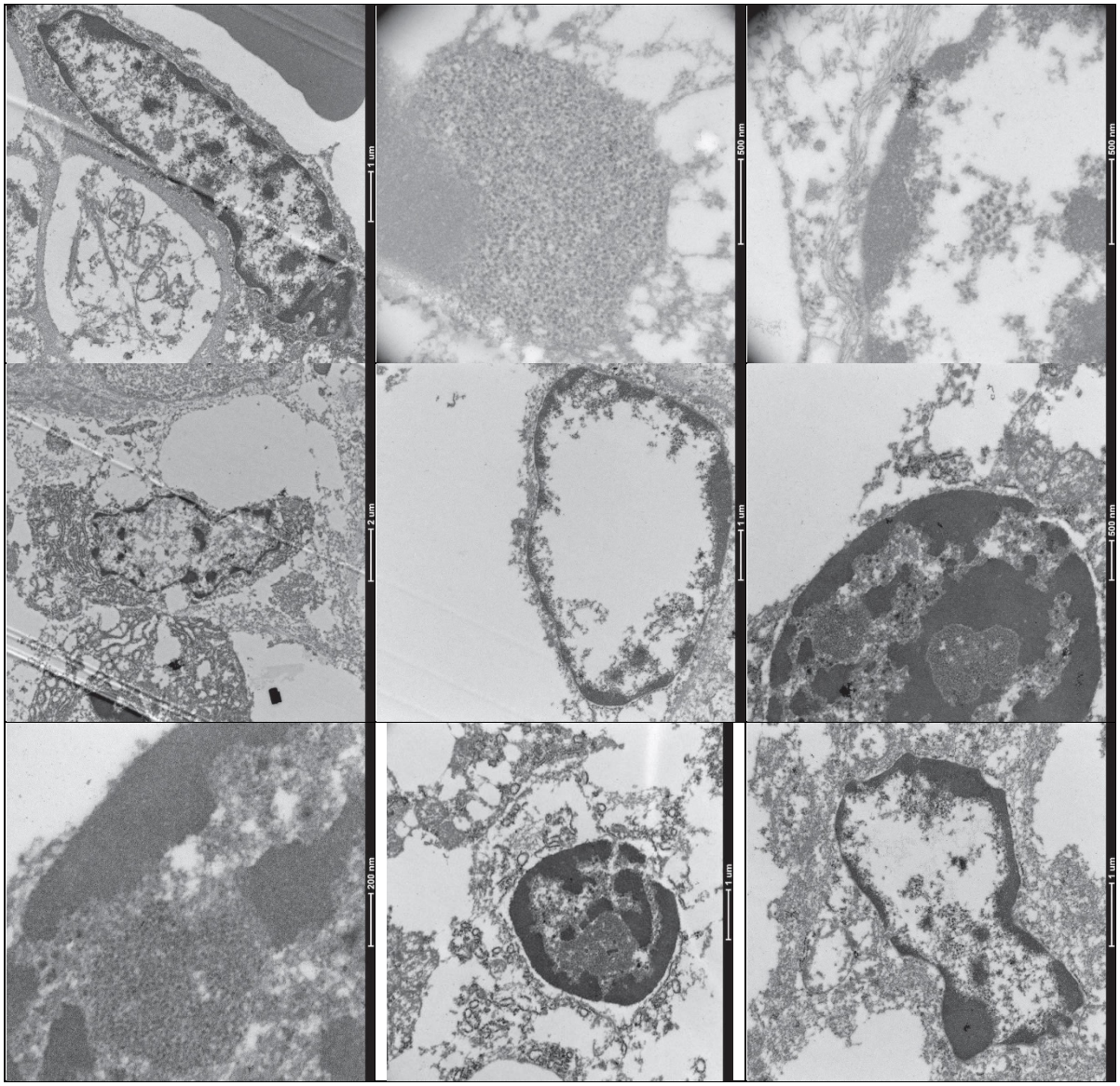

Figure 8. -- Selected initial proofs of potential polyomavirus in the mammary gland from CAHFS. No proofs of the negative brain EM were included.

Viral Culture and Transmission Electron Microscopy

Possible, but not definitive signs of CPE were visualized in BWK and Vero.DogSLAMtag cells inoculated from mammary tissue in viral transport media including occasional rounding and appearance of vacuoles in the cytoplasm post infection consistent with CPE in other polyomaviruses (Lednicky et al. 2013). Cells were harvested for PCR and sequencing attempts. Subsequent PCR targeting the VP1 gene of polyomavirus yielded a product, however, sequencing of that product did not yield a viral sequence consistent with polyomavirus. Subsequent efforts to obtain viral sequence from cultured cellular product, as well as a sample of the original mammary tissue via high throughput screening (HTS) also did not yield sequence consistent with any polyomavirus.

DISCUSSION

While polyomaviruses in mammals are generally subclinical, there are two documented cases of polyomaviruses associated with possible disease in marine mammals, both in the Pacific Ocean (Colgrove et al. 2010, Anthony et al. 2013). CIB are a critically endangered species with multifactorial possible causes of the decline (Marine Mammal Commission 2021). Several recent necropsies have suggested possible immunocompromise including animals with infections with multiple pathogens (Burek-Huntington et al. 2015).

Histopathology suggested the presence of virus in both the mammary gland and brain as indicated by possible inclusion bodies. Virus isolation of polyomaviruses in primary cells is possible (Gardner et al. 1989), however, it can be very difficult to detect because these viruses rarely cause visible CPE in primary cells, thus the study of newly emerging polyomaviruses is limited by the lack of suitable cell culture systems (White et al. 2013). Viral culture of mammary gland samples in this case resulted in subtle, suspect CPE in BWK cells and initial PCR positive results seemed to corroborate this finding.

Additionally, the initial reported presence of a polyomavirus-type structure on TEM examination of the paraffin-embedded block led us to believe we had confirmed the presence of viral structures in the mammary gland lesions. For these reasons, we pursued

the diagnosis of a polyomavirus in this animal. We were thus disappointed that sequencing of the PCR products and next generation viral sequencing failed to identify a polyomavirus in our sample. This information, however, prompted the recut and re- interpretation of the EM for this case, illuminating the true negative result.

With the initial encouraging results for polyomavirus we followed this diagnoses to the furthest degree. Because CIB are critically endangered, any possible lead on health concerns is considered an important pursuit. Since polyomaviruses can cause disease in immunocompromised animals and humans, and since we have had some suspicions of possibly compromised immune function in these animals, polyomavirus was of interest even though the lesions did not indicate they were involved in the death and significant illness of this animal.

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APPENDIX 1: CAFHS Initial Report

California Animal Health & Food Safety Laboratory System
PO Box 1770
Davis, CA 95617
(530) 752-8700

FINAL REPORT

This report supersedes all previous reports for this case

CAHFS Case #: D1613807
Referral #: V14-092 #61/#32
Date Collected:
Date Received: 10/31/2016
Case Coordinator: Leslie Woods, DVM, PhD, Dipl. ACVP
Electronically Signed and Authorized By: Woods, Leslie on 4/5/2017 4:08:21PM

Email To:
Goldstein, Tracey
tgoldstein@ucdavis.edu

Collection Site:

AK

Specimens Received: 2 Tissue Block;

Comments: 2 slides and 2 blocks

Case Contacts

Submitter	Goldstein, Tracey	754-795-3	Wildlife Health Center	Davis	CA	95616
Bill To	WILDLIFE HEALTH CENTER	530-752-4896	Tb 128 Old Davis Rd	Davis	CA	95616

Specimen Details

Animal/Source	ID Type	Taxonomy	Gender	Age
1	CAHFS Internal ID	Beluga Whale	Female	Adult

Laboratory Findings/Diagnosis

Polyomavirus detected in mammary tissue by TEM

Clinical History

Blocks from two Beluga with accompanying slides for the EM lab. Histopath report attached.
Please return blocks and slides to Dr. Goldstein.

THIN SECTION EM FROM WAX HISTOBLOCKS

Animal/Source	Specimen	Specimen Type	Results
1	(1) V14-092 #61	Tissue Block	Done
1	(2) V14-092 #32	Tissue Block	Done

APPENDIX 2: CAFHS Amended Report

EM on old possible polyomavirus case

Kathy Burek Huntington <avps.kbh@gmail.com> Mon, May 17, 2021 at 11:30AM
To: Natalie Rouse <avps.natalierouse@gmail.com>, Anibal Guillermo Armien Medianero <agarmien@ucdavis.edu>

Hi Anibal
I talked to Leslie the other day and she said she would talk to you about this case. It is a beluga with possible polyomavirus inclusions in the mammary gland. A previous EM there at UC Davis indicated the EM was positive, however the photomicrographs were terrible. See attached. We had a possible virus isolate, and initial PCR was thought to be positive and then that was changed to negative on sequencing. We are trying to write a brief paper / summary and would like to see if we could confirm this with a good photomicrograph. Leslie said it would be redone atno cost since the last one was so bad. The case number is DL1402 CA case ID D1613807. We are just wondering what the timing frame would be. Thanks much, Kathy

Kathy Burek Huntington DVM, MS, DACVP
Alaska Veterinary Pathology Services (AVPS)
23834 The Clearing Dr., Eagle River, AK 99577
cell: 907 242-2566 Fax: 907 696-3565
https://veterinarypathologyservicesalaska.com/

Anibal Guillermo Armien Medianero <agarmien@ucdavis.edu> Wed, May 19, 2021 at 9:49AM
To: Kathy Burek Huntington <avps.kbh@gmail.com>, Natalie Rouse <avps.natalierouse@gmail.com>, Leslie Willis Woods <lwwoods@ucdavis.edu>

Hi Kathy and Natalie,
I did recut your samples from the beluga whale. Unfortunately, **I did not find intranuclear Polyomavirus or other DNA virus replication and assembly (intranuclear inclusion on H&E)**. The mammary gland is autolyzed, but the tissue preservation was fair to unequivocal identified a virus, if present. I did just see intranuclear accumulation of pleomorphic granular material of about 18 to 30 nm which suggest with Beta-glycogengranules (normally 20-40 nm). **You will be not charged for these recuts.** If you want to pursuit this, I will suggest asking the virologist to send me fresh isolate for virus identification by directEM and fixed cell with cytopathy effect for tissue EM examination to validate their findings. Let me know if this work for you. Best, Anibal

Anibal G. Armien, DVM, MSc, PhD, Diplomate, ACVP
Professor of Diagnostic Pathology, Electron Microscopy Section Head,
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School of Veterinary Medicine, University of California, Davis
620 W. Health Sciences Drive, Davis, CA 95616
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U.S. Secretary of Commerce
Gina M. Raimondo

Under Secretary of Commerce for Oceans and Atmosphere
Dr. Richard W. Spinrad

Assistant Administrator, National Marine Fisheries Service. Also serving as Acting Assistant Secretary of Commerce for Oceans and Atmosphere, and Deputy NOAA Administrator
Janet Coit

September 2021

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