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Authors: Saliki, Jeremiah T., Cooper, Emily J., Rotstein, David S., Caseltine, Shannon L., Pabst, D. Ann, et al.

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A Novel Gammaherpesvirus Associated with Genital Lesions in a Blainville's Beaked Whale (*Mesoplodon densirostris*)

Jeremiah T. Saliki,^{1,5,6} Emily J. Cooper,¹ David S. Rotstein,² Shannon L. Caseltine,¹ D. Ann Pabst,³ William A. McLellan,³ Pamela Govett,³ Craig Harms,³ Kara A. Smolarek,⁴ and Carlos H. Romero⁴

¹ Oklahoma Animal Disease Diagnostic Laboratory, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078, USA; ² Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37996, USA; ³ Department of Biology and Marine Biology, University of North Carolina, Wilmington, North Carolina 28403, USA; ⁴ Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610, USA; ⁵ Current address: Athens Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Georgia: Athens, Georgia 30602, USA;

⁶ Corresponding author (email: jsaliki@uga.edu)

ABSTRACT: An adult male Blainville's beaked whale (*Mesoplodon densirostris*) was found stranded on the Atlantic coast of the USA on 28 January 2004. Necropsy revealed a focal papilloma-like penile lesion, the cells from which revealed single 4–6 µm basophilic intranuclear inclusions. Total DNA extracted from lesion material was tested using a pan-herpesvirus PCR assay that targets the DNA polymerase gene and found to be positive. When the amplified DNA fragment was cloned, sequenced, and compared to GenBank-deposited herpesvirus DNA polymerase sequences, the detected virus was determined to be a distinct member of the *Gammaherpesvirinae* subfamily of herpesviruses. This new virus, tentatively named Ziphiid herpesvirus type 1, was associated with but not determined to be the cause of genital disease in the Blainville's beaked whale.

Key words: Beaked whale, gammaherpesvirus, genital papilloma, *Mesoplodon densirostris*, PCR.

The *Herpesviridae* family contains over 140 distinct viruses infecting a variety of birds, fish, mammals, and reptiles. The family is divided into three subfamilies named *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Some herpesvirus infections have not been associated with any clinical disease, while others have been associated with, or determined to be the cause of, a wide variety of conditions in their host species. Herpesviruses tend to be host specific, and most animal species have a distinct herpesvirus. Few reports have described the occurrence of herpesviruses in cet-

ceans. Herpesvirus or herpes-like infections have been associated with encephalitis in a harbor porpoise (*Phocoena phocoena*) in Sweden (Kennedy et al., 1992), dermatitis in a beluga whale (*Delphinapterus leucas*) in Canada (Martineau et al., 1988; Barr et al., 1989), and skin lesions in dusky dolphins (*Lagenorhynchus obscurus*) in the South Pacific (Van Bressem et al., 1994). Recently two apparently distinct alphaherpesviruses were found to be associated with fatal disseminated infections in two Atlantic bottlenose dolphins (*Tursiops truncatus*) off the east coast of the USA (Blanchard et al., 2001). This report describes the detection of a novel gammaherpesvirus in a genital lesion from a stranded Blainville's beaked whale (*Mesoplodon densirostris*).

An adult male Blainville's beaked whale (*M. densirostris*) (WAM 593), measuring 423 cm in length and weighing 940 kg, was found stranded at Kure Beach, North Carolina, USA (34°01'00"N, 77°54'30"W) on 28 January 2004 in clear weather with an air temperature of approximately 14 C. The carcass was fresh at the time of discovery and was graded with a Smithsonian Condition Code of 2 (Geraci and Lounsbury, 1993). A complete necropsy examination was performed. Selected tissues were fixed in 10% neutral buffered formalin, routinely paraffin embedded, sectioned at 4 µm, and stained with hematoxylin and eosin for microscopic

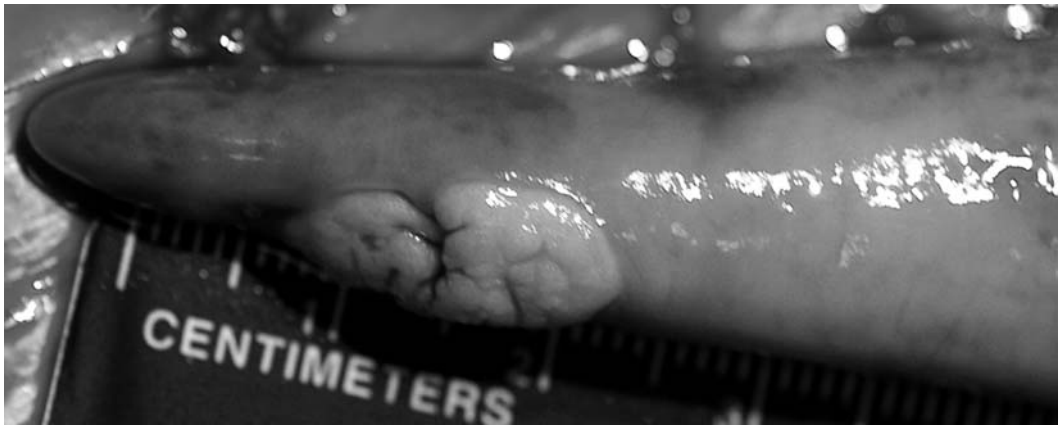


FIGURE 1. Penile mass of Blainville's beaked whale (*M. densirostris*); gross appearance.

examination. Gross findings were observed in the hepatobiliary, urogenital, integumentary, respiratory, and musculoskeletal systems. Specifically, there were thin-walled, multiloculated cavities ob-

served in the liver, subcapsular hemorrhage in the right and left kidneys, pulmonary hemorrhage, ventral reddening (lividity), and multiple green stick fractures. The most remarkable gross finding

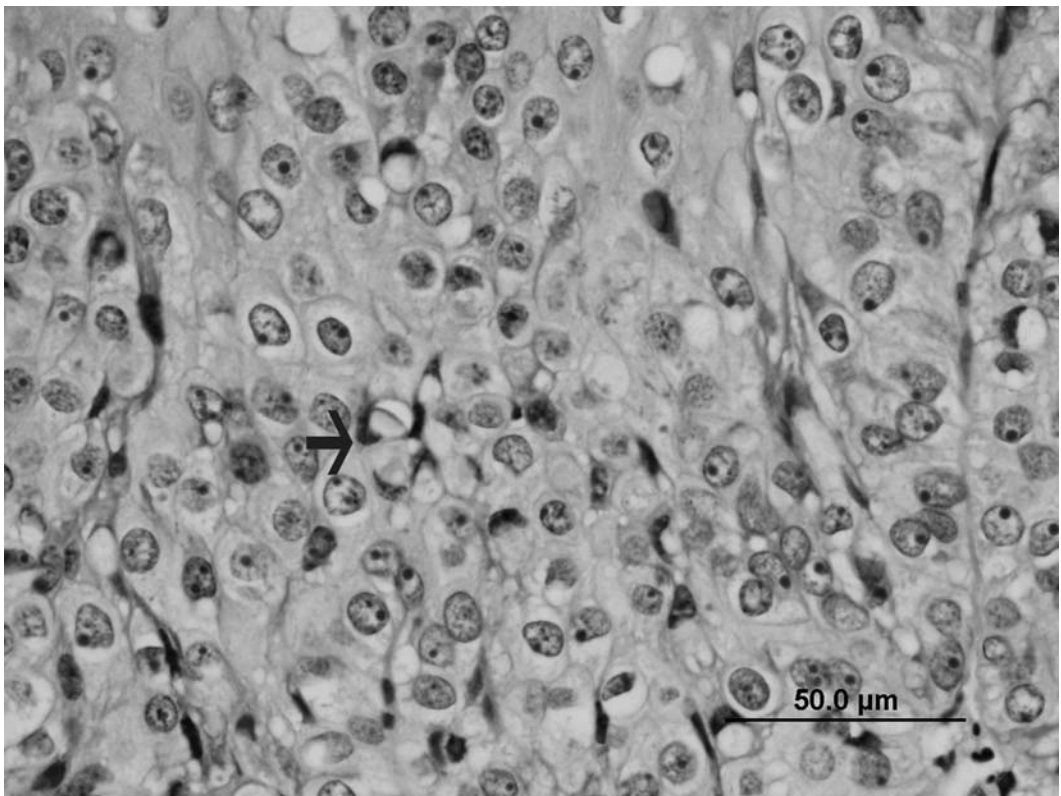


FIGURE 2. Penile mass of Blainville's beaked whale (*M. densirostris*). Epithelial cells are hyperplastic with prominent nucleoli. Occasional cells contain acidophilic inclusions (arrow). H&E. Bar=50 μ m.

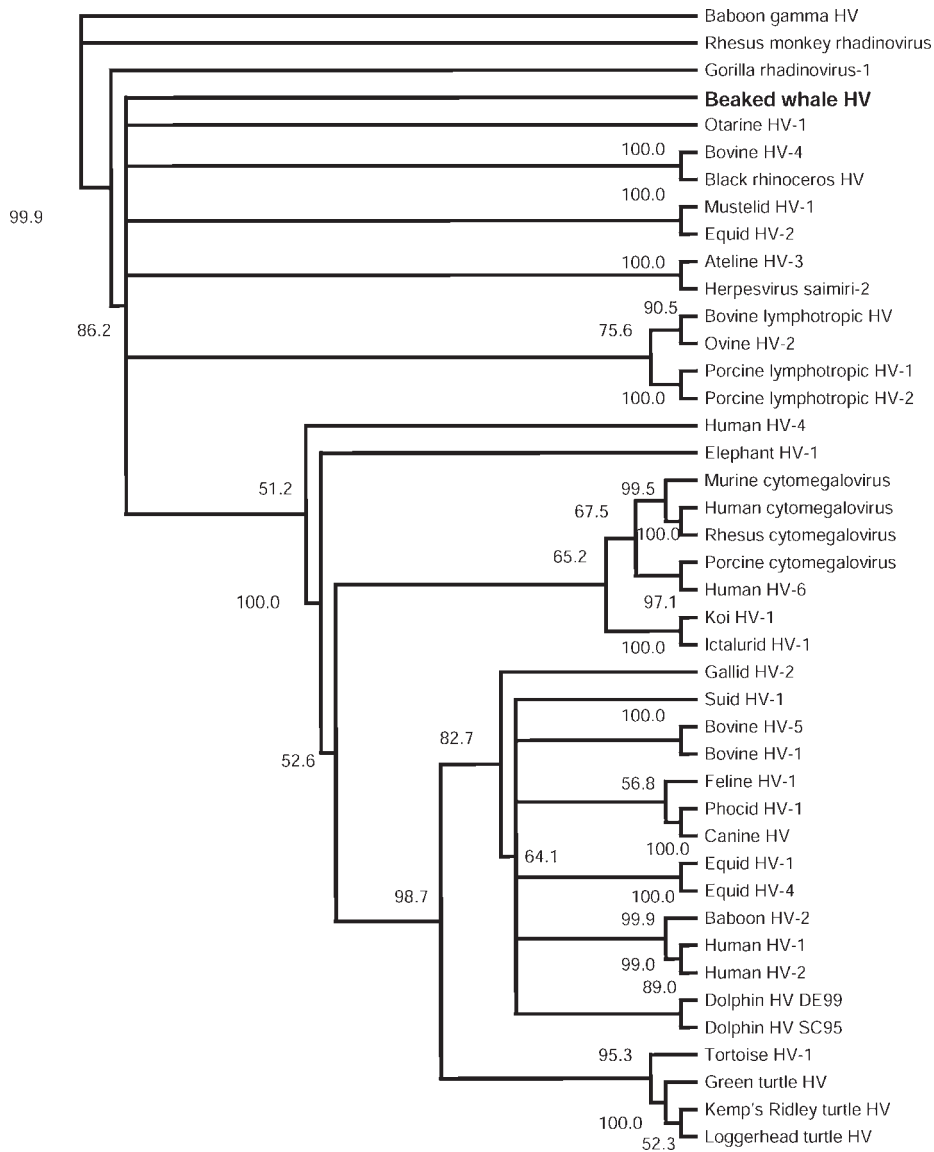


FIGURE 3. Neighbor-joining phylogenetic tree of the deduced amino acid sequences of the herpesvirus DNA polymerase gene fragment. The tree was generated by PAUP 4.0 software using ClustalW slow and accurate function and Gonnnet 250 residue weight table, gap penalty of 11, gap extension penalty of 0.2, and 1,000 bootstrap replications. The GenBank accession numbers of the DNA polymerase gene fragments used for the construction of this tree are the following: *Alphaherpesvirinae* subfamily: AF196646–Dolphin herpesvirus (HV) SC95, AF245443–Dolphin HV DE99, NC_001847–Bovine HV-1, NP_954917–Bovine HV-5, NC_006151–Suid HV-1, AJ224971–Feline HV-1, U92269–Phocid HV-1, AY949827–Canine HV, AY464052–Equid HV-1, AF030027–Equid HV-4, AB070848–Human HV-1, AY038367–Human HV-2, AY168637–Baboon HV-2, AF147806–Gallid HV-2, AB047545–Tortoise HV-1, AY646888–Loggerhead turtle HV, AY646894–Kemp's Ridley turtle HV, AF239684–Green turtle HV. *Gammapherpesvirinae* subfamily: AF236050–Otarine HV-1, AJ507799–Human HV-4, AY949828–Blainville's beaked whale HV, AF083424–Ateline HV-3, NC_001350–Herpesvirus saimiri-2, AF250886–Gorilla rhadinovirus-1, AY270026–Baboon gamma HV, AF029302–Rhesus monkey rhadinovirus, NC_002665–Bovine HV-4, AF287948–Black rhinoceros HV, AF376034–Mustelid HV-1, NC_001650–Equid HV-2, AF327830–Bovine lymphotropic HV, AF327831–Ovine HV-2, AF118399–Porcine lymphotropic HV-1, AF118401–Porcine lymphotropic HV-2.

was a 1.5-cm-in-diameter, raised, tan, fleshy mass present near the tip of the penis (Fig. 1). Histologically, this mass was composed of hyperplastic to dysplastic epithelial cells forming deeply penetrating dermal cords. Affected cells had single 4–6 μm basophilic intranuclear inclusions (Fig. 2). Lymphocytes and plasma cells were present at the epidermal-dermal junction in the affected region and in the remaining penis. Other histological findings included pulmonary hemorrhage, erosive cystitis, renal interstitial hemorrhage, verminous colitis, intramysial protozoa, testicular atrophy, and hepatic venular ectasia.

Based on the association of herpesviruses with skin lesions in other marine mammal species (Barr et al., 1989; Van Bressem et al., 1994), an attempt was made to detect herpesvirus in the penile lesion by virus isolation and polymerase chain reaction (PCR). About 0.5 g of fresh unfixed tissue was homogenized in 4.5 ml of minimum essential medium to obtain a 10% suspension of the tissue. After centrifuging at $1,000 \times G$ for 10 min, the supernatant was used for DNA extraction and for inoculation of primary dolphin skin and kidney cell lines (kindly provided by Bobby Middlebrooks, University of Southern Mississippi, Hattiesburg, Mississippi, USA). Inoculated cells were examined for evidence of virus-induced cytopathic effects for 8 days and subcultured twice for 2 additional 8-day passages. For PCR, DNA was extracted from the tissue suspension using the High Pure PCR Template Kit (Roche Diagnostics Corporation, Indianapolis, Indiana, USA, catalog no. 1796828) following the manufacturer's protocol for mammalian tissue.

A nested PCR assay was performed with

two forward and one reverse primers in the first reaction and one forward and one reverse primer in the second reaction (VanDevanter et al., 1996; Ehlers et al., 1999). Primer sequences for the first reaction were FP1-5'-GAY TTY GCI AGY YTI TAY CC-3', FP2-5'-TCC TGG ACA AGC AGC ARI YSG CIM TIA A-3', RP1-5'-GTC TTG CTC ACC AGI TCI ACI CCY TT- 3'. Primers for the second reaction were FP3-5'-TGT AAC TCG GTG TAY GGI TTY ACI GGI GT-3', RP2-5'-CAC AGA GTC CGT RTC ICC RTA IAT-3'. These primers are known to direct the amplification of DNA polymerase gene fragments 215–235 bp in length for most herpesviruses and 315 bp for cytomegaloviruses. Total DNA preparations extracted from cell monolayers of Madin Darby canine kidney (MDCK) cultures infected with canine herpesvirus 1 (CHV-1) and from cell monolayers of eastern box turtle (*Terrapene carolina*) heart (TH-1) cell cultures infected with tortoise herpesvirus 1 (THV-1) were used as positive control templates for PCR. In the first PCR, approximately 500 ng of the lesion DNA was used as an amplification template. The first PCR mixture contained 400 nM of each primer, 100 mM of each dNTP, 10 mM KCl, 10 mM $(\text{NH}_4)_2 \text{SO}_4$, 20 mM Tris-HCl, 2 mM MgSO_4 , 0.1% Triton X-100 at pH 8.8, and 1 unit of Taq DNA polymerase (New England BioLabs, Ipswich, Massachusetts, USA). Cycling conditions for the first and second PCR were the following: initial incubation at 94 C for 2 min followed by 55 cycles at 94 C for 20 sec, 46 C for 30 sec, and 72 C for 30 sec. A final extension step at 72 C for 10 min finished the cycling. In the second PCR, mixtures were identical to those in the first PCR but contained

←

Betaherpesvirinae subfamily: AF268040–Porcine cytomegalovirus, NC_001664–Human cytomegalovirus, AY186194–Rhesus cytomegalovirus, P27172–Murine cytomegalovirus, CAA58372–Human HV-6. Unclassified viruses: AF322977–Elephant HV-1, AY572853–Koi HV-1, NC_001493–Ictalurid HV-1.

400 nM of each of the second reaction primers and 2 μ l of the first PCR product as DNA template. Approximately 20 μ l from the second PCR were resolved by horizontal gel electrophoresis in 1.0% agarose containing ethidium bromide (0.5 μ g/ml) and the DNA fragments visualized by UV light transillumination and photographed using a gel documentation system (Bio-Rad Laboratories, Hercules, California, USA).

Amplified DNA fragments of the expected size (220 bp) were cloned into the plasmid vector pCR2.1 TOPO T/A (Invitrogen, Carlsbad, California, USA) and sequenced (100 fmol) with the forward and reverse M13 primers, using the CEQ 2000 XL (Beckman Coulter, Fullerton, California, USA) sequencing instrument, following the manufacturer's protocol. Sequences were reviewed manually using the Chromas 2.3 software (Technelysium, Tewantin, Queensland, Australia) and exported into the Seqed function of the University of Wisconsin Package version 10.2, Genetics Computer Group (GCG), Madison, Wisconsin, USA. Sequences were analyzed using the Gap, Translate, and Lineup functions of this software and assembled using SeqMan, SeqEd, and MegAlign (DNASTar, Lasergene software, Madison, Wisconsin, USA). The BLAST Local Alignment Search Tool of the National Center for Biotechnology Information, Bethesda, Maryland, USA, was used to identify herpesvirus sequences more closely related to those of the Blainville's beaked whale herpesvirus. A phylogenetic tree was generated by the ClustalW (<http://www.ebi.ac.uk/clustalw>) slow and accurate function using a Gonnet residue weight table, gap penalty of 11, and gap extension penalty of 0.2 (Ganova-Raeva et al., 2004). The tree was based on the amino acid sequences deduced from DNA polymerase gene fragments from 41 members of the alpha, beta, and gamma subfamilies of herpesviruses. Trees of this DNA polymerase region generated with other algorithms (e.g., nearest neighbor)

do not always accurately assign known viral sequences to the established subfamilies (VanDevanter et al., 1996).

The virus isolation attempt did not yield any virus. The nested PCR assay targeting the DNA polymerase gene of herpesviruses detected herpesvirus genome sequences in total DNA from the genital lesion. DNA fragments were of the expected molecular size (220 bp) and were similar in size to homologous fragments amplified from the extracted DNA of CHV-1 and THV-1 (data not shown). Sequences from two DNA fragments from the beaked whale lesion were identical, 221 nucleotides in length, and translated into a protein of 73 amino acids, beginning from the third nucleotide of the top DNA strand. Comparisons with homologous sequences obtained from the GenBank database using the Gap function of the GCG software demonstrated that the Blainville's beaked whale herpesvirus DNA polymerase gene fragment was most closely related to the California sea lion gammaherpesvirus, with which it shared identities of 58.8% and 61.9% at the nucleotide and amino acid levels, respectively. Similar comparisons to the only two GenBank-deposited homologous sequences from cetacean alphaherpesviruses (Blanchard et al., 2001) uncovered identities of 48.9% and 36.8% at the nucleotide and amino acid levels, respectively, for one virus (GenBank accession number AF196646), and 40.8% and 34.5% for the second virus (AF245443). The herpesvirus DNA polymerase gene fragment sequence of the Blainville's beaked whale has been deposited in the GenBank database under accession number AY803337. Phylogenetic analysis of multiple amino acid sequence alignments using 41 members of the *Herpesviridae* analyzed by the slow and accurate ClustalW method showed that the Blainville's beaked whale herpesvirus belongs in the *Gammaherpesvirinae* subfamily of herpesviruses (Fig. 3).

The data presented in this report confirm the detection of a novel gamma-

herpesvirus associated with genital lesions in a cetacean, Blainville's beaked whale (*M. densirostris*). There may be two reasons for the negative virus isolation results. First, the cultured sample had been frozen and thawed multiple times before testing, and this could have rendered any virus present in the sample to become nonviable. Second, the cell cultures used might not have been sensitive to this new virus. The PCR assay used in this study relies on the knowledge that the functional domains of the DNA-directed DNA polymerase enzyme of eukaryotes and some DNA viruses are highly conserved (Ito and Braithwaite, 1991; Heringa and Argos, 1994). Analysis of the amplified 221-bp DNA fragment of the polymerase gene clearly identified the herpesvirus present in the genital lesion as a novel member of the *Gammaherpesvirinae* subfamily, within the *Herpesviridae* family. This virus is tentatively designated Ziphiid herpesvirus type 1 (ZHV-1). The ZHV-1 is clearly distinct from two previously identified alphaherpesviruses of cetaceans (Atlantic bottlenose dolphins) that were associated with fatal disseminated infections (Blanchard et al., 2001), as shown by nucleotide and amino acid sequence analysis, and by its clading with other members of the *Gammaherpesvirinae* subfamily on the phylogenetic tree (Fig. 3). The new virus is also distinct from the two known marine gammaherpesvirus described in harbor seals (Harder et al., 1996) and California sea lions (Lipscomb et al., 2000; King et al., 2002).

Of the two marine gammaherpesviruses that have been molecularly characterized, the seal gammaherpesvirus, phocid herpesvirus-2, has not been associated with any known clinical condition (Harder et al., 1996), whereas the California seal lion virus, Otarine herpesvirus 1, has been clearly associated with urogenital carcinomas (Lipscomb et al., 2000; King et al., 2002). Although herpesvirus or herpesvirus-like particles have been associated

with skin disease in other cetacean species (Martineau et al., 1988; Barr et al., 1989; Van Bresseem et al., 1994), no phylogenetic information is available from those cases. In this report, the presence of herpes-like intranuclear inclusions and detection of gammaherpesvirus genomic sequences from the penile lesion suggest that the ZHV-1 is associated with the observed lesion. However, because no cause-effect relationship was established between this herpesviral infection and the stranding of the beaked whale, it is not possible to determine if the virus contributed to the stranding of the animal. Nevertheless, the data herein, together with data from previous reports (Lipscomb et al., 2000; King et al., 2002), suggest that gammaherpesviruses might be possible causes of genital disease in some pinnipeds and cetaceans. For Blainville's beaked whales, the finding of a possible sexually transmitted disease warrants the screening of stranded members of the species to determine the prevalence of ZHV-1 and other possible herpesviruses. The previously described PCR assay used in this study is known to detect most members of the *Herpesviridae* family and would be a good tool for this purpose.

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