DOI: 10.7589/2017-09-224

Journal of Wildlife Diseases, 54(3), 2018, pp. 592–596

Identification of Novel Gammaherpesviruses in a South American Fur Seal (*Arctocephalus australis*) with Ulcerative Skin Lesions

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ABSTRACT: There are few studies on pathogens affecting free-ranging pinnipeds from South America. We employed molecular techniques to identify a gammaherpesvirus infection by two putative novel herpesvirus species: *Otariid herpesvirus* 5 (OtHV-5), possibly associated with ulcerative cutaneous lesions, and *Otariid herpesvirus* 6 (OtHV-6) in a wild South American fur seal (*Arctocephalus australis*) that stranded alive in Santa Catarina state, southern Brazil. Here we provide new information regarding pinniped herpesviruses, important for the design of future disease surveillance studies.

Key words: Brazil, glycoprotein B, herpesvirus, pathology, pinnipeds, ulcerative skin lesions, virology.

Herpesviruses are linear, enveloped, and large double-stranded DNA viruses within the *Herpesviridae* family (McGeoch et al. 2006). At least thirteen different herpesviruses have been described in captive, rehabilitating, or free-ranging pinnipeds in Europe and North America: one (in phocids) from the *Alphaherpesvirinae* subfamily (Harder et al. 1996) and twelve (seven in phocids, four in otariids, and one in odobenids) from the *Gammaherpesvirinae* subfamily (Melero et al. 2014; Bodewes et al. 2015; Wright et al. 2015; Bellehumeur et al. 2016). The *Otariid herpesvirus* 1 (OtHV-1) has been associated with urogenital carcinoma in a captive South American fur seal (*Arctocephalus australis*) and in California sea lions (*Zalophus californianus*) under rehabilitation (King et al. 2002; Dagleish et al. 2013). The OtHV-3 was identified in California sea lions in an individual with esophageal ulcers and B-cell lymphoblastic lymphoma, in an animal with T-cell lymphoma, in healthy wild animals, and in rehabilitating individuals with pneumonia (Venn-Watson et al. 2012). Both OtHV-2 and OtHV-4 were detected in ocular lesions of California sea lions (Maness et al. 2011; Wright et al. 2015). A new gammaherpesvirus sequence also named OtHV-4 has been recently identified in vaginal swabs of apparently healthy northern fur seals (*Callorhinus ursinus*) from Alaska and California, despite a high similarity (95% nucleotide identity in the polymerase and glycoprotein B genes) with OtHV-1 (Cortés-Hinojosa et al. 2016). Here we report herpesvirus infection in a wild South American fur seal, identifying two novel gammaherpesvirus species, one of them possibly associated with ulcerative skin disease.

A juvenile male South American fur seal (FAI249) stranded alive in Florianópolis (27°45′13″S, 48°29′59″W), Santa Catarina state, southern Brazil, on 21 October 2015. The animal was rescued by Associação R3 Animal through the Coastal Monitoring Program–Santos Basin, operated by the Brazilian Institute of the Environment and Renewable Natural Resources and Petróleo Brasileiro S.A. (PETROBRAS). The animal died during transport and was immediately necropsied. Tissue samples were placed in 10% buffered formalin and frozen at –20 C. Formalin-fixed tissues were routinely processed, sectioned at 5 µm, and stained with H&E.

We extracted DNA from manually homogenized frozen tissues (skin, lung, spleen, stomach, pancreas, and intestines) using the DNeasy Blood and Tissue Kit (Qiagen®, Valencia, California, USA), following the

manufacturer's specifications. Due to the macroscopic similarities between the observed ulcerative skin lesions and those described by Goldstein et al. (2006) in herpesvirus infection, we performed a universal herpesvirus PCR to amplify DNA polymerase (VanDevanter et al. 1996) and terminase genes (Hargis et al. 1999), and an assay to detect a 500 base pair fragment of gammaherpesvirus glycoprotein B (Ehlers et al. 2008). Positive samples were confirmed by direct sequencing.

Phylogenetic analysis of the obtained sequences was performed by selecting the 151 glycoprotein B gene deduced amino acid sequences we obtained, previously detected pinniped herpesvirus, alpha-, beta-, and gamma-herpesviruses identified in other taxa sequences presenting at least the same length (100% coverage) available at GenBank, and the *Ictalurid herpesvirus* 1 as the outgroup. Glycoprotein B gene multiple sequence alignment was made by MUSCLE algorithm (Edgar 2004), as implemented in MEGA version 7.0 (Kumar et al. 2016), and an amino acid maximum likelihood tree of 1,000 bootstrap replicates was subsequently generated with MEGA version 7.0 (Kumar et al. 2016).

The animal was in poor body condition, weighing 12.7 kg. Grossly, several multifocal, circumscribed, light-tan-colored, ulcerative skin lesions approximately 0.5 cm in diameter were observed on the fore and rear limbs (Fig. 1). The animal also had pale spleen and oral mucosa, an empty stomach with mucosal congestion, and foamy fluid in the tracheal lumen. Histologically, skin lesions presented predominantly fibrinonecrotic vasculitis and moderate to severe, multifocal to coalescent, acute, fibrinonecrotic dermatitis, caused mainly by neutrophils with fewer macrophages, lymphocytes, and plasmatic cells. No herpesvirus inclusion bodies were observed (Fig. 1). Other significant histopathological findings were observed in the lungs (mild to moderate diffuse congestion), liver (mild to moderate loss of the normal cord-like arrangement of hepatocytes with mild to moderate diffuse congestion and mild to moderate diffuse hydropic degeneration), lymph nodes (moderate lymphoid hyperplasia with expansion of the germinal center and mantle, expansion and confluence of the lymphoid follicles in the lymph node), spleen (mild to moderate lymphocytolysis of the mantle, mild to moderate hyperplasia of lymphoid follicle and expansion of the germinal center), and intestine (moderate diffuse lymphoplasmacytic enteritis). No significant findings were observed in the pancreas, muscle, heart, brain, thyroid, adrenal glands, and bladder.

The PCRs for the DNA polymerase and terminase genes produced weak bands, which we were unable to sequence. However, strong bands were obtained with the glycoprotein B PCR from skin lesion, spleen, stomach, intestine, and lung samples. We obtained two different sequences of 453 base pair (excluding primers): one from skin lesion and spleen samples, and another from samples of stomach and intestines. The novel herpesvirus glycoprotein B sequences were submitted to GenBank under accession numbers MF496135 and MF924392.

The two novel herpesvirus sequences obtained from skin and spleen samples, and from samples of stomach and intestines were classified as gammaherpesviruses (Fig. 2) with, respectively, the highest glycoprotein B gene nucleotide (86.5% and 86.8%) and amino acid (95.4% and 96.0%) identities to the herpesvirus obtained from a blood sample of a harp seal (*Pagophilus groenlandica*) from Canada (KP136799.1). When compared, both South American fur seal sequences presented 83% nucleotide and 92.7% amino acid identities. The two herpesvirus sequences obtained in this study clustered with other pinniped herpesvirus sequences and members of the genus *Percavirus* obtained from carnivores (Fig. 2).

One gammaherpesvirus was amplified from a skin ulcer. Cutaneous lesions are usually observed in gammaherpesvirus infections in members of the suborder Caniformia (Goldstein et al. 2006; Gagnon et al. 2011; Bodewes et al. 2015). Intranuclear inclusion bodies

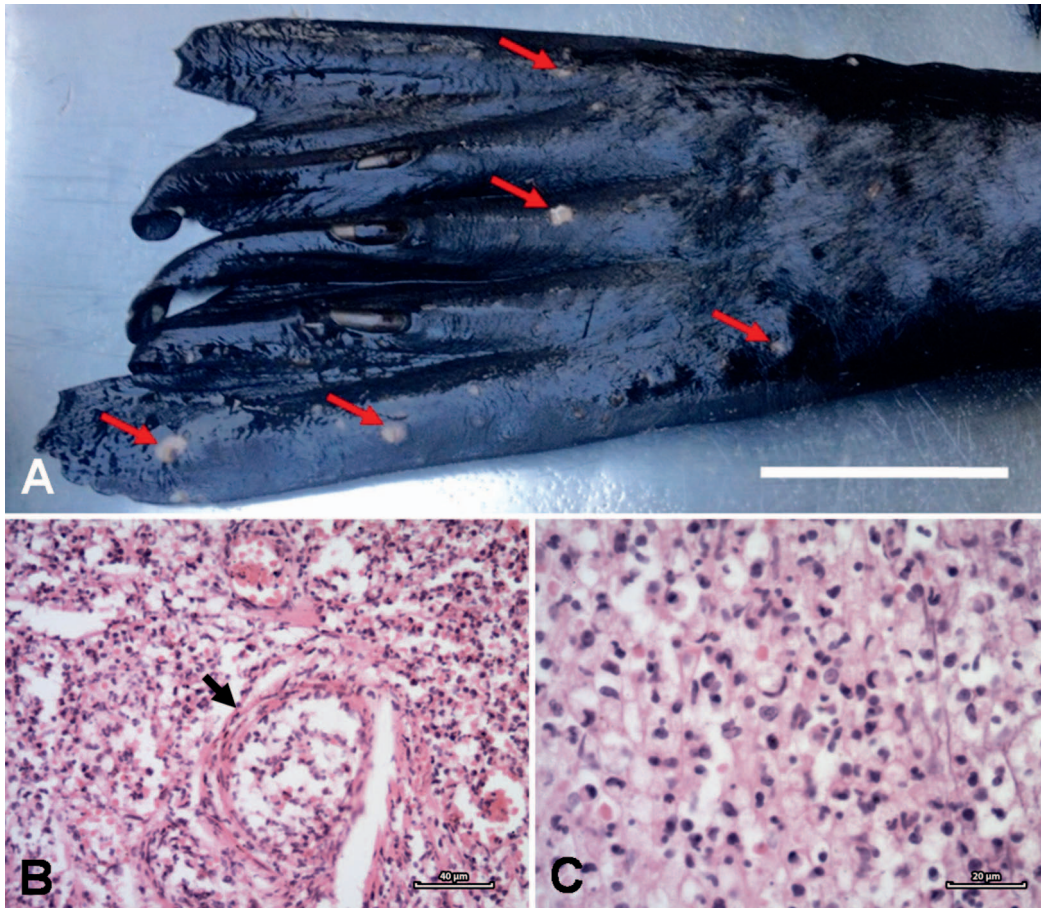

FIGURE 1. Skin lesions in a South American sea lion skin (*Arctocephalus australis*) that stranded in October 2015 along the coast of Santa Catarina state, Brazil. (A) Ulcerative skin lesions seen grossly (red arrow). Bar=5 cm. (B) Fibrinonecrotic vasculitis. H&E stain. (C) Mixed inflammatory infiltrate, composed mainly of neutrophils. H&E stain.

(INIBs) were not observed in the skin lesion upon histopathology, which does not rule out herpesvirus infection. The absence of INIBs has been described in advanced herpesviral infections (Caswell and Williams 2007). Additionally, intranuclear inclusion bodies were not observed in harbor seals infected by gammaherpesvirus, either in oral nor in skin ulcers (Bodewes et al. 2015) nor in tissues from infected animals during a PhHV-1 outbreak (Borst et al. 1986). Although we were not able to exclude an asymptomatic or a concomitant infection (e.g., by calicivirus), our findings supported the hypothesis that the gammaherpesvirus herein identified could have been involved in the development of the observed skin lesions, despite the lack of INIBs. The association between the herpesviruses identified in this study and the lesions observed in the spleen, stomach, and intestines remains unclear.

Our novel sequences have considerable differences between them and to the conserved region of the herpesvirus glycoprotein B sequence of a harp seal from Canada. Based on the International Committee on Taxonomy of Viruses criteria (Pellett et al. 2012), and their distinct gene sequence composition, we suggest two novel herpesvirus species tentatively named OtHV-5 and OtHV-6. The phylogenetic tree showed that these novel herpesviruses are probably members of the

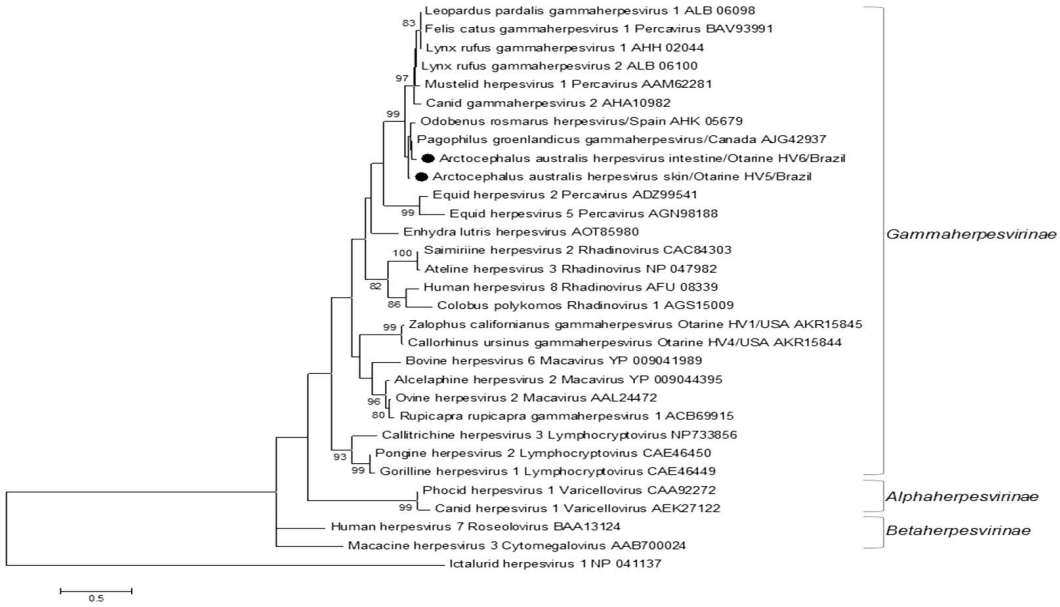

FIGURE 2. Maximum-likelihood phylogram using the MEGA7 (Kumar et al. 2016) of the alignment 151 deduced amino acid sequences of the herpesviral DNA glycoprotein B strain found in this study (circle), 28 alpha-, beta-, and gammaherpesvirus sequences obtained from GenBank, and the *Ictalurid herpesvirus 1* (GenBank accession no. NP–041137) as outgroup. Taxon names are presented as herpesvirus species, genus (when assigned), country of origin (for herpesvirus sequences detected in pinnipeds), and accession number. The reliability of the tree was tested by bootstrap analyses with 1,000 bootstrap replicates. Bootstrap values lower than 70% were omitted. The scale bar indicates the number of substitutions per site.

Percavirus genus, *Gammaherpesvirinae* subfamily. Another gammaherpesvirus (OtHV-1) has been previously identified in a wild-born South American fur seal; however, this individual could have been infected by California sea lions while in captivity (Dagleish et al. 2013).

We thank Janaina Lorenço, Marzia Antonelli, and staff of Associação R3 Animal for their collaboration and support. We also thank Cíntia Maria Favero, Marco Aurélio Gattamorta, Silmara Rossi, Pedro Enrique Navas Suárez, Sândara Sguario, and Jorge Oyakawa at the Universidade de São Paulo for their technical support and assistance. This study was funded by the Coordination for the Improvement of Higher Level Personnel (CAPES) and PETROBRAS through the Beach Monitoring Project of the Santos' Basin (PMP-BS) project. The authors declare this study presents no conflicts of interest.

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Submitted for publication 11 September 2017.
Accepted 20 December 2017.