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Novel herpesviruses in riverine and marine cetaceans from South America

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ABSTRACT

Herpesvirus (HV) infections in cetaceans are frequently associated with skin and mucosal lesions. Although HV infections have been reported worldwide, their occurrence in southern Atlantic marine mammals is still poorly understood. We tested skin, oral and genital mucosal beta-actin PCR-positive samples from 109 free-ranging Brazilian cetaceans using a universal herpesvirus DNA polymerase PCR. Herpesvirus-positive skin samples from a Guiana dolphin (*Sotalia guianensis*), a dwarf sperm whale (*Kogia sima*), a Bolivian river dolphin (*Inia boliviensis*), and a lingual sample from an Atlantic spotted dolphin (*Stenella frontalis*) were histologically evaluated. Additional tissue samples from these animals were also PCR-positive for HV, including a novel sequence obtained from the dwarf sperm whale's stomach and mesenteric lymph node. Four novel HV species were detected in the Guiana dolphin (one), the dwarf sperm whale (two) and the Bolivian river dolphin (one). The cutaneous lesions (marked, focally extensive, chronic proliferative dermatitis) of the Guiana dolphin and the Bolivian river dolphin were similar to previous HV reports in cetaceans, despite the absence of intranuclear inclusion bodies. This is the largest HV survey in South American cetaceans and the first detection of HV infection in riverine dolphins worldwide.

1. Introduction

Dermatopathology is a valuable diagnostic tool to assess and monitor the health status of cetaceans; direct observation of cutaneous lesions (sometimes the only available resource in field studies) can reveal systemic and/or infectious diseases (Pettis et al., 2004; Sacristán et al., 2018a). Herpesviruses (HVs) are able to induce cutaneous and mucosal lesions in mammals, and may cause persistent and latent infections potentially activated by host immunosuppression, with periodic or continuous shedding of infectious virus (Siegal et al., 1981; Roizmann et al., 1992). These viruses are comprised within the family *Herpesviridae*, order *Herpesvirales*, formed by single, linear, double-stranded DNA viruses, currently subdivided into *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* subfamilies (ICTV, 2017).

Martineau et al. (1988) reported the first HV infection in cetaceans 30 years ago, detected by electron microscopy in skin lesions of beluga whales (*Delphinapterus leucas*). With the advent of molecular diagnostics, Blanchard et al. (2001) identified the first cetacean HV DNA sequences in stranded Atlantic bottlenose dolphins (*Tursiops truncatus*). Since then, HV sequences have been identified in at least six odontocete

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families: Delphinidae, Kogiidae, Ziphiidae, Physeteridae, Monodontidae, and Phocoenidae (Smolarek-Benson et al., 2006; Arbelo et al., 2010; Miyoshi et al., 2011; Bellehumeur et al., 2015; Van Beurden et al., 2015); and one mysticete family, the Balaenopteridae (Melero et al., 2015). To date, all identified cetacean HVs were classified either into *Alphaherpesvirinae* or *Gammaherpesvirinae* subfamilies (Davison et al. 2017a, b). Although some HV infections have not been linked to clinical disease (Bellière et al., 2010; Casalone et al., 2014; Van Elk et al., 2016), both alphaherpesvirus (α-HV) and gammaherpesvirus (γ-HV) can cause cutaneous and mucosal lesions in cetaceans (Saliki et al., 2006; Smolarek-Benson et al., 2006; Van Elk et al., 2009). In addition, α-HVs have been associated with severe localized and fatal systemic infections in cetaceans (Blanchard et al., 2001; Arbelo et al., 2010; Arbelo et al., 2012; Burek-Huntington et al., 2015), as well as encephalitis and meningoencephalitis in the same clade (Sierra et al., 2014; Van Elk et al., 2016).

Herpesviruses have been identified in cetaceans worldwide; in the Mediterranean Sea, northwestern and northeastern Atlantic Ocean, Sea of Japan, and northwestern, central, northeastern, and southeastern Pacific Ocean (Martineau et al., 1988; Van Bressem et al., 1994; Esperón et al., 2008; Bellière et al., 2010; Miyoshi et al., 2011; West et al., 2013; Burek-Huntington et al., 2015). In South America, HV-like particles were detected ultrastructurally in a dusky dolphin (*Lagenorhynchus obscurus*) from Peru presenting few black dots on its rostrum (Van Bressem et al., 1994); however, to the authors' knowledge, there is only one report of HV molecular detection in South American cetaceans (Seade et al., 2017). This study evaluated the presence of HV in skin, oral and genital mucosal samples from cetaceans of Brazil, including riverine and marine species.

2. Material and Methods

2.1. Samples

We evaluated 166 frozen samples (skin, oral and genital mucosa) from 115 individuals of seven cetacean families. Out of 115 animals, 112 were found stranded or bycaught along the Brazilian coast between 2005 and 2015, and three were riverine dolphins species assessed during capture and release expeditions in 2015 (one Amazon river dolphin [*Inia geoffrensis*] from Rio Negro and two Bolivian river dolphins [*I. boliviensis*] from Rio Guaporé). Additional data on the number of evaluated specimens, species, sex, age class, and tissue type are listed in Table S1. Necropsies were performed following standard procedures (Geraci and Lounsbury, 2005). Selected tissue samples were collected and fixed in 10% formalin, preserved in 70% ethanol or frozen at -20 °C or −80 °C. Carcass and tissue conditions were established according with Geraci and Lounsbury (2005), that classified from code 1 (alive or just died) to code 5 (mummified or skeletal remains). All procedures, for both necropsies and capture and release activities, were performed according to the Ethical Committee of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (process number: 2951280914).

2.2. Molecular diagnostics

After manually homogenization by sterile surgical blades, total DNA extraction from frozen skin and mucosal samples was performed with the DNeasy Blood & Tissue kit (Qiagen®, Valencia, CA, USA), following the manufacturer's protocol. To validate the DNA extraction, we tested the housekeeping beta-actin gene PCR assay with the primers described by Behrens et al. (1998), at a melting temperature of 55 °C. Validated DNA samples (beta-actin positive) were subsequently analyzed by the universal HV PCR protocol described by Vandevanter et al. (1996) for DNA polymerase gene. In HV-positive cases, additional available tissues aside from skin, and oral or genital mucosal were subsequently evaluated by the above described method (Table 1, Table S2). Positive samples were confirmed through direct sequencing of amplicons, generating an approximately 200 bp-long fragment, excluding primer sequences. In an attempt to obtain longer sequences, following the external PCR techniques described by Vandevanter et al. (1996), the positive cases were tested by heminested PCRs using different combinations of external and internal primers described by the same authors: DFA (external forward) and IYG (internal reverse), ILK (external forward) and IYG (internal reverse), TGV (internal forward) and KG1 (internal reverse). Additionally, sequences of γ-HV were also specifically screened for glycoprotein B subfamily detection (Ehlers et al., 2008). Proliferative skin and mucosal lesions were additionally tested for the presence of papillomavirus using conventional PCRs with different primer sets that amplify segments of the E1 or F1 papillomavirus-genes (Forslund et al., 1999; Iftner et al., 2003; Rector et al., 2005). Diethylpyrocarbonate (DPEC) water was used as negative control, while Magellanic penguin (*Spheniscus magellanicus*) and chamois (*Rupicapra rupicapra*) (KJ720217 and KP260923) herpesvirus-positive respiratory tissue samples were used as positive controls of alpha- and gammaherpesvirus, respectively. A human papillomavirus-positive sample was selected as positive control for papillomavirus PCRs.

The DNA polymerase multiple sequence alignment was made by MUSCLE algorithm (Edgar, 2008), selecting the deduced amino acid (aa) DNA polymerase gene sequences we obtained, and previously detected cetacean herpesvirus and α-, β- and γ-HV aa sequences from different animal families with at least the same length (100% coverage) available on GenBank. After that, a maximum likelihood phylogenetic tree of 1000 bootstrap replicates was generated, using *Salmonid herpesvirus* 3 as outgroup. Sequence identities were calculated based on the p-distance. Sequence analyses were performed with MEGA software, version 6.0. (Tamura et al., 2013).

2.3. Histological examination

Histological evaluation was performed by light microscopy in formalin-fixed tissues embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin-eosin.

3. Results

3.1. Molecular findings

Beta-actin was amplified in 145 of 151 evaluated skin samples and 15 of 15 oral or genital mucosal samples, corresponding to 109 of 115 individuals.

Overall, HV DNA was detected in 4.8% (7/145) of the skin- and 6.7% (1/15) of the oral or genital mucosal beta-actin positive samples, corresponding to 3.7 % (4/109) of the analyzed beta-actin positive animals: a Guiana dolphin, a dwarf sperm whale (*Kogia sima*), a Bolivian river dolphin, and an Atlantic spotted dolphin (*Stenella frontalis*). Additional information regarding each positive individual is shown in Table 2.

Additional HV sequences were found in other tissues, aside from the skin, in the Guiana dolphin (in kidney, liver and blood, the same sequence previously reported in the skin of this animal), the dwarf sperm whale (coinfected with two different HVs, one found in the lung, liver and intestine, identical to the sequence identified in the skin, and one novel sequence in the stomach and mesenteric lymph node) and the Bolivian river dolphin (blood, a sequence identical to that found in this animal's skin) (Table 1). The five different nucleotide (nt) DNA polymerase gene fragment sequences ranged from 206 to 207 bp. We were not able to amplify longer sequences with the heminested PCR for the DNA polymerase gene, and neither glycoprotein B gene nor papillomavirus amplifications were detected in the analyzed skin and oral mucosa samples of the HV positive specimens. The five novel HV DNA polymerase nt sequences obtained from the positive Guiana dolphin, the dwarf sperm whale (one from the skin and a different one from the

Table 1
Gross and histologic findings on PCR-positive tissues of the herpesvirus-positive individuals.

ID	Tissue	Gross	Histologic
Bolivian river dolphin	Skin	Focal, 8 mm in diameter, well-demarcated, raised verrucous nodule on the right pectoral fin.	Marked, focally extensive, chronic proliferative dermatitis with lipokeratinocyte hydropic degeneration.
	Blood	-	-
Dwarf sperm whale	Skin	Multiple *antemortem* skin lacerations and deep oval wounds in the head, dorsum and peduncle, compatible with inter-specific interaction. Some of them inflicted by cookie cutter shark, *Isistius brasiliensis*; mild cutaneous infestation by *Monorygma* sp. plerocercoids.	Marked, multifocal, chronic ulcerative and fibrinosuppurative dermatitis and panniculitis with hemorrhage, thrombosis, intralesional bacteria and granulation tissue.
	Lung	Severe pulmonary edema with hemorrhage; Bilateral atelectasia.	Marked, diffuse congestion, edema, hemorrhage and fibrin with rare fibrinocellular thrombi; diffuse atelectasia; mild, multifocal lymphoplasmacytic bronchitis and bronchiolitis.
	Liver	Severe, diffuse congestion and hemorrhage; zonal hepatopathy.	Severe, diffuse, acute centrilobular congestion and hemorrhage with hepatocellular atrophy, degeneration and loss; periportal hepatocellular vacuolar change with hyaline and eosinophilic globules.
	Lymph nodes*	NSFO**	Periaortic: moderate sinus erythrocytosis and erythrophagocytosis; mild medullary plasmacytosis. Pancreatic: Moderate sinus erythrocytosis and erythrophagocytosis and mild hemosiderosis. Unidentified origin: Mild, diffuse lymphoid reactive hyperplasia with medullary plasmacytosis, sinus erythrocytosis and mild hemosiderosis; mild, diffuse sinus edema; multifocal, acute hemorrhage; moderate, diffuse lymphoid reactive hyperplasia with plasmocytosis, erythrophagocytosis, chronic interstitial fibrosis, rare sinus multinucleate giant cells; moderate, diffuse histiocytosis with follicular hyalinosis. Unidentified origin: Moderate, diffuse sinus histiocytosis with mild hemosiderosis and moderate lymphoid depletion with follicular hyalinosis.
	Stomach	Ulcerative gastritis with numerous intralesional anisakid nematodes in all gastric compartments.	Moderate, focally extensive, chronic ulcerative gastritis with intralesional nematode debris (keratinized compartment); mild to moderate, multifocal, chronic lymphoplasmacytic and granulomatous gastritis with intralesional nematodes, ulceration and fibrosis (glandular compartment); mild, multifocal, chronic lymphoplasmacytic gastritis with scattered submucosal granulomas (pyloric compartment)
	Small intestine*	NSFO	NSFO
	Large intestine*	NSFO	Moderate, diffuse, transmural edema.
Atlantic spotted dolphin	Tongue	-	Advanced autolysis.
Guiana dolphin	Skin	Nine 1 to 3 cm, white, well-demarcated, circular or oval white skin lesions, one of them ulcerated and other two with a red ulcerated punctiform center, were present in the peduncle. Net marks.	Marked, focally extensive, chronic proliferative dermatitis, with irregular epidermal hyperplasia, mainly involving the basal and intermedium layers and formation of prominent rete pegs that were often fused. Multifocally, lipokeratinocytes in the basal and intermediate layers had cytoplasmic hydropic and ballooning degeneration with occasional nuclear clearing and peripherally marginalized chromatin, and mild intercellular edema. Scattered intraepidermal keratin pearls with mild lipokeratinocyte dyskeratosis, cellular debris and exocyted degenerate neuthophils, and also rare apoptotic lipokeratinocytes.
	Kidney	Congestion.	NSFO
	Liver	NSFO	NA***
	Blood	NSFO	NA

* Identification of these HV-PCR positive lymph nodes or intestine segments was not available.
** NSFO: no significant findings observed.
*** NA: not available.

stomach and intestines), the Bolivian river dolphin, and the Atlantic spotted dolphin were submitted to GenBank database under accession numbers MF999151 to MF999155, respectively.

The HV phylogenetic lineages were determined based on a phylogenetic tree (Fig. 1). The Atlantic spotted dolphin, Guiana dolphin, and dwarf sperm whale sequences were classified as α-HVs. The Bolivian river dolphin sequence was closer to γ-HV. The Atlantic spotted dolphin HV presented a nt identity of 96.1% and a deduced aa of 98.5% to a sequence obtained in a striped dolphin (*Stenella coeruleoalba*) from the Canary Islands, Spain (KJ156332.1). The Guiana dolphin sequence had 86.9% and 89.7% nt and aa identity, respectively, to a striped dolphin sequence, also from the Canary Islands (KJ156330.1). The two HV sequences (skin and stomach) obtained from the dwarf sperm whale presented the highest nt and aa identity among them (90.8% nt and 94.2% aa) and 68.2% aa identity to a sequence from a Risso's dolphin (*Grampus griseus*) from the Spanish Mediterranean coast (ALP00299.1). The Bolivian river dolphin HV sequence only presented an aa identity of 65.1% to the closest HV: γ-HV sequences (ALH21051.1, ALH21053.1, ALH21057.1) obtained from bats in China. The closest cetacean HVs to the sequence obtained in the Bolivian river dolphin, with aa identity of 54.1%, were the γ-HV obtained in a bottlenose dolphin (AAX55679) and a rough-toothed dolphin (*Steno bredanensis*) (APG38166.1), both from the US.

3.2. Gross and histological findings

Herein we summarized the gross and histological findings observed in skin and tongue samples of the HV-positive animals (Fig. 2).

Table 2
Herpesvirus-positive animals: identification (ID), institution of origin, species, age class (C = calf, J = juvenile, A = adult), sex (M = male, F = female, U = unidentified), total body length, status (captured alive, stranded alive, found dead), tissue condition (code 1 to code 5), cutaneous or oral mucosal tissue evaluated for HV by PCR, and stranding/capture location and date.

ID	Origin (Institution[1])	Species	Sex	Age class	Total body length (cm)	Status and tissue condition	Location[2]	Date of stranding/ capture	Sample type and number	HV result
Boto 20	INPA	Bolivian river dolphin	M	A	222	Captured alive. Code 1	Guaporé river (RO) 12.48 °S, 64.13°W	27-Sep-15	Nodular verrucous skin lesion (1)	+
MM579	AS	Dwarf sperm whale	M	J	200	Stranded alive. Code 3	Santos (SP) 23.99 °S, 46.31°W	04-May-14	Skin lesions (2)	+
									Healthy skin (2)	+
MM595, UNI#353	UNI	Atlantic spotted dolphin	U	A	190	Found dead. Code 4	São Francisco do Sul (SC) 26.18°S, 48.53 °W	29-Jun-12	Tongue (1)	+
									Skin (1)	-
MM731 05C1422/335	IBJ	Guiana dolphin	F	C	116	Found dead. Code 3	Linhares (ES) 19.25 °S, 39.70 °W	06-Jan-15	Skin lesions (2)	+

Notes: Sex (M = male, F = female, U = unknown); age class (N: newborn, C: calf, J: juvenile, A: adult). NA = data not available.
[1] Institution of origin: AS: Aquário de Santos; IBJ: Instituto Baleia Jubarte; INPA: Instituto Nacional de Pesquisas da Amazônia; UNI: Universidade da Região de Joinville (UNIVILLE) - Projeto Toninhas.
[2] Brazilian Federal State: ES: Espírito Santo, RO: Rôndonia, SP: São Paulo, SC: Santa Catarina. *NA = data not available.

Additional information is available in Table 1 and Table S2.

Guiana dolphin. Grossly, at least nine well-demarcated, 1 to 3 cm in diameter, circular or oval light tanned skin lesions - one ulcerated and two presenting red ulcerated punctiform centers, were observed in the peduncle. Additionally, the specimen presented net marks throughout the body. On histopathology, the two HV-positive skin lesions (two light tanned lesions, one of them ulcerated) were characterized by marked, focally extensive, chronic proliferative dermatitis (Fig. 2a). Both presented irregular epidermal hyperplasia, mainly involving the basal and intermedium layers, forming prominent, often fused rete pegs. Multifocally, cytoplasmic hydropic and ballooning degeneration with occasional nuclear clearing and peripherally marginalized chromatin, and mild intercellular edema was observed in lipokeratinocytes of the basal and intermediate layers. Scattered intraepidermal keratin pearls with mild lipokeratinocyte dyskeratosis, cellular debris and exocytosed degenerate neutrophils, and rare apoptotic lipokeratinocytes were also observed. No viral inclusion bodies were observed.

Dwarf sperm whale. Grossly, the animal presented multiple (at least 16) parallel *antemortem* cutaneous lacerations throughout the head, dorsum and peduncle, likely caused by interspecific interaction (compatible with bite wounds inflicted by killer whales (*Orcinus orca*) or sharks). Four well-demarcated, yellow to green, necrotic ulcers were present on the left lateral flank (ranging from 3.5 x 2 cm, 7 x 3.5 cm, and 8 x 4 cm) and on the peduncle (4 x 2.5 cm), possibly caused by cookie cutter shark (*Isistius brasiliensis*) bites. An identical HV sequence was obtained from a 3 x 2 cm avulsive and torn purulent lesion on the dorsal fluke, from an unidentified lesion and from healthy skin. Histologically, such lesions were characterized by marked, multifocal, chronic ulcerative and fibrinosuppurative dermatitis and panniculitis with hemorrhage, thrombosis, intralesional mixed bacteria and granulation tissue. No viral inclusion bodies were observed.

Bolivian river dolphin. Grossly, the animal presented an 8 mm in diameter, well-demarcated, raised verrucous skin nodule on the right pectoral fin (Fig. 2e). Histologically, this nodule was diagnosed as marked, focal, chronic proliferative dermatitis with lipokeratinocyte hydropic and ballooning degeneration, mainly on the stratum intermedium. No viral inclusion bodies were observed.

Atlantic spotted dolphin. No relevant lesions were observed grossly. Advanced autolysis prevented further histopathological evaluation.

4. Discussion

We detected HV in 3.7% (4/109) of the evaluated beta actin-positive animals – in 4.8% (7/145) of the skin samples and 6.7% (1/15) of the mucosal samples. These percentages are lower than those reported for γ-HV in skin lesions of harbor porpoises (*Phocoena phocoena*) (8.3%, 5/60) from the Netherlands (Van Beurden et al., 2015); α-HV in skin samples from 18 delphinids (11.1%, 2/18) from Spain (Sierra et al., 2014); and γ-HV in genital mucosal samples from a captive population of Atlantic bottlenose dolphins (25%, 9/36) from France and the Netherlands (Van Elk et al., 2009).

To our knowledge, this is the first report of HVs in Atlantic spotted dolphins and Bolivian river dolphins, adding a new cetacean family (Iniidae) to those already known to be susceptible to HV infection. We also report the first α-HV in a dwarf sperm whale and a Guiana dolphin, species with previous reports of γ-HV (AY949830 and KU666557, respectively) in genital slit lesion (Smolarek-Benson et al., 2006, Seade et al., 2017). We identified α-HV in the tongue of an Atlantic spotted dolphin, in skin lesions of a Guiana dolphin, and in healthy and lesional skin of a dwarf sperm whale (Table 1). In cetaceans, α-HVs have been previously identified in cutaneous and mucosal samples – in a sample of ulcerative stomatitis (Bellehumeur et al., 2015), in skin lesions (Manire et al., 2006; Smolarek-Benson et al., 2006; Burdett-Hart et al., 2012; Burek-Huntington et al., 2015), in skin and penile mucosal samples (Melero et al., 2015), in proliferative and ulcerative genital lesions (Bellehumeur et al., 2015) and on a genital swab (Van Elk et al., 2016) -

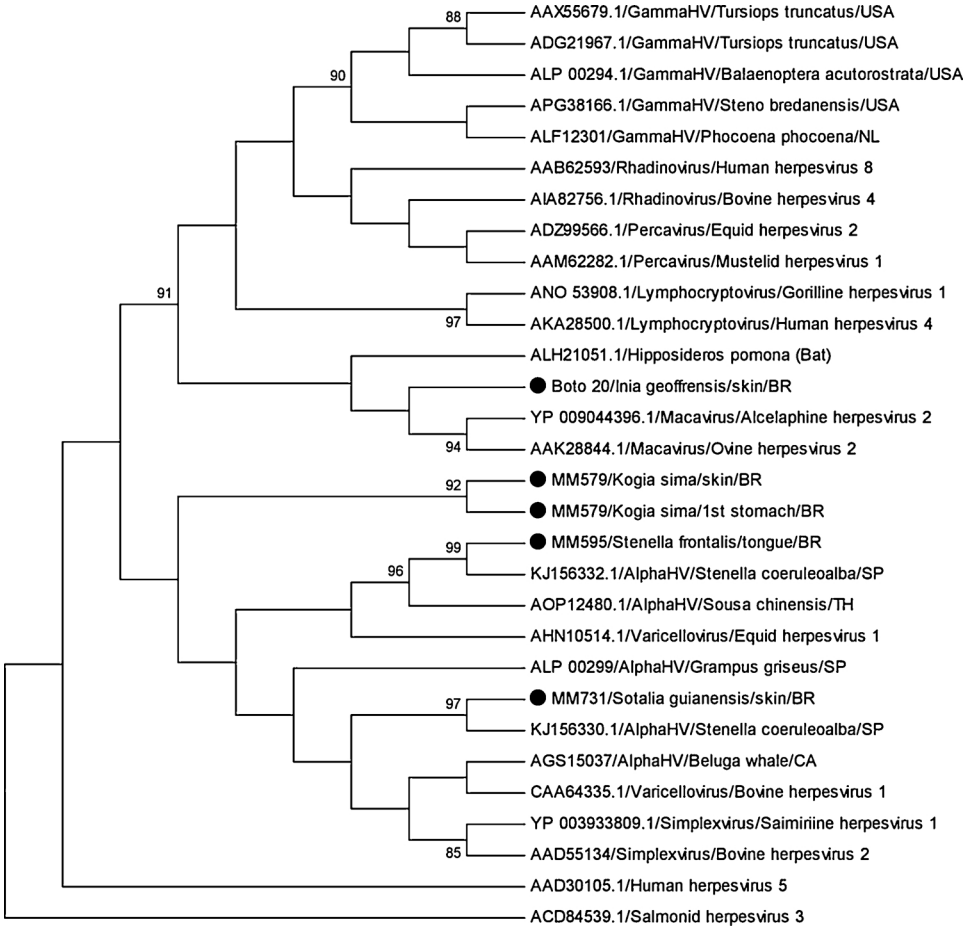

Fig. 1. Maximum-likelihood phylogram of the alignment of the herpesviral DNA polymerase strain deduced amino acid sequences found in this study (circle) and 25 herpesvirus sequences of this gene. The reliability of the tree was tested by bootstrap analyses with 1000 bootstrap replicates. Bootstrap values lower than 70% were omitted. Abbreviations: BR = Brazil; CA = Canada; NL = The Netherlands; SP = Spain; TH = Thailand; USA = United States of America.

but this is the first molecular report in tongue samples. HV PCR screening in lingual samples have been scarcely described, only reported for γ-HV (AY952779) in an Atlantic bottlenose dolphin (Smolarek-Benson et al., 2006). Additionally, viral particles and intranuclear inclusion bodies (INIBs) compatible with HV were also described in the tongue of an Atlantic bottlenose dolphin with α-HV-positive cardiac tissue (Blanchard et al., 2001).

The HV herein detected in an Atlantic spotted dolphin is related to that found by Sierra et al. (2014) (KJ156332.1) in the skin of a striped dolphin from the Canary Islands. Different *Stenella* species are known to interact and co-exist in social groups (Psarakos et al., 2003), and also hybridize (Silva et al., 2005; Amaral et al., 2014), which could facilitate HV transmission. Another possibility is the virus-host coevolution from a herpesvirus-infected common ancestor into the *Stenella* genus. However, in order to define their origin, longer DNA sequences are required along with a definition of the molecular clock to make a comparison between our sequence and the sequences found in the Canary Islands.

The HV identified in the Bolivian River dolphin's foci of proliferative dermatitis was more similar to the subfamily *Gammaherpesvirinae*, previously associated with skin lesions in cetaceans (Burdett-Hart et al., 2012; Van Beurden et al., 2015), but especially mucosal lesions (mainly genital and oral) (Saliki et al., 2006; Smolarek-Benson et al., 2006; Rehtanz et al., 2012; Cruz et al., 2014; Van Elk et al., 2016). However, our sequence presented low amino acid identity to the nearest HV sequences (ALH21051.1, ALH21057.1, ALH21053.1) detected in bats by Zheng et al. (2016). Likewise, Pei et al. (2012) detected an ultrastructurally herpes-like virus in the liver of an Indo-Pacific finless porpoise (*Neophocaena phocaenoides*) from a freshwater population of the Yangtze River, China, with only 22% identity to other HVs' DNA polymerase gene fragment. Unfortunately, the sequence is not available in public databases for comparison. The Bolivian river dolphin is part of the polyphyletic group of 'river dolphins', comprised of four different odontocete families: Iniidae, Pontoporiidae, Lipotidae, and Platanistidae (Cassens et al., 2000; Hamilton et al., 2001; Hrbek et al., 2014). These families are probably relict representatives of originally diverse marine taxons, previous to the radiation of the Delphinidae that remained in riverine ecosystems or coastal water, in this latter case, Pontoporiidae (Cassens et al., 2000). This evolutionary context could have promoted a separate coevolution of Bolivian river dolphin populations and their herpesviruses for thousands of years. The comparison between sequences from known *Herpesviridae* subfamilies and the ones described in this study shows great amino acid differences, which could justify their classification into a novel genus within the γ-HV subfamily. The significant HV sequence differences observed in the Bolivian river dolphin could possibly be explained by (1) host-virus coevolution with a relict species, geographically isolated millions of years ago and/or (2) scarcity of viral studies (including HV) in riverine dolphin species.

Despite the amplified fragment's short length, we believe that the HVs herein identified in Guiana dolphin, dwarf sperm whale and Bolivian river dolphin are in accordance with all biological and epidemiological characteristics and conditions required to describe a novel HV species (Davison, 2010): (1) an elevated percentage of differences in genome composition to the closest previously described HV sequences (at least 10.3%, 32.3% and 34.9%, respectively), possibly corresponding to independent replicating lineages, and (2) the first identification of alphaherpesvirus (Guiana dolphin and dwarf sperm whale) and gammaherpesvirus (Bolivian river dolphin) subfamilies infecting these cetacean species. Cetacean HVs were tentatively named following ICTV guidelines, using terms derived from the order and host family of the discovered virus in question (Davison et al., 2009): *Delphinid herpesvirus 10*, *Kogiid herpesvirus 2*, *Kogiid herpesvirus 3* and *Iniid herpesvirus*

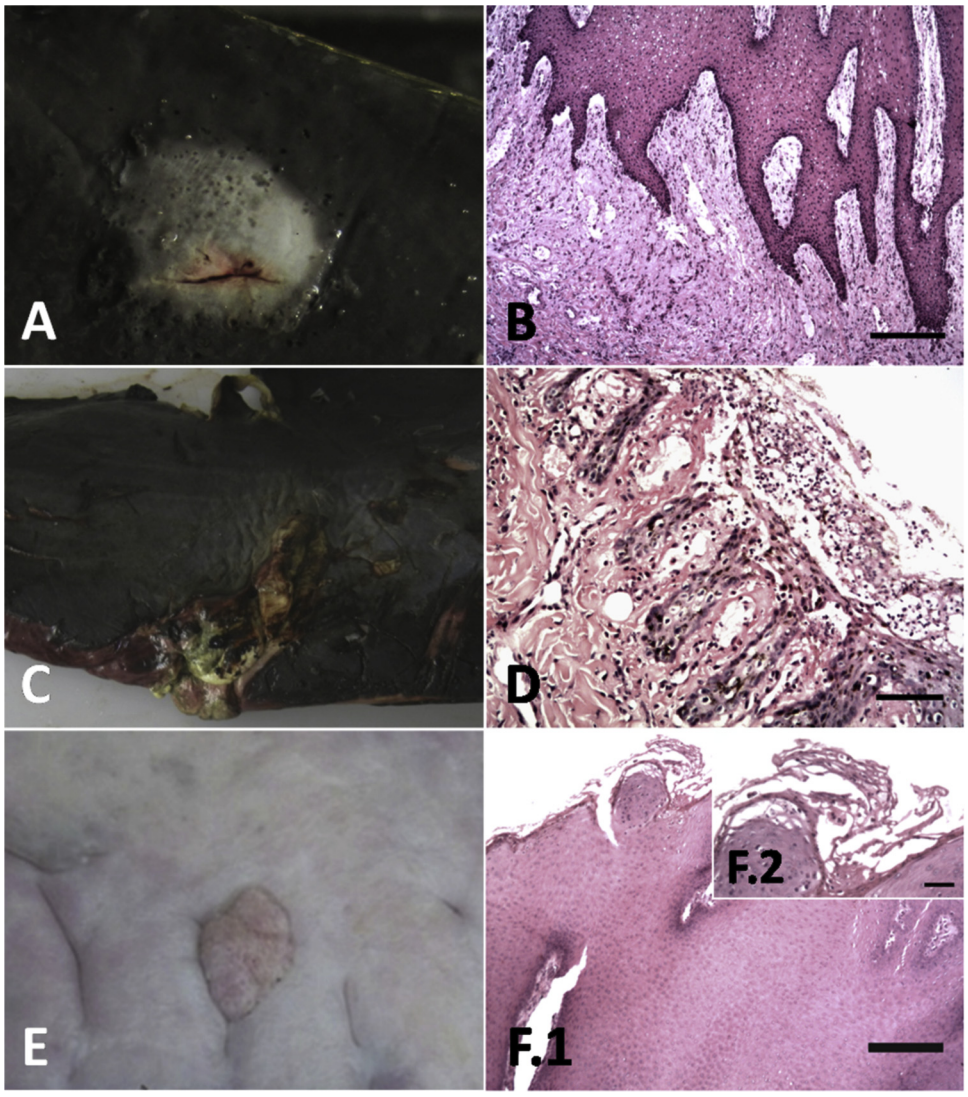

Fig. 2. Gross and histologic aspects of the skin lesions observed in the herpesvirus positive specimens. Guiana dolphin: **(A)** White stippled ulcerated skin lesion; **(B)** Discrete superficial dermatitis, prominent irregular fused epidermal rete pegs, hydropic and ballooning degeneration, HE, 4 × . Dwarf sperm whale: **(C)** Purulent exudate-draining lacerated skin lesion on the right dorsal area of the fluke; **(D)** Ulcerative and fibrinosuppurative dermatitis in a cookie cutter shark bite lesion, HE. 4 × . Bolivian river dolphin: **(E)** Raised verrucous skin nodule; **(F.1)** Chronic proliferative dermatitis, HE, 4X; **(F.2)** detailed view of the inflammatory exudates, HE, 20 × .

1.

Microscopically, despite the absence of viral INIBs suggestive of HV infection, the lesions observed in the Bolivian river dolphin and the Guiana dolphin are in accordance with gross findings previously associated with herpesviral infection in marine mammals (Manire et al., 2006; Van Elk et al., 2009; Van Beurden et al., 2015) or presumptive HV-associated skin lesions (Blanchard et al., 2001), whereas the skin lesions found in the dwarf sperm whale could be associated with predation. Nevertheless, the presence of herpesviral DNA in these skin lesions does not necessarily imply that the virus caused skin disease. From a diagnostic point of view, the presence of intranuclear herpesviral inclusion bodies may be helpful. However, it is an inconsistent finding, mainly observed in very early stages of the infection, but rarely after that (Caswell and Williams, 2007). The absence of herpesviral INIB on histopathology of PCR-positive animals have been documented in ulcerative skin lesions of pinnipeds (Bodewes et al., 2015; Sacristán et al. 2018b) and in cetaceans, in a beluga whale with an oral ulcer and five penile lesions positive for HV (Bellehumeur et al., 2015). The detection of herpesviruses in several organs of three positive animals suggests their reactivation from latency, although no herpesvirus-compatible lesions were observed in positive tissues aside from the Guiana dolphin and the Bolivian river dolphin skin samples, commented above. It is important to highlight that active herpesviral infections may not cause clinical disease (Grinde, 2013).

Papillomavirus is a major differential viral etiology for proliferative lesions in cetaceans, especially in genital mucosa (Rehtanz et al., 2006; Rehtanz et al., 2012). Concurrent papillomavirus and HV infections have been detected in mucosal lesions of free-ranging Atlantic bottlenose dolphins from Cuba (Cruz et al., 2014). However, in the present study, the Bolivian river dolphin's verrucous skin nodule was negative to papillomavirus.

In conclusion, we detected four new cases of HV infection in skin and oral mucosa of an Atlantic spotted dolphin, a Guiana dolphin, a dwarf sperm whale, and a Bolivian river dolphin from Brazil. Four of the detected sequences are possibly novel species, tentatively named *Delphinid herpesvirus 10*, *Kogiid herpesvirus 2*, *Kogiid herpesvirus 3* and *Iniid herpesvirus 1*. This is the largest herpesvirus survey in South American cetaceans and constitutes the first molecular report of HV infection in Atlantic spotted dolphins and riverine dolphins worldwide. Additionally, gross and histologic findings in the Guiana dolphin and Bolivian river dolphin HV-positive skin lesions were in agreement with previous reports of herpesvirus-associated lesions in cetaceans. However, future studies are necessary to clarify the pathogenic role of the herpesviruses detected in this study. The significant nucleotide and amino acid differences observed in the HV sequence described in the Bolivian river dolphin could be associated with viral-host coevolution. Further monitoring of Iniidae dolphins is needed to elucidate the pathogenic potential of the detected HV.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2018.11.021.

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