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Molecular identification and microscopic characterization of poxvirus in a Guiana dolphin and a common bottlenose dolphin, Brazil

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ABSTRACT: The poxviruses identified in cetaceans are associated with characteristic tattoo or ring skin lesions. However, little is known regarding the prevalence and progression of these lesions and the molecular characterization of cetacean poxviruses in the Southern Hemisphere. This manuscript describes the progression of poxvirus-like skin lesions in 5 free-ranging Guiana dolphins *Sotalia guianensis*. Additionally, 151 skin samples from 113 free-ranging cetaceans from Brazil, including 4 animals with tattoo skin lesions, were selected for poxvirus testing. Poxviral DNA polymerase gene PCR amplification was used to detect the virus in β-actin-positive samples (145/151). DNA topoisomerase I gene PCR was then used in *Cetaceanpoxvirus* (CePV)-positive cases (n = 2), which were further evaluated by histopathology and electron microscopy. Based on photo-identification, adult Guiana dolphins presented regressing or healed poxvirus-like lesions (2/2), while juveniles presented persistent (2/3) or healed and progressive lesions (1/3). CePV DNA was amplified in a common bottlenose dolphin *Tursiops truncatus* and in a Guiana dolphin. Intracytoplasmic inclusion bodies and viral particles consistent with poxvirus were identified by histology and electron microscopy, respectively. CePV-specific amino acid motifs were identified through phylogenetic analysis. Our findings corroborate previous studies that suggest the placement of poxviruses from cetaceans within the novel CePV genus. This is the first molecular identification of poxvirus in South American odontocetes.

KEY WORDS: Cetacean poxvirus · Tattoo lesion · Pathology · Virology · *Sotalia guianensis* · *Tursiops truncatus* · South America

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INTRODUCTION

Poxvirus infections in cetaceans were first reported in the 1970s in common bottlenose dolphins *Tursiops truncatus* and an Atlantic white-sided dolphin *Lagenorhynchus acutus* (Flom & Houk 1979, Geraci et al. 1979). Such viruses have been tentatively classified into a novel genus, *Cetaceanpoxvirus* (CePV) (Bracht et al. 2006, Blacklaws et al. 2013), with at least 2 described groups: CePV-1 in odontocetes and CePV-2 in mysticetes (Bracht et al. 2006, Blacklaws et al. 2013, Fiorito et al. 2015). In cetaceans, poxviruses have been associated with round or elliptical flat or slightly raised grayish skin lesions (rings) or with irregularly shaped skin lesions with dark margins and a punctiform stippled pattern (tattoo) (Geraci et al. 1979, Van Bressem et al. 2003). Histological findings in these lesions include thickened stratum corneum, ballooning degeneration and intracytoplasmic inclusions containing viral particles within stratum intermedium cells (Geraci et al. 1979). Poxvirus infections apparently do not impact the general health status of the affected individual; however, 1 common bottlenose dolphin died after developing generalized tattoo lesions (Sweeney & Ridgway 1975).

CePV has a worldwide distribution, with confirmed reports in the Atlantic (Geraci et al. 1979, Fiorito et al. 2015) and Pacific oceans (Van Bressem et al. 1993, Van Bressem & Van Waerebeek 1996, Bracht et al. 2006) and the North Sea (Blacklaws et al. 2013). In South America, pox-like particles were ultrastructurally identified in odontocetes and mysticetes (Van Bressem et al. 1993, Van Bressem & Van Waerebeek 1996, Fiorito et al. 2015), while CePV infection was confirmed by PCR in mysticetes (Fiorito et al. 2015). Nevertheless, no molecular techniques have been used to detect poxvirus-related skin lesions in South American odontocetes. Our goals were to (1) describe and evaluate the progression of poxvirus-like skin lesions in free-ranging cetaceans, (2) identify poxvirus through molecular diagnostics and electron microscopy, and (3) describe the histopathology of CePV PCR-positive skin lesions.

MATERIALS AND METHODS

Visual assessment

Since 1995, the Guiana dolphin population of Guanabara Bay (22.83° S, 43.17° W, Rio de Janeiro, southeastern Brazil) has been monitored by photo-identification, based on skin marks (e.g. lesions caused by intraspecific and/or anthropogenic interactions and skin lesions of unknown etiology). The progression of poxvirus-like skin lesions of 5 of these Guiana dolphins was evaluated on boat surveys (139) from July 2008 through February 2012. Once a group of Guiana dolphins was sighted, photographs were taken with a digital camera (Canon 40D) with 100 to 400 mm zoom lenses and analyzed in Windows Image Visualizer to identify group members individually and register any skin lesions. The poxvirus-like lesions were classified as persistent (the lesion remained, increased in size, eventually got darker and presented dark margins), regressing (the lesion got lighter in color, and the dark margin was less evident or disappeared), or healed (the lesion was barely visible and occasionally identified as a slightly lighter area). Poxvirus-like skin lesions (2 samples, 1 from each animal) were sampled during the necropsy of the 2 members that were found stranded dead.

Samples

Overall, 148 skin samples of 110 cetaceans stranded along the Brazilian coast between 2005 and 2015 were evaluated (6 different families: Delphinidae [n = 60], Pontoporiidae [n = 34], Kogiidae [n = 3], Physeteridae [n = 1], Balaenopteridae [n = 10] and Balaenidae [n = 2]). Additionally, 3 skin samples from 3 free-ranging Iniidae specimens captured in 2015 and immediately released were included in the study (Table S1 in the Supplement at www.int-res.com/articles/suppl/d130p177_supp.pdf). Six of 151 skin samples were identified as tattoo skin lesions, presented by 4 individuals (2 Guiana dolphins, 1 common bottlenose dolphin and 1 spinner dolphin *Stenella longirostris*) (Table S1).

Necropsies followed standard procedures (Geraci & Lounsbury 2005). Tissue samples (skin and other tissues) were collected and frozen at –20 or –80°C or fixed in 10% formalin. Age determinations of specimen MM610 (common bottlenose dolphin) and Guiana dolphins were based, respectively, on the examination of dental enamel (Hohn 1990) and on total body length (Rosas & Monteiro-Filho 2002). The postmortem decomposition code was based on Geraci & Lounsbury (2005).

Molecular diagnostics

Total DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen) in blubber-free, manu-

ally homogenized samples of frozen skin, according to manufacturer's protocol. DNA was tested with a housekeeping β-actin gene PCR assay with a primer set (Behrens et al. 1998) at a melting temperature of 55°C. DNA polymerase primers (Bracht et al. 2006) were employed to detect the agent in β-actin PCR-positive samples. CePV-positive cases were subsequently tested for DNA topoisomerase I gene PCR (Bracht et al. 2006). Both techniques were performed at a melting temperature of 43°C. In CePV-positive cases, all available tissues, aside from skin, were extracted by the same technique described above and tested by DNA polymerase PCR. Positive samples were identified through purification and direct sequencing of amplicons. Amino acid CePV sequences obtained in this study were aligned with those of similar size available at GenBank, and several representative species in the *Chordopoxvirinae* subfamily recognized by the International Committee on Taxonomy of Viruses (ICTV), using ClustalW software. Maximum likelihood phylogenetic trees of 1000 bootstrap replicates were generated. Amino acid alignment was also employed to detect specific amino acid motifs. The identity of the obtained sequences to the most closely related sequences was established based on the p-distance. Sequence analyses were performed with MEGA 6.0 software.

Electron microscopy

Transmission electron microscopy (TEM) was performed in 2 tattoo samples from a pair of CePV PCR-positive individuals, initially fixed in formalin and subsequently in Karnovsky solution. Samples were washed in 0.1 M cacodylate buffer (CaCo) and post-fixed in 1% osmium tetroxide (in 0.1 M CaCo buffer). After a gradient step dehydration with increasing volumes of ethanol, samples were embedded in Epon-Araldite, which polymerized over 48 h at 60°C. Ultrathin sections were then obtained. Micrographs were taken in an FEI Morgagni 268 transmission electron microscope, and images were recorded with an Olympus Veleta charge-coupled device camera.

Histological examination

Histological evaluation of the CePV PCR-positive specimens was performed on formalin-fixed tissues embedded in paraffin, sectioned at 5 µm and stained with H&E.

RESULTS

Visual assessment

Poxvirus-like skin lesions were identified and monitored by photo-identification in 5 Guiana dolphins (Table A1 in the Appendix, Fig. S1 in the Supplement): BG#34, BG#47, BG#89, MM499/CalfBG#66 and MM672/BG#81. Two adult Guiana dolphins (BG#34, BG#47) presented regressing or healed poxvirus-like lesions, 2 juveniles showed persistent lesions (MM672/BG#81, MM499/CalfBG#66), and a third juvenile presented healed and persistent lesions (BG#89) (Table A1, Fig. S1). Only lesions from MM499/CalfBG#66 and MM672/BG#81 were collected upon stranding. The 159 cm long juvenile male MM672/BG#81 was found dead on October 17, 2009, in Ilha do Governador, Rio de Janeiro, Rio de Janeiro state (22.82° S, 43.20° W). The animal was in good body condition, with moderate autolysis (Code 3), and had fresh entanglement marks. The 151 cm long juvenile MM499/CalfBG#66 was found dead on February 16, 2012, a little further offshore in Guanabara Bay, Rio de Janeiro state (22.82° S, 43.20° W). The animal was emaciated and with moderate autolysis (Code 3).

Molecular findings

The β-actin gene was amplified in 107 of 113 specimens and in 145 of 151 skin samples. Three of 4 specimens presenting tattoo lesions were β-actin positive (2 Guiana dolphins [MM672/BG#81, MM 499/CalfBG#66] and a common bottlenose dolphin [MM610]).

CePV DNA polymerase and DNA topoisomerase I genes were amplified in 2 of 107 β-actin-positive specimens: a common bottlenose dolphin (MM610) and a Guiana dolphin (MM672/BG#81), both presenting tattoo lesions (3 and 1, respectively, all PCR positive). Two 497 nt sequences of the DNA polymerase gene and two 302 nt sequence fragments of the DNA topoisomerase I gene were obtained, excluding primers. The new CePV DNA polymerase and DNA topoisomerase I sequences were submitted to GenBank under accession numbers KU726611 and KU726612 for the common bottlenose dolphin and MF458199 and MF458200 for the Guiana dolphin. We were not able to amplify poxvirus DNA in the other 2 animals that also presented tattoo lesions, the Guiana dolphin MM499/CalfBG#66 and a β-actin-negative spinner dolphin. Tissue samples aside from tattoos of CePV-positive common bottlenose dolphin (apparently

healthy skin, brain, laryngeal tonsil, lung, thymus, spleen, pancreas, liver, kidney, and prescapular, pulmonary, mesenteric and rectal lymph nodes) and Guiana dolphin (liver, kidney, muscle) were also negative.

In the common bottlenose dolphin, the DNA polymerase gene presented the highest nucleotide identity (95.5%) with a sequence from an Indo-Pacific bottlenose dolphin *Tursiops aduncus* from Hong Kong (AY463006). The DNA topoisomerase I gene presented a nucleotide identity of 92.4% with the Guiana dolphin from Brazil reported here. The deduced amino acid sequences showed high identity of DNA polymerase (98.8%) and DNA topoisomerase I (95.9%) genes with sequences from a rough-toothed dolphin *Steno bredanensis* from the USA (AY463004 and AY952949, respectively) and our Guiana dolphin sequence for the DNA polymerase gene. All CePV sequences, including the novel ones from Brazil, clustered together, separately from the other analyzed poxvirus genera, in the phylogenetic trees of the poxvirus DNA polymerase and DNA topoisomerase I genes (Fig. 1).

The poxvirus DNA polymerase gene fragment obtained from our positive Guiana dolphin presented a 94.1% nucleotide identity to CePVs from a rough-toothed dolphin from the USA (AY463004) and an Indo-Pacific bottlenose dolphin from Hong Kong (AY463006). The highest DNA topoisomerase I gene nucleotide identities (93.4%) were with CePV sequences from a short-beaked common dolphin *Delphinus delphis* (KC409060) and a striped dolphin *Stenella coeruleoalba* (KC409051) from the UK. The CePV identified in the Guiana dolphin showed the highest DNA polymerase gene and DNA topoisomerase I gene amino acid identity (98.8% and 95.9, respectively) to sequences from a rough-toothed dolphin from Florida, USA (AY463004 and AY952949), and further 98.8% identity for the DNA polymerase gene between the Guiana dolphin CePV sequence and the one from the common bottlenose dolphin (KU726612) from Brazil.

Comparison between the DNA polymerase sequences obtained in this study and sequences available at GenBank of (1) CePV of similar size available in the literature and (2) sequences from different genera within the *Chordopoxvirinae* subfamily recognized by the ICTV revealed 3 CePV genus-specific amino acid sequence motifs across the DNA polymerase catalytic subunit. When compared with the *Vaccinia virus* reference strain (*Orthopoxvirus* genus), CePV amino acid sequence motifs differ from the *Vaccinia* motif at residue 526, where *Vaccinia* has phenylalanine (F) and CePV sequences have leucine (L). Additionally, amino acid sequence motifs comprised between residues 525 and 526 (of *Vaccinia*) presented lysine (K) (in CePV-1) or leucine (L) (in CePV-2) insertion (Table 1).

TEM findings

Both CePV PCR-positive cases presented lipokeratinocytes with abundant smooth-surfaced, brick-shaped viral particles of variable size (400 to 600 nm in diameter), consistent with poxvirus (Fig. 2). Some lipokeratinocytes containing viral particles presented irregular clear cytoplasmic vacuoles (Fig. 2D).

Table 1. Specific amino acid motifs of poxvirus in different genera according to positions 524, 570 and 593 of the *Vaccinia virus* (*Orthopoxvirus* genus) genome. na: not applicable

Genus	No. of taxa	524	570	593
Avipoxvirus	4	V(R/K)-YP	Variable	Variable
Capripoxvirus	3	NK-YH	SVFVANN	PPPRYISIHCEPRC
Cervidpoxvirus	1	NK-FP	CVFVANN	PSPKYIAVHCEPRS
Cetaceanpoxvirus	na	Q(Q/K)(K/L)LP[a]	GVVVSNN	PSPRYI(V/I)VHCEPRF[b]
Crocodylidpoxvirus	1	PRAHH	FVLVNRN	PFPDYVHVETSTAE
Leporipoxvirus	2	NK-YP	CVFVANN	PGPRYISVQCEPRS
Molluscipoxvirus	1	AR-YT	GVVGNAH	PEPAFLHVLCEARA
Orthopoxvirus	10	QK-FP	GVVVS(T/S)N	P(P/S)(P/H)RYITV(H/R)CEPRL
Parapoxvirus	4	SK-(Y/F)(F/C)	GVVVSDN	PAPRYIAV(A/P)CEPR(S/A)
Suidpoxvirus	1	NK-FP	CVFIANN	PPPRYISVHCEPRS
Unassigned (squirrelpox virus)	1	TK-FL	GVMVSGN	RPPRFLCIECEPRS
Yatapoxvirus	2	(T/N)K-FP	GVFVSNN	PPPRYISINCEPRS

[a]*Cetaceanpoxvirus* (CePV)-1: QQKLP; CePV-2: QKLLP
[b]CePV-1: PSPRYIVVHCEPRF; CePV-2: PSPRYIIVHCEPRF

Fig. 1. Maximum likelihood phylogram of poxvirus amino acid sequences obtained in this study (black dots) and those selected from GenBank for: (A) the DNA polymerase gene, (B) the DNA topoisomerase I gene. The reliability of the tree was tested by bootstrap analyses with 1000 bootstrap replicates. Bootstrap values lower than 70% were omitted. The *Cetaceanpoxvirus* sequences obtained in this study are marked with black dots. The *Cetaceanpoxvirus* sequences selected are expressed as follows: tentative name of the virus, year of detection, place of detection (AR: Argentina; BR: Brazil; HK: Hong Kong), host species and GenBank accession number. The remaining sequences are from recognized poxvirus species and include their GenBank accession numbers

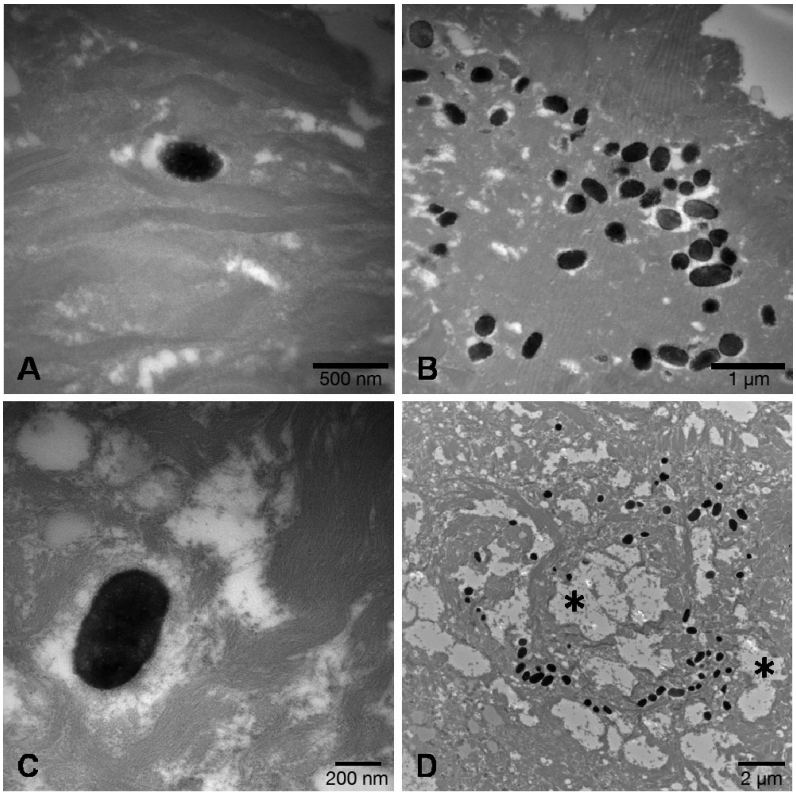

Fig. 2. Transmission electron microscopy of tattoo skin lesions from the *Cetaceanpoxvirus* PCR-positive cases: (A,B) Guiana dolphin, (C,D) common bottlenose dolphin. Viral ovoid particles of approximately 440 nm in diameter were observed (A,C) as well as viral aggregates formed by particles of variable sizes (B,D). Note numerous irregular vacuoles (asterisk) along with viral particles (D)

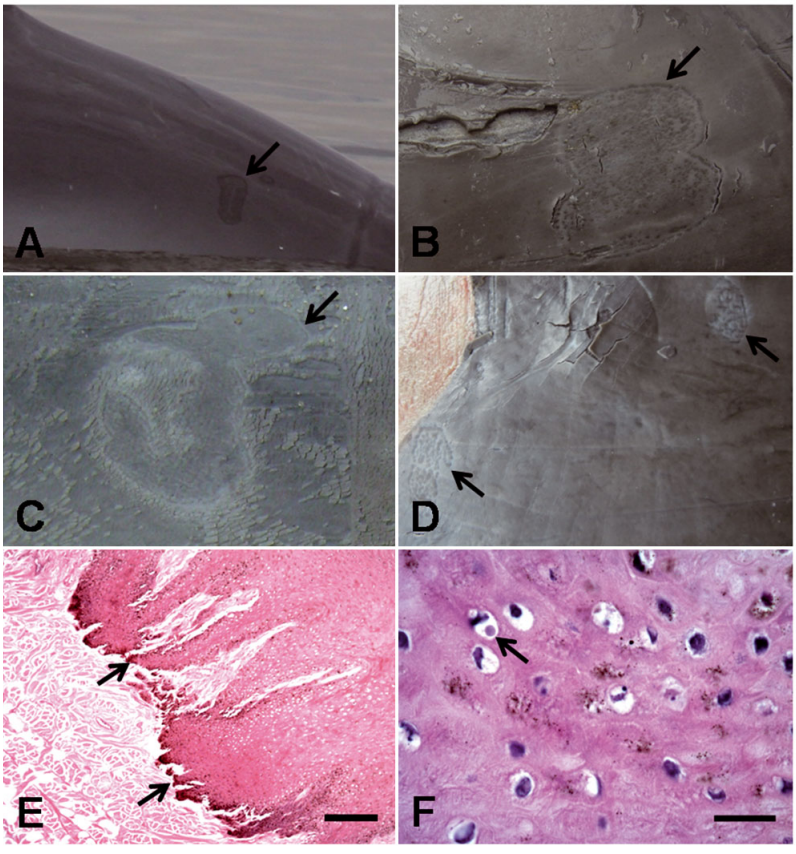

Fig. 3. Skin of specimens affected by *Cetaceanpoxvirus*. Macroscopic aspect of skin lesions (arrow): (A,C) Guiana dolphin, (B,D) common bottlenose dolphin. (E) Irregular aspect and hyperplasic epidermal papillae of a tattoo skin lesion from the Guiana dolphin (arrow), H&E, scale bar = 400 µm. (F) Presence of amphophilic intracytoplasmic inclusion bodies in the epidermis of the common bottlenose dolphin (arrow), H&E, scale bar = 50 µm

Macroscopic and microscopic findings of CePV-positive animals

Common bottlenose dolphin (MM 610): A 257 cm juvenile male of approximately 5 yr, in good body condition and with moderate autolysis, was found dead in the Laguna estuary, Santa Catarina state (28.46° S, 48.79° W), Brazil, on January 18, 2014. Linear marks on the left flipper and rostrum were suggestive of fishing interaction. Macroscopically, 4 round to oval, 2 to 4 cm in diameter skin lesions presenting well-defined dark gray irregular margins and pale light gray stippled interiors, consistent with tattoo lesions, were observed in the flank and peduncle. Three of these lesions were collected during necropsy (Fig. 3B,D).

Guiana dolphin (MM672/BG#81): Macroscopic findings and visual assessment (photo-identification) are described in 'Visual assessment' and in Fig. 3A,C, Table A1 in the Appendix, and Fig. S1 in the Supplement.

Histopathologically, skin lesions from both animals presented moderate epidermal hyperplasia, irregular and occasionally fused epidermal rete pegs,

hydropic and ballooning degeneration (mainly in the stratum spinosum), and lipokeratinocytes containing irregularly shaped, compressed and marginalized nuclei and small, homogeneous, amphophilic intracytoplasmic inclusions (Fig. 3E,F). In the common bottlenose dolphin, the other significant microscopic finding was a moderate, multifocal to coalescing, verminous, purulent pneumonia.

DISCUSSION

Poxvirus-like skin lesions progressed differently in the photo-identified animals; adult Guiana dolphins presented regressing tattoo lesions, while juveniles presented apparently active lesions and both healed and progressive lesions, as described by previous studies (Smith et al. 1983, Van Bressem et al. 2003, Barnett et al. 2015). Juveniles seem to be more susceptible to CePV infections (Barnett et al. 2015). Poxvirus-like skin lesions (tattoo-like lesions) persist from at least 2 to 14 mo, according to previous studies (Van Bressem et al. 2003). The presence of tattoo skin lesions has been proposed as an indicator of cetacean health (Blacklaws et al. 2013).

We amplified CePV DNA in 2 odontocetes with tattoo lesions: Guiana dolphin and common bottlenose dolphin. CePV has been previously amplified in a common bottlenose dolphin from the USA (Bracht et al. 2006), and tattoo lesions have been observed in Guiana dolphins (Van Bressem et al. 2007); however, to our knowledge, this is the first molecular, TEM and histopathological description in the latter species. This represents the first molecular report of poxvirus infection in odontocetes in Brazil and in South America. In the Guiana dolphin, CePV was detected over 1 yr after the first tattoo lesion detection, possibly because of persistent infection or reinfection. The lack of CePV amplification on tattoo skin lesions from another 2 cases was likely due to a low number of CePV DNA copies (e.g. healed lesions) or poor DNA quality associated with their degradation by autolysis, linked to the low sensitivity of the available PCR techniques (conventional PCRs), or because a different etiological agent or agents originated the lesions.

Our data confirm a relative stability between the studied CePV sequences for 2 relatively conserved genes obtained from Delphinidae specimens, despite their geographic distance. Further molecular studies are required to clarify the CePV species infecting odontocete cetaceans.

The identification of tree CePV amino acid-specific motifs is consistent with the clustering of all cetacean poxviruses into a new and unique genus, in accordance with our phylogenetic trees (Fig. 1) and previous studies (Bracht et al. 2006, Blacklaws et al. 2013, Barnett et al. 2015, Fiorito et al. 2015). Amino acid-specific motifs have been previously used to classify viruses into their genus (Sauvage et al. 2012).

The viral particles observed by TEM in CePV PCR-positive cases presented a similar morphology to those described by Barnett et al. (2015) for CePV. The size variation observed in the viral particles may in part be explained by different orientation and maturation stages (mature and immature) (Geraci et al. 1979, Van Bressem et al. 1993). Unfortunately, it was not possible to perform negative contrast, which prevented evaluation of poxvirus' internal features.

Macroscopically, the lesions of CePV-positive animals were compatible with tattoo lesions (Geraci et al. 1979, Smith et al. 1983, Van Bressem et al. 2003). Histopathological findings were consistent with previous descriptions (Geraci et al. 1979, Fiorito et al. 2015). However, the intracytoplasmic inclusion bodies we observed are amphophilic, similar to B-type inclusions, associated with viral replication and usually found in all poxvirus-infected cells (Fenner 1992), and not eosinophilic, as described in previous studies (Geraci et al. 1979, Fiorito et al. 2015). Additionally, the microscopic findings in the common bottlenose dolphin indicated that the respiratory process was possibly the cause of death.

In this study we (1) evaluated the progression of poxvirus-like skin lesions in 5 live Guiana dolphins, (2) identified 2 new cases of CePV infection (in common bottlenose dolphin and Guiana dolphin of inshore waters) and provided the first CePV sequences in odontocetes from South America, and (3) described specific CePV amino acid motifs that reinforce the potential classification of CePV into a novel genus of the *Chordopoxvirinae* subfamily.

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Appendix

Table A1. Record date, location and size of the poxvirus-like skin lesions of the photo-identified Guiana dolphins. (–) data not available

Individual ID	Record date (month and year)	Location of skin lesion	Size of skin lesion	Description of skin lesion
BG#34	July 2010	Adjacent to right side of blowhole	Approx. 5 cm in diameter	Round tattoo lesion with very well delimited and dark margins; light-colored inner area, with a uniform pattern
	October 2010	Adjacent to right side of blowhole	Approx. 5 cm in diameter	Round tattoo lesion with very well delimited and dark margins; light-colored inner area, with stippled pattern
	December 2010	Adjacent to right side of blowhole	Approx. 5 cm in diameter	Lesion apparently did not change in size, still presenting dark margins, but with a lighter halo around the center of the lesion
	January 2011	Adjacent to right side of blowhole	Approx. 5 cm in diameter	Dark margins disappeared, but light halo around the center of the lesion remained
BG#47	July 2010	Left side of dorsum	–	Two adjacent very well delimited large circular dark gray stippled skin lesions
	October 2010	Left side of dorsum	–	Lesions appeared slightly enlarged and coalescent, still presenting dark gray coloration and an even more evident white stippled pattern, this time with focal inner grayish areas and parallel linear marks adjacent to the most ventral lesion, possibly caused by intraspecific interactions
	June 2011, February 2012	Left side of dorsum	–	Lesions were barely visible, characterized by slightly clear, uniform and poorly delimited areas

Continued on next page

Table A1 (continued)

Individual ID	Record date (month and year)	Location of skin lesion	Size of skin lesion	Description of skin lesion
BG#89	February 2009	Cranial and medial left side of dorsal fin	–	Two circular coalescing and 1 single circular tattoo skin lesions; all 3 lesions were well delimited, presenting dark margins and dark gray inner areas
	October 2009	–	–	No lesions were observed
	July 2010	Right side and top of head	–	Two new well-delimited circular skin lesions with light-colored stippled centers and dark margins
	October 2010, December 2010	Right side and top of head	–	Lesions apparently did not increase in size, but the inner areas became progressively darker and more uniform
MM672/ BG#81	March 2009, August 2009	Right side of dorsum	–	Two small oval tattoo lesions characterized by dark margins, located next to a linear depressed mark between the dorsal fin and the head (probably a healed wound inflicted by fishing gear)
	October 2009	Right side of dorsum	Approx. 4 × 3 cm	Upon necropsy, the 2 previously observed tattoo lesions now coalescent; two novel lesions observed (right flank and adjacent to the blowhole); all presented light-colored interiors surrounded by a darker halo, light margins and gray stippled interiors with marked dark gray raised edges
		Right flank and adjacent to blowhole	2 × 2 cm and 4 × 3 cm	
MM499/ CalfBG#66	December 2010	Left and right sides of dorsum; head, caudal to blowhole	–	Three dark uniform small tattoo skin lesions well delimited by dark margins, of circular shape (on the dorsum) and irregular shape (on the head)
	December 2011	Right side of head	–	Two circular lesions, characterized by dark margins and lighter stippled interiors
	February 2012	Left and right sides of dorsum and on head, caudal to blowhole	3 × 2 and 6 × 4 cm (tattoo lesions reported first in December 2011, on right side of head)	When alive, the 2 circular lesions reported in December 2011 were still well delimited by darker margins but presented more uniform and light-colored centers
		Unreported tattoo lesion, cranial to left eye	12 × 12 cm (close to the blowhole, reported for the first time in December 2010)	Upon necropsy, the 2 circular lesions described above were well delimited by dark raised margins, with light-colored stippled interiors and circular shape; lesions were apparently larger than previously estimated; another lesion, caudal to the blowhole (reported in December 2010), well delimited by dark raised margins, with light-colored stippled interiors and irregular shape, was observed, apparently larger than previously estimated; a previously unreported tattoo lesion, well delimited by dark raised margins, with light-colored stippled interiors and circular shape, cranial to the left eye was observed
			1.2 × 1.2 cm (novel tattoo lesion cranial to left eye)	

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