CARLOS SACRISTÁN YAGÜE

Research and characterization of selected pathogens of cutaneous and mucocutaneous lesions in cetaceans from the Brazilian coast

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Departamento:

Patologia

Área de concentração:

Patologia Experimental e Comparada

Orientador:

Prof. Dr. José Luiz Catão Dias

De acordo:_____

Orientador

São Paulo
2017

Obs: A versão original se encontra disponível na Biblioteca da FMVZ/USP

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Programa de Pós-Graduação: Patologia Experimental e Comparada.

Área de concentração: Patologia Experimental e Comparada.

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Orientador: Prof. Dr. José Luiz Catão Dias.

1. Pathology. 2. Dermatology. 3. Herpesvirus. 4. Poxvirus. 5. *Paracoccidioides brasiliensis*. I. Título.

São Paulo, 03 de julho de 2017
CEUA N 2951280914

Ilmo(a). Sr(a).
Responsável: José Luiz Catão Dias
Área: Patologia
José Luiz Catão Dias (orientador)

Título da proposta: "Pesquisa e caracterização de patógenos cutâneos e mucocutâneos selecionados em cetáceos da costa brasileira. ".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais FMVZ/USP

A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** o Relatório Final (versão de 12/junho/2017) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Pesquisa e caracterização de patógenos cutâneos e mucocutâneos selecionados em cetáceos da costa brasileira. CEUA 2951280914. 1. Os objetivos propostos foram todos cumpridos? Resp: Sim. 2. Os resultados obtidos propiciaram a criação de novos projetos? Resp: Sim, os resultados deste estudo propiciaram a criação do projeto de iniciação científica do estudante Eduardo Ferreira Machado, sob a orientação do Prof. José Luiz Catão Dias. Além disso, foram feitas contribuições aos projetos de Pedro Enrique Navas Suarez, Samira Costa Silva e Angélica María Sánchez Sarmiento, todos eles alunos de pós-graduação da Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo. 3. O N amostral proposto inicialmente foi suficiente? Qual o N amostral total alocado? Resp: Sim, foram analisadas amostras de pele e/ou mucosa oral e genital de 115 cetáceos, um número inferior aos 300 animais projetados inicialmente. Desses animais 109 apresentavam uma qualidade suficiente do DNA para poder realizar as determinações moleculares subsequentes. Nos casos positivos para herpesvírus (n=4), poxvírus (n=3), ou leveduras do gênero Onygenales(n=4) além das amostras de pele e/ou mucosa oral e genital, se analisaram todos os tecidos congelados disponíveis do animal. O número total de amostras analisadas, incluindo pele, mucosa oral e genital e outros tecidos, foi de 214. 4. Houve perdas? se sim, quantas? Resp: não houve perdas. 5. Ocorreu algum evento adverso durante a condução do estudo? Resp: não ocorreram eventos adversos durante a condução do estudo. 6. Resultados já apresentados em congresso? Resp: sim, os resultados foram apresentados durante o desenvolvimento do projeto, com 3 trabalhos como primeiro autor já apresentados em congressos internacionais e o aceite de mais 4 (3 como primeiro autor), que serão apresentados ao longo de 2017. Tais congressos foram/serão: - II Congresso da Wildlife Diseases Association- Latin America (WDA-LA), Bogotá, Colômbia, 24-27 de Setembro de 2015. - 21st Biennial Conference on the Biology of Marine Mammals, San Francisco, Estados Unidos da America, 13- 18 de Dezembro de 2015. - 12th Conferência da European Wildlife Disease Association (EWDA). Berlim, Alemanha, 26-31 de Agosto de 2016. - 66th Conferência anual da Wildlife Diseases Association (WDA), Terceira Conferência bienal da Wildlife Diseases Association Latin America (WDA-LA), Quinto Congresso Internacional KalaanKab em Ecologia das Doenças, a ser realizado em San Cristóbal de las Casas, México, nos dias 23-28 de Julho de 2017. - 22nd Biennial Conference on the Biology of Marine Mammals, 22-27 de Outubro de 2017, Halifax, Canadá. 7. Resultados já publicados? Resp: Sim, resultados parciais já foram publicados (Sacristán et al. 2016, Diseases of Aquatic Organisms 117: 229235) e cinco trabalhos mais estão sendo elaborados, pendentes de submissão. ".

Comentário da CEUA: "O experimento foi realizado como descrito no projeto de pesquisa, sendo suficiente para a conclusão do estudo um número de cetáceos menor (n=115) do que o previsto inicialmente (n=300). ".

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 48279-1	Data da Emissão: 23/04/2015 11:13	Data para Revalidação*: 22/05/2016

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Katia Regina Groch	CPF: 739.751.419-72
Título do Projeto: Pesquisa e Caracterização da Morbilivirose em Cetáceos do Brasil	
Nome da Instituição : Faculdade de Medicina Veterinária e Zootecnia USP	CNPJ: 63.025.530/0019-33

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Análise dos resultados	04/2015	03/2018
2	Redação de trabalhos e divulgação dos resultados	04/2015	03/2018
3	Análises laboratoriais	04/2015	03/2019
4	Colheita e transporte de tecidos de mamíferos marinhos do litoral brasileiro	04/2015	12/2019

Observações e ressalvas

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Outras ressalvas

1	Os pesquisadores: Angélica María Sánchez Sarmiento e CARLOS SACRISTAN YAGUE possuem vinculo de Programa de bolsas ou auxilio à pesquisa patrocinado pela CAPES. Dispensados de autorização do Ministério da Ciência, Tecnologia e Inovação.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	José Luiz Catão Dias	Supervisor	029.597.888-00	8914397-8 SSP-SP-SP	Brasileira
2	Angélica María Sánchez Sarmiento	Pesquisadora colaboradora	234.040.368-59	V663133-A DPF-SP	Estrangeira
3	CARLOS SACRISTAN YAGUE	Pesquisador colaborador		V932606W DPF-SP	Estrangeira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo

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1		AL	Litoral	Fora de UC Federal
2		BA	Litoral	Fora de UC Federal
3		CE	Litoral	Fora de UC Federal
4		ES	Litoral	Fora de UC Federal
5		PB	Litoral	Fora de UC Federal
6		PE	Litoral	Fora de UC Federal
7		RJ	Litoral	Fora de UC Federal
8		RS	Litoral	Fora de UC Federal
9		SC	Litoral	Fora de UC Federal
10		SE	Litoral	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de amostras biológicas in situ	Otariidae, Mustelidae, Cetacea, Phocidae, Sirenia

Material e métodos

1	Amostras biológicas (Carnívoros)	Fragmento de tecido/órgão, Animal encontrado morto ou partes (carcaça)/osso/pele, Sangue
2	Amostras biológicas (Mamíferos Aquáticos:cetáceos, sirênios e pinípedes)	Animal encontrado morto ou partes (carcaça)/osso/pele, Fragmento de tecido/órgão, Sangue
3	Método de captura/coleta (Carnívoros)	Outros métodos de captura/coleta(necropsias e amostras em acervo)
4	Método de captura/coleta (Mamíferos Aquáticos:cetáceos, sirênios e pinípedes)	Outros métodos de captura/coleta(necropsias e amostras em acervo), Captura manual

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	Faculdade de Medicina Veterinária e Zootecnia USP	coleção

Código de autenticação: 69718769

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Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº 03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

* Identificar o espécime no nível taxonômico possível.

Código de autenticação: 69718769

FOLHA DE AVALIAÇÃO

Autor: SACRISTÁN YAGÜE, Carlos

Título: Research and characterization of selected pathogens of cutaneous and mucocutaneous lesions in cetaceans from the Brazilian coast.

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Data: _____/_____/_____

Banca Examinadora

Prof. Dr._____

Instituição:_____Julgamento:_____

Prof. Dr._____

Instituição:_____Julgamento:_____

Prof. Dr._____

Instituição:_____Julgamento:_____

Prof. Dr._____

Instituição:_____Julgamento:_____

Prof. Dr._____

Instituição:_____Julgamento:_____

Prof. Dr._____

Instituição:_____Julgamento:_____

Este trabajo está

dedicado a mis abuelos:

Natividad Velasco García,

Segundo Sacristán Centeno,

Marcelo Primitivo Yagüe Calonge

y Ramona Ferrer Oliván,

y a mi tía abuela Andresa Ferrer Oliván.

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A **Cetacean Society International**, pela ajuda financeira para o desenvolvimento desse projeto.

Tudo passa e tudo fica,
Porém o nosso é passar,
Passar fazendo caminhos,
Caminhos sobre o mar,

Nunca persegui a glória,
Nem deixei na memória
Dos homens minha canção
Eu amo os mundos subtis,
Leves e gentis,
Como bolas de sabão

Gosto de vê-las pintar-se
De sol e grená, voar
Debaixo do céu azul, tremer
Subitamente e quebrar-se...

Nunca persegui a glória

Caminhante, são tuas pegadas
O caminho e nada mais;
Caminhante, não caminho,
Se faz caminho ao andar

Ao andar se faz caminho
E ao voltar a vista atrás
Se vê a senda que nunca
Se há de voltar a pisar

Caminhante não há caminho,
Senão sulcos no mar...

Antonio Machado

RESUMO

SACRISTÁN YAGUE, C. **Pesquisa e caracterização de patógenos cutâneos e mucocutâneos selecionados em cetáceos da costa brasileira.** [Research and characterization of selected pathogens of cutaneous and mucocutaneous lesions in cetaceans from the Brazilian coast]. 2017. 181 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Cetáceos são sentinelas do ambiente marinho, atualmente ameaçados por diversos fatores, principalmente antropogênicos. Os processos de pele e mucosas externas são os mais facilmente identificados, bons indicadores do estado de saúde em cetáceos. Lesões cutâneas e mucocutâneas já foram amplamente relatadas em cetáceos de vida livre e de cativeiro, mas pouco se sabe a respeito dos fatores etiológicos envolvidos, evolução das lesões dermatológicas e suas consequências sistêmicas. Vírus são os agentes mais comumente envolvidos em lesões cutâneas e mucocutâneas, especialmente os herpesvírus (HV), associados a lesões de morfologias variáveis em mucosas, e poxvírus dos cetáceos (Cetacean Poxvirus – CePV), principalmente associados a lesões de pele "tatuagem" ou "anel" características. Agentes fúngicos também podem causar doenças dermatológicas em cetáceos, como por exemplo a paracoccidioidomicose ceti, caracterizada por lesões esbranquiçadas elevadas e proliferativas, causada por leveduras não-cultiváveis de *Paracoccidioides brasiliensis* (ordem Onygenales). Apesar de mundialmente reportados, a ocorrência desses agentes etiológicos em cetáceos do Atlântico Sul ainda é pouco compreendida. O objetivo desse estudo foi identificar e caracterizar patógenos cutâneos e mucocutâneos selecionados (HV, CePV e *P. brasiliensis*) de cetáceos brasileiros de vida livre e desenhar métodos diagnósticos mais sensíveis para sua detecção. Todos os animais estudados encalharam ao longo da costa brasileira, entre 2005 e 2015, exceto por três botos-cor-de-rosa que foram fisicamente imobilizados e liberados após a coleta de amostras. Para atingir tais objetivos, empregamos técnicas moleculares e histológicas, e ocasionalmente de imunohistoquímica e microscopia eletrônica. A presença de HV e CePV foi avaliada, respectivamente, em amostras cutâneas e de mucosa oral e genital de 115 espécimes, e amostras de pele de 113 indivíduos; enquanto a presença de membros da ordem Onygenales foi avaliada em quatro espécimes que apresentavam lesões macroscópicas compatíveis. Amostras de pele ou de mucosa oral de quatro animais foram positivas para a PCR de HV: uma lesão ulcerada de pele de coloração esbranquiçada de um boto-cinza (*Sotalia guianensis*), uma amostra de tecido lingual de um golfinho-pintado-do-Atlântico (*Stenella frontalis*), lesões ulcerativas e amostras de pele saudável de um cachalote-anão (*Kogia sima*), e uma lesão proliferativa de pele em boto-vermelho-boliviano (*Inia boliviensis*). Os primeiros três animais estavam infectados com alphaherpesvirus. Uma sequência mais similar com gammaherpesvírus foi obtida da lesão proliferativa de pele do boto-vermelho-boliviano. A sequência do boto-vermelho-boliviano possivelmente pertence a um novo gênero de gammaherpesvírus. Ademais, todas as outras amostras de tecidos disponíveis dos espécimes HV-positivos, à parte de pele e mucosa oral, também foram avaliadas por técnicas de PCR e histológicas. Uma sequência diferente de alphaherpesvírus foi encontrada no estômago e em um linfonodo mesentérico do

cachalote-anão. Achados microscópicos em dois animais HV-positivos (dermatites proliferativas em boto-vermelho-boliviano e boto-cinza) eram compatíveis com HV. CePV foi identificado em lesões de pele do tipo “tattoo” de um golfinho-nariz-de-garrafa (*Tursiops truncatus*) e de um boto-cinza por meio de técnicas moleculares estabelecidas, e observação de partículas de poxvírus por microscopia eletrônica. Animais CePV-positivos apresentavam degeneração balonosa epidérmica e ocasionais inclusões intracitoplasmáticas anfofílicas ou eosinofílicas compatíveis com CePV. Motivos aminoácidos específicos para todos os CePVs também foram identificados, reforçando a sugestão de um novo gênero, chamado *Cetaceanpoxvirus*. Nesse estudo também foram desenvolvidas novas técnicas de PCR convencional e real-time com SYBR® Green, significativamente mais sensíveis do que os métodos atualmente disponíveis em literatura. Um boto-cinza, inicialmente negativo segundo os métodos de PCR previamente conhecidos foi diagnosticado positivo para CePV por meio das novas técnicas aqui descritas. Leveduras refratáveis (4−9 µm de diâmetro) foram observadas á microscopia sob a forma de lesões de pele granulomatosas moderadas e necróticas em quatro golfinhos-nariz-de-garrafa, e pela primeira vez, em um abscesso muscular (esse último um indício do potencial invasivo desse agente). Leveduras de Onygenales sp. foram identificadas em lesões de pele por meio de imunohistoquímica e uma sequência de *P. brasiliensis* mais semelhante (100% de identidade de nucleotídeos) áquela descrita em um gofinho-nariz-de-garrafa de Cuba do que a casos de humanos e mamíferos descritos no Brasil, foi encontrada em lesões de pele de um dos espécimes. Esse estudo relata a primeira identificação molecular de HV em cetáceos da América do Sul e em golfinhos de rio no mundo. Além disso, descrevemos a primeira amplificação de CePV e *P. brasiliensis* em odontocetes da América do Sul, confirmando a etiologia desse tipo de lesões. Quatro das cinco novas sequências de HV identificadas são possivelmente novas espécies, provisoriamente chamadas *Delphinid HV-10*, *Kogiid HV-2*, *Kogiid HV-3* e *Iniid HV-1*.

Palavras-chave: Patologia. Dermatologia. Herpesvírus. Poxvírus. *Paracoccidioides brasiliensis*.

ABSTRACT

SACRISTÁN YAGUE, C. **Research and characterization of selected pathogens of cutaneous and mucocutaneous lesions in cetaceans from the Brazilian coast.** [Pesquisa e caracterização de patógenos cutâneos e mucocutâneos selecionados em cetáceos da costa brasileira]. 2017. 181 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Cetaceans are sentinels of the marine environment, currently threatened by many factors, mainly anthropogenic. The most easily identified compromising conditions are those affecting the skin and external mucosae - good indicators of the cetacean's health status. Cutaneous and mucocutaneous lesions have been extensively reported in wild and captive cetaceans, but little is known about the involved etiological factors, evolution of the dermatological lesions and their systemic consequences. Viruses are the most commonly involved agents in cutaneous and mucocutaneous lesions, especially herpesviruses (HV) and, associated with skin and mucosal lesions with varying morphologies, and cetacean poxviruses (CePV), mainly associated with characteristic "tattoo" or "ring" skin lesions. In addition, fungal agents are also recognized as causative agents of dermatological disease in cetaceans, especially in the process known as paracoccidioidomycosis ceti, observed as raised proliferative whitish lesions, caused by non cultivable yeast of *Paracoccidioides brasiliensis* (order Onygenales). Despite being reported worldwide, the occurrence of these etiological agents in southern Atlantic cetaceans is still poorly understood. The goal of this study was to identify and characterize selected cutaneous and mucocutaneous pathogens (HV, CePV and *P. brasiliensis*) of free-ranging cetaceans from Brazil, and to design more sensitive diagnostic methods for their detection. All the studied cetaceans stranded along the coast of Brazil, between 2005 and 2015, except three wild riverine dolphins that were physically contained and released after sample collection. In order to achieve our goals, we employed molecular, histopathological, and occasionally immunohistochemical and electron microscopy techniques. The presence of HV and CePV was evaluated in cutaneous, and oral and genital mucosal samples from 115 specimens and skin samples from 113 individuals, respectively; whereas the presence of members of the genus *Onygenales* sp. was evaluated in four specimens presenting macroscopic compatible lesions. Skin or oral mucosal samples from four animals were HV PCR-positive: a whitish ulcerated skin lesion from a Guiana dolphin (*Sotalia guianensis*), a lingual sample from an Atlantic spotted dolphin (*Stenella frontalis*), ulcerative lesions and healthy skin samples from a dwarf sperm whale (*Kogia sima*), and a proliferative skin lesion from a Bolivian river dolphin (*Inia boliviensis*). The tree first animals were infected with alphaherpesvirus. A sequence more similar to gammaherpesvirus was obtained from the Bolivian river dolphin's proliferative skin lesion. The Bolivian river dolphin sequence could possibly be a member of a new gammaherpesvirus genus. Additionally, all other available tissue samples from HV-positive specimens, aside from skin and oral mucosa, were also tested by PCR and histologically evaluated. A different alphaherpesvirus sequence was found in the stomach and in a mesenteric

lymph node of the dwarf sperm whale. Microscopic findings in two HV-positive animals (chronic proliferative dermatitis in Bolivian river dolphin and Guiana dolphin) were compatible with HV. CePV was identified in “tattoo” skin lesions of an Atlantic bottlenose dolphin and a Guiana dolphin by established molecular methods, and poxviral particles were observed by electron microscopy. CePV-positive animals presented epidermal ballooning degeneration and occasionally small, pale eosinophilic or amphophilic intracytoplasmic inclusions, compatible with CePV. Specific amino acid motifs for all CePV were also identified, reinforcing the suggestion of the new *Cetaceanpoxvirus* genus. We also designed novel SYBR® Green real-time and conventional CePV PCR methods significantly more sensitive than those currently available in the literature. An additional Guiana dolphin, previously negative based in established PCR methods was diagnosed positive for CePV through these new techniques. Refractile yeasts (4−9 µm in diameter) were observed under light microscopy in mild granulomatous and necrotic skin lesions of four Atlantic bottlenose dolphin, and for the first time, in a skeletal muscle abscess (the former possibly indicating the invasive potential of the agent).*Onygenales* sp. yeasts were identified in skin lesions by immunohistochemistry and a sequence of *P. brasiliensis*, more similar (100% nucleotide identity) to the one described in an Atlantic bottlenose dolphin from Cuba than to human or any other terrestrial mammals cases in Brazil, was obtained from the skin lesion of one of the specimens, confirming the etiological agent of these type of lesions. Herein we report the first molecular identification of HV in South American cetaceans and in riverine dolphins worldwide. This study also describes the first amplification of CePV and *P. brasiliensis* in odontocetes from South America. Four of the five novel herpesvirus sequences herein identified are possibly novel species, tentatively named *Delphinid HV-10*, *Kogiid HV-2*, *Kogiid HV-3* and *Iniid HV-1*.

Keywords: Pathology. Dermatology. Herpesvirus. Poxvirus. *Paracoccidioides brasiliensis*.

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SUMMARY

1 GENERAL INTRODUCTION

The ecosystem health could be defined as the lack of signs of ecosystem distress, its resilience (defined as the ability to recover rapidly and complexly from an injury), and/or the absence of risk or threats towards the ecosystem's composition, structure and/or function (RAPPORTS, 1995). In order to maintain biodiversity, preserve healthy ecosystems is necessary (HILTY; MERENLENDER, 2000).

One of the most used methods to assess ecosystem health is the measurement of the effects of a phenomenon or substance of interest over a species used as a sensor, known as indicator species - usually invertebrates, but also of animals of upper trophic level (HILTY; MERENLENDER, 2000; CARIGNAN; VILLARD, 2002; HEINK; KOWARIK, 2010).

Cetaceans are important indicator species, used as sentinels of marine and riverine environments due to their long life spans, high position at the food chain, storage ability of their large fat deposits (e.g., anthropogenic pollutants) and shared sensitivity to certain pathogens (e.g. *Toxoplasma gondii*), toxins and chemicals with humans (REDDY et al., 2001; WELLS et al., 2004; MOORE et al. 2008; BOSSART, 2011; WISE et al., 2009; GIBSON et al., 2011). The study of resident populations, e.g., Atlantic bottlenose dolphin (*Tursiops truncatus*), Guiana dolphin (*Sotalia guianensis*), beluga whale (*Delphinapterus leucas*), provides additional information on specific geographical areas of the marine environment (DE GUISE; LAGACÉ; BÉLAND, 1994; SIMÕES-LOPES; FABIAN, 1999; AZEVEDO et al., 2017). Cetaceans are charismatic aquatic megafauna species that arise very strong human empathy, easily observed at their natural habitat (BOSSART, 2011), subject of a growing whale and dolphin watching tourism. Cetacean-associated economical activities also include hunting, either for commercial or livelihood purposes (REEVES, 2002; TRYLAND et al., 2014; DA SILVA JÚNIOR, 2017), a commonly controversial issue, also strongly related with cultural values.

Two clades are recognized in the unranked Cetacea taxon: Odontoceti (dolphins, porpoises and toothed whales) and Mysticeti (baleen whales), respectively comprised of by ten and four families (Box 1).

Box 1 - Clades (Odontoceti and Mysticeti), families and cetacean species.

CLADE		FAMILIY	SPECIES
Cetacea	Odontoceti	Physeteridae	sperm whale (*Physeter macrocephalus*)
		Kogiidae	pygmy sperm whale (*Kogia breviceps*) dwarf sperm whale (*K. sima*)
		Ziphiidae (beaked whales)	e.g., Hector's beaked whale (*Mesoplodon hectori*)
		Platanistidae	Indian river dolhin (*Platanista gangetica*)
		Iniidae (pink river dolphins)	e.g. Bolivian river dolphin (*Inia boliviensis*)
		Lipotidae	baiji (*Lipotes vexillifer*)
		Pontoporiidae	Francisca/toninha (*Pontoporia blainvillei*)
		Monodontidae (belugas and narwhals)	beluga (*Delphinapterus leucas*) narwhal (*Monodon monoceros*)
		Delphinidae (true dolphins)	e.g., Atlantic bottlenose dolphin (*Tursiops truncatus*)
		Phocoenidae (true porpoises)	e.g., Burmeister's porpoise (*Phocoena spinipinnis*)
	Mysticeti	Balaenidae (right whales and bowhead whale)	e.g., southern right whales (*Eubalaena australis*) bowhead whale (Balaena mysticetus)
		Neobalaenidae	pygmy right whales (*Caperea marginata*)
		Eschrichtiidae	grey whale (*Eschrichtius robustus*)
		Balaenopteridae (rorquals)	e.g., Bryde's whale (*Balaenoptera brydei*)

All of these families are part of a larger group, the order Cetartiodactyla, which also comprises the Artiodactyla (e.g., suborders Suina [pigs and boars], Tylopoda [camels and llamas], and Ruminantia [deer, giraffes and cows]) (TRUJILLO et al., 2010; GRAVENA et al., 2014; COMMITTEE ON TAXONOMY, 2016). The divergence between cetaceans and their terrestrial ancestors occurred approximately 53 Million years ago (ARNASON; GULLBERG; JANKE, 2004). Since then, these organisms have adapted to an obligate aquatic life cycle through special adaptive mechanisms, such as echolocation (odontocetes), filter-feeding (mysticetes), presence of a

vestigial pelvis girdle, loss of hind limbs at the end of the fetal development, and other modifications in organs, such as kidneys, lungs, gonads, internal ears and skin (e.g., absence of sebaceous or apocrine glands) (ROMMEL; LOWENSTINE, 2001; THEWISSEN et al., 2006; DINES et al., 2014; PYENSON, 2017). A high number of cetacean species is present in Brazil, a megadiverse country, with at least 48 riverine, coastal or pelagic described cetacean species (LODI; BOROBIA, 2013; GRAVENA et al., 2014; CYPRIANO-SOUZA et al., 2016). This rich diversity is promoted by three main marine currents: Malvinas/Falklands, North Brazil, and Brazil, and two riverine basins (Amazonas and Tocantins), creating different ecosystems (LODI; BOROBIA, 2013).

The study of wildlife health, through the evaluation of the etiology, occurrence and prevalence of diseases, is a very important branch of conservation management, not to mention the application of such processes as sensitive indicators of anthropogenic impacts (DEEM; KARESH; WEISMAN, 2001). Cetacean health studies may have even further significance, especially when it comes to raising the general public's awareness about the deterioration of the marine environment (BOSSART, 2011).

The Health Concept comprises not only the absence of disease, but also the interaction among environmental, biological and social parameters that influence the organisms' adaptation to environmental changes and populations' resilience (STEPHEN, 2014). Due to ethical and logistic issues, the most common way to assess the health status of free-ranging cetacean populations is through the study of stranded animals. For instance, the first identification of morbillivirus in cetaceans was performed in stranded dead striped dolphins (*Stenella coeruleoalba*) (DOMINGO et al., 1990; GERACI; LOUNSBURY, 2005). Other alternative methods adapted to the study of cetaceans' health include exhaled breath analysis and skin biopsies (NOREN; MOCKLIN, 2012; RAVERTY et al., 2017). These studies have clarified many of the current cetacean morbidity and mortality causes, such as: (1) fishing interaction and competition, (2) hunting, (3) pollution: debris, heavy metals, organic pollutants (e.g., the high of neoplasm prevalence in the St. Lawrence River's beluga whale [*Delphinapterus leucas*] population in Canada, associated with polycyclic aromatic hydrocarbons, and reproduction impairment in odontocetes from European waters associated with polychlorinated biphenyls), (4) ship collision and acoustic pollution, (5) biotoxins, (6) the effects of climate change (e.g., oceanic acidification,

alterations in the food chain), and (7) infectious diseases (DE GUISE; LEGACE; BÉLAND, 1994; MARTINEAU et al., 2002; WRIGHT et al., 2007; FERNÁNDEZ et al., 2008; PARSONS et al., 2008; MARIGO et al., 2010; KAPLAN et al., 2013; LITZ et al., 2014; JEPSON; DEAVILLE; LAW, 2016; UNGER et al., 2016).

Diseases can affect wild population's reproductive trends, survival and dispersal (GULLAND; HALL, 2006). Initially, wildlife infectious diseases were only valued when the wildlife-livestock interface was involved or when considered potential zoonoses. Nevertheless, the concern over their impact and threat to the biodiversity is currently increasing (DASZAK; CUNNINGHAM; HYATT, 2000; DE CASTRO; BOLKER, 2004). One must also consider the differences between the terrestrial and aquatic environments. In the marine environment, some diseases may spread at a faster rate than on the terrestrial environment, as seen in certain viral epizootics reported in fish (Australian pilchard, *Sardinops sagax neopilchardus*) by herpesvirus (10,000 km/year), and phocids and cetaceans by morbillivirus (3,000 km/year) (MCCALLUM; HARVELL; DOBSON, 2003; WHITTINGTON et al., 2008).

Diseases in cetaceans, including of several emerging pathogens, e.g., morbillivirus, poxvirus, herpesvirus, *Brucella ceti*, *T. gondii*, *Paracoccidioides brasiliensis*, have been increasingly reported (ESPERON; FERNANDEZ; SANCHEZ-VIZCAINO, 2008; VAN BRESSEM et al., 2009; BELLIERE et al., 2011; GONZALES-VIERA et al., 2013; GROCH et al., 2014; SIMEONE et al. 2015; VILELA et al., 2016). However, assessing cetacean diseases' spatiotemporal trends, as observed in other marine species, such as corals, is very challenging (SIMEONE et al., 2015). Cetaceans are long-live animals that generally produce only one calf per year; therefore, infectious diseases that compromise reproductive success and/or fertility (e.g., brucellosis, toxoplasmosis, sarcocystosis by *Sarcocystis neurona*) could negatively impact cetacean populations (MILLER et al., 1999; JARDINE; DUBEY, 2002; BARBOSA et al., 2015). In addition, physiological stress - increasingly associated with anthropogenic impact over the marine and riverine environments - can impair the cetacean immune system, increasing its susceptibility to infectious diseases (WRIGHT et al., 2007; REIF et al., 2009; JEPSON; DEAVILLE; LAW, 2016).

The study of infectious diseases affecting the skin and oral and genital mucosa of cetaceans is considered especially relevant for several reasons: (1) the skin is directly evaluated through visual assessment, which is sometimes the only or

the main available resource in field studies; (2) although infectious cutaneous and mucosal diseases are usually not fatal, and often self-limiting, they may serve as entry routes for other pathogens; (3) several systemic diseases cause cutaneous and oral mucosal alterations (e.g., herpesvirus, erysipelas); and (4) cutaneous and oral and genital mucosal alterations may be good indicators of cetaceans' health status (BOSSART; EIMSTAD, 1988; SCHULMAN; LIPSCOMB, 1999; PETTIS et al., 2004; BOSSART et al., 2008; REIF et al., 2009; VAN BRESSEM et al., 2009; SIERRA et al., 2014). Cutaneous and mucocutaneous alterations have been observed both in wild and in captive cetaceans, since the 50's (SIMPSON; WOOD; YOUNG, 1958); however, most of the time, the involved etiological agents were not identified (MALDINI et al., 2010; FURY; REIF, 2012; GROCH, 2014).

Cutaneous and mucocutaneous lesions are usually benign, although they may occasionally be fatal, and present different distributions, characteristics, sizes and presentations (unique or multiple lesions). The main etiological agents known to affect cetacean's skin, oral and genital mucosa are viruses (e.g., herpesvirus, poxvirus and papillomavirus) and fungal agents (*P. brasiliensis*) (SWEENEY; RIDGWAY, 1975; VAN BRESSEM; VAN WAEREBEEK; RAGA, 1999; VAN BRESSEM et al., 2009; ESPERON et al., 2012; VILELA et al., 2016).

Herpesviruses present a wide distribution and affect most animal species (PELLETT; ROIZMAN, 2007). The first cases of herpesviral infection in cetaceans were reported in the 80's by electron microscopy (MARTINEAU et al., 1988; BARR et al., 1989). Since then, with the advent of molecular techniques based on the employment of universal primers, novel sequences related to cutaneous and mucocutaneous processes have been described (SMOLAREK-BENSON et al., 2006; VAN ELK et al., 2009; SIERRA et al., 2014). In cetaceans, herpesviruses may cause skin, oral and genital lesions, occasionally characterized by loss of pigmentation or proliferative nodules (VAN BRESSEM et al., 1994; HART et al., 2012). This latter manifestation of the disease is currently surrounded by controversy on whether they are caused by gamma-herpesvirus or by papillomavirus (REHTANZ et al., 2012).

Papillomaviruses cause tumors (papillomas) - mainly in the oral and genital mucosa - in several cetacean species (LAMBERTSEN et al., 1987; REHTANZ et al., 2006; VAN BRESSEM et al., 2007; RECTOR et al., 2008). Large oral papillomas may affect feeding, whereas genital papillomas could compromise reproduction (VAN BRESSEM; VAN WAEREBEEK; RAGA, 1999; VAN BRESSEM et al., 2009).

Poxviruses have been reported in cetaceans since the late 70's (FLOM; HOUK, 1979; GERACI; HICKS; ST AUBIN, 1979; BRACHT et al., 2006), associated with characteristic skin lesions presenting melanic margins and a stippled interior, known as "tattoo"-lesions (GERACI; HICKS; ST AUBIN, 1979; BRACHT et al., 2006).

Paracoccidioidomycosis ceti, previously known as lobo's disease, lacaziosis, lacaziosis-like disease, lobomycosis or lobomycosis-like disease, is a cetacean cutaneous disease firstly identified in the 70's (MIGAKI et al., 1971), caused by *P. brasiliensis* non-cultivable yeasts of the order Onygenales (ROTSTEIN et al., 2009; ESPERON et al., 2012; UEDA et al., 2013; VILELA et al., 2016), closely related to *Lacazia loboi*, the etiological agent of lacaziosis in humans (VILELA et al., 2016). Clinical signs include chronic cutaneous, well demarcated, firm, proliferative, ulcerative or verrucous, whitish- to grayish colored, and occasionally pink lesions (MIGAKI et al., 1971).

In Brazil, papillomatosis and poxvirus-like lesions have been reported, respectively, in a rough-toothed dolphin (*Steno bredanensis*) (GONZALES-VIERA et al., 2011; GONZALES-VIERA et al., 2012) and in Guiana dolphins (GONZALES-VIERA et al., 2012). Skin lesions caused by yeast similar to those observed in paracoccidioidomycosis ceti cases were also described in the country in 1993, in an Atlantic bottlenose dolphin (SIMÕES-LOPES et al., 1993), and suggestive macroscopic lesions have been observed in an Atlantic bottlenose dolphin population (DAURA-JORGE; SIMÕES-LOPES, 2011; VAN BRESSEM et al., 2015) and in Guiana dolphins (VAN BRESSEM; SANTOS; OSHIMA, 2009).

In Brazil, studies of infectious agents related to cetacean cutaneous and mucocutaneous lesions are scarce, with limited histopathological descriptions and complete absence of immunohistochemical and/or molecular identification.

2. LITERATURE REVIEW

2.1 CETACEAN'S SKIN, ORAL AND GENITAL MUCOSA

In vertebrates, the skin or integument is the largest organ, and along with its normal microbiota constitutes a barrier against the environment, including injuries and/or infections (GINN; MANSELL; RAKICH, 2007; NESTLE et al., 2009). Aside from innate and adaptive immune function, the skin is a major sensory organ that plays several important roles, including: homeostasis, thermoregulation, photoprotection, metabolism of nutrients, steroidogenic function (synthesis or metabolism of various steroid hormones), detoxification pathways of xenobiotics, and in some cases, patterns or secreting substances that camouflage, attract, or repel aggressors (MONTAGNA, 1967; GINN; MANSELL; RAKICH, 2007; TIMAR et al., 2007; MONTIE et al., 2008; MANTEL, 2012).

The integument comprises the epidermis, dermis, hair follicles, adnexal glands, and subcutaneous tissue (GINN; MANSELL; RAKICH, 2007). In most mammal species, the epidermis is formed by four main layers (from the deepest to the most superficial): stratum basale or germinative (a single-cell layer formed by cuboid to slightly columnar cells in contact with the basal membrane, and presenting mitotic activity), stratum spinosum or intermedium (variable number of polyhedral to moderate flattened cell layers, some of them specialized in the production of proteins), stratum granulosum (one or two cell layers of flattened cells, present only in some species), and stratum corneum (with a variable number of layers, usually formed by anucleate flattened dead cells, cornified, physically sustained by keratinized cells and extracellular lipids) (GINN; MANSELL; RAKICH, 2007). Approximately 90% of the epidermal cells are keratinocytes, which form a physical barrier and act as immune sentinels. Melanocites are also important cells, responsible for the production of the melanin pigment, and Langerhans cells; this latter presenting immune function - once activated, is able to phagocytize antigens and migrate to the lymph nodes to activate the adaptive immune system (NESTLE et al., 2009). The dermis is composed mainly by collagen and elastic fibers embedded in a ground substance, nerves, blood and lymphatic vessels, and a small quantity of

mixed cells (e.g., mast cells). In some mammal species (e.g., pigs, humans) the dermis contacts with the epidermis through the dermal papillae, which carries innervation, and vascular and lymphatic irrigation; additionally most mammal species present sebaceous and sweat glands, and hair follicles (GINN; MANSELL; RAKICH, 2007). The subcutaneous tissue below the dermis is formed basically by lipocytes, small vessels and collagen bands (GINN; MANSELL; RAKICH, 2007).

The cetacean skin presents some specific modifications, acquired through millions of years, related to their evolutionary adaptation to the aquatic environment (ARNASON; GULLBERG; JANKE, 2004). Due to their strictly aquatic life, cetacean skin faces constant water friction, large heat losses (compared to air, water is approximately 800 times denser, 60 times more viscous and conducts the heat 24 times faster) and increased pressure when diving, although there are variations between the different types of water (DEJOURS, 1987; PISCITELLI et al.; 2010). Furthermore, the aquatic environment harbors a remarkably large number of microorganisms (DAS; LYLA; KHAN, 2006).

In response to such conditions, cetaceans have developed especially adapted epidermal, dermal and hypodermal layers (REEB; BEST; KIDSON, 2007). For instance, moulting or ecdysis, the periodically replacement of the outer epidermis, has been described in some species of mysticetes (REEB; BEST; KIDSON, 2007). However, the most evident characteristic is the lack of fur, promoting an easy visualization of the cetacean epidermis, and consequently, of its alterations (HARZEN; BRUNNICK, 1997, REIF et al., 2009). Nonetheless, the absence of hair follicles is partial; they can be observed on the upper lip of newborn odontocetes and on the head of adult southern right whales (*Eubalaena australis*), presenting innervation and blood sinuses, similar to tactile vibrissae from terrestrial animals (PALMER; WEDDEL, 1963; ROMMEL; LOWENSTINE, 2001). From the outer to the inner layers, the epidermis is organized in three strata: stratum externum (a preferred term over corneum, given that it is parakeratinized rather than keratinized), intermedium (spinosum) and basale (germinativum), lacking the granulosum stratum present in most terrestrial mammals (ROMMEL; LOWENSTINE, 2001; REEB; BEST; KIDSON, 2007; ZABRA, ROMANO, 2003).

The stratum externum is parakeratinized (ZABRA, ROMANO, 2003), incompletely cornified, the opposite of what is observed in terrestrial mammals, and formed by flattened cells with nuclei and organelles, and extensive intracellular

keratin fibers and intracellular lipid droplets (MOUTON; BOTHA, 2012). The surface of the stratum externum is coated by a gel that promotes unfavorable conditions for microorganism, although bacteria, fungi, and biofilm-forming diatoms have been described as part of the commensal microbiota (NEMOTO, 1920; BAUM et al., 2001; APPRILL et al., 2014; REEB et al., 2010). The stratum intermedium, the thickest layer, formed by migrating keratinocytes, and the single-cell thick stratum basal form deep and highly convoluted projections called “rete pegs”, that interdigitate with dermal papillae into the dermis, increasing its surface area (SOKOLOV et al., 1969; ROMMEL; LOWENSTINE, 2001; ZABRA, ROMANO, 2003).

The cetacean epidermis is thick (e.g., approximately 1.5 mm thick in bottlenose dolphins [*Tursiops truncatus*]), up to 20 times thicker than terrestrial mammals’, due to its high cell production rate (in Atlantic bottlenose dolphins the mitotic activity is estimated around 35-40 times greater than in terrestrial mammals), and a prolonged lipokeratinocyte life span (BROWN et al. 1983; HICKS et al. 1985). The main cell type present in cetacean epidermis is the lipokeratinocyte, distinct from the keratinocytes observed in terrestrial mammals, which not only produce keratin, but lipid droplets that contribute to the mechanical strength, buoyancy, and insulation of cetacean skin (MENON et al. 1986), and peptides (e.g., pro-inflammatory cytokines - β-defensin-2 and -3 and lysozyme) that act as a non-specific defense against bacteria, fungi, algae and ectoparasites (MEYER; SEEGARS, 2004). The melanocytes are similar to those described in terrestrial mammals. Microscopic calcareous concretions are also present in the odontocete epidermis (BEHRMANN, 1996).

The dermis is formed by a collagenous stroma, well vascularized and innervated, also presenting lymphatic vessels, but lacking adnexal structures, e.g., sebaceous or sweat glands (ZABRA, ROMANO, 2003). Specialized cells, such as Langerhans-like cells, dermal dendritic cells and/or macrophages are also present in the dermis (ZABRA, ROMANO, 2003). In cetaceans, the hypodermis is known as “blubbler”, a specialized fatty layer disposed between the dermis and the musculature, formed by adipocytes surrounded by vasculature, nerves, and collagen and elastin structural fibers, separated from the muscle by the subcutis (ROMMEL; LOWENSTINE, 2001).

In mammals, the oral mucosa (the mucous membrane lining the interior of the mouth) is formed by an inner epithelium and a middle lamina propria in contact with

the underliying connective tissue. The epithelium is stratified squamous, keratinized in certain places, e.g., as tongue, hard palate and check; and present duct of gland, e.g., salivary glands. The tongue has various papillae, mainly on its dorsal surface, some of them associated to the sense of taste (BACHA; BACHA, 2000a). The genital mucosa of the penis and the vagina is composed by a stratified squamous epithelium, presenting different glands, and a lamina propria, in contact with the submucosa (BACHA; BACHA, 2000b; MESCHER, 2013a, b).

The cetaceans' oral and genital mucosa is composed by three strata (stratum externum, intermedium and basal) (VAN BRESSEM et al., 1996; SIERRA et al., 2015; GUIMARÃES et al., 2011). The epidermis of the female genital slit vulva is lined by a stratified squamous epithelium, with different cell types; the vagina is lined by transitional-type epithelium, presenting vaginal glands (ROMMEL; LOWENSTINE, 2001; BECEGATO et al., 2015). In the tongue, the stratification and keratiniztion of the lingual epithelium is a common characteristic for most of the mammals, including cetaceans, and present three layers: basal, spinous granular and corneous; salivary gland ducts are also observed (GUIMARÃES et al., 2011, 2012). Differences in the number, size, shape and functions of the lingual papillas could be observed between different cetacean species; papillary projections of the lingual margins are usually present in young animals (GUIMARÃES et al., 2012). According to Zhu et al. (2014), cetaceans have loss all taste modalities (sour, sweet, bitter and umami), except salt.

Cutaneous and oral and genital mucosa diseases have been reported in wild and captive cetaceans all over the world (SIMPSON; WOOD; YOUNG, 1958; BOSSART; EIMSTAD, 1988; SIMÕES-LOPES et al., 1993; BOSSART et al., 2008; FURY; REIF, 2012; UEDA et al., 2013), and the number of reports in some species have apparently increased, possibly due to changes in the aquatic ecosystem (MOUTON; BOTHA, 2012). In the following chapter we will discuss some of the most recognized cutaneous and/or mucocutaneous infectious agents in cetaceans: herpesvirus, poxvirus and *Paracoccidioides brasiliensis*.

2.2 SELECTED INFECTIOUS AGENTS

2.2.1 A brief introduction on herpesvirus

The etymology of herpesvirus (HV) originated from the Greek word "*herpein*", which means "to creep", originally used to name spreading skin lesions, such as those caused by erysipelas or smallpox, and possibly herpes varicella zoster virus (BESWICK, 1962). The order *Herpesvirales* comprises linear, double stranded and relatively large DNA viruses, ranging between 124-295 kilobases, containing between 70 and 200 protein-coding genes. HVs present a typical virion morphology: a core (viral DNA), an icosahedral nucleocapsid of 125 nm in diameter containing 161 capsomeres surrounded by amorphous tegument, and an outer lipid bilayered-envelope with viral glycoprotein spikes (PELLETT, ROIZMAN, 2013; ICTV, 2017).

The order *Herpesvirales* comprises three known families, capable of infecting different host species: *Malacoherpesviridae* (in bivalves), *Alloherpesviridae* (in fishes and amphibians), and *Herpesviridae* (in reptiles, birds and mammals) (PELLET; ROIZMAN, 2013; ICTV, 2017). Three subfamilies are recognized in the *Herpesviridae* family: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*, and a genus not assigned to any of them, which contains the *Iguanid HV 2* (ICTV, 2017). The creation of a new subfamily, *Deltaherpesvirinae*, containing elephant-infecting HVs previously assigned to the *Betaherpesvirinae*, has been proposed (RICHMAN et al., 2014). The subfamily *Alphaherpesvirinae*, divided into five genera: *Iltovirus*, *Mardivirus*, *Scutavirus*, *Simplexvirus* and *Varicellovirus*, affects a variety of hosts, mostly establishing latency in sensory ganglia and presenting a relatively short reproductive cycle. *Betaherpesvirinae* have a restricted host range and a long reproductive cycle, comprising four genera: *Cytomegalovirus*, *Muromegalovirus*, *Proboscivirus* and *Roseolovirus*. The *Gammaherpesvirinae* subfamily also presents four genera: *Lymphocryptovirus, Macavirus*, *Percavirus* and *Rhadinovirus* and one unassigned genus*,* infecting species within their natural host's family or order (PELLET; ROIZMAN, 2013; ICTV, 2017). Some authors postulate that the divergence of the different *Herpesviridae* subfamilies occurred over 181 to 221 million years ago (Ma), before the evolutionary radiation of placental mammals, which

took place around 105 Ma (MCGEOCH et al.,1995; SPRINGER et al., 2003). Viral and host speciation likely occurred concomitantly during the species' coevolution (MCGEOCH; RIXON; DAVISON, 2006). Herpesviruses and their specific/natural hosts have coevolved along millions of years, mutually adapting, thus causing generally mild disease in adapted natural hosts (DAVISON, 2002; PELLET; ROIZMAN, 2013). Nevertheless, HV infections in non-natural hosts within the same family or order have been described: in a Grauer's gorilla (*Gorilla beringei graueri*) presenting vesicles on the lips and gingival mucosa, caused by human herpes simplex virus type 1 (HSV-1) (GILARDI et al., 2014); the potentially fatal human infection by *Macacine HV 1* (B virus) (previously known as *Cercopithecine HV 1*) from non-human primates of the genus *Macaca* (HUFF; BARRY, 2003); and malignant catarrhal fever in cattle and bison caused by *Ovine HV 2* (DUNOWSKA et al., 2001). In addition, some alphaherpesviruses, e.g., *Equine HV 1* (EHV-1), *Suid HV type 1* (responsible for pseudorabies/Aujeszky disease) and *Gallid HV 2* and *3* (the etiological agents of Marek's disease) are able to infect and cause disease in a broad range of hosts from different genera and/or orders (WOŹNIAKOWSKI; SAMOREK-SALAMONOWICZ, 2015).

Most of the studied vertebrate species have at least one, and often several, endemic HVs (PELLET; ROIZMAN, 2013). *Betaherpesvirinae* and *Gammaherpesvirinae* have only been identified in mammals (ICTV, 2017), whereas *Alphaherpesviridae*, considered the least specific among the members of the *Herpesvirinae* subfamily, has been reported in reptiles, birds and mammals (MCGEOCH; RIXON; DAVISON, 2006). Herpesvirus transmission occurs mainly through aerosols, but may also occur through close oral (e.g., bites) and sexual contact, inhalation of feather dust, and vertical transmission (GOLDSTEIN et al., 2004; LI et al., 2004; GOLDSTEIN et al., 2005; GARLAND; STEBEN, 2014; SANMARTÍN et al., 2016; ICTV, 2017). In addition, long term stability and infectivity of HV in water has been recently described, constituting an additional potential route of infection (DAYARAM et al., 2017).

HVs are able to establish a quiescent stage of infection, also known as latency, characterized by the continuous presence of HV DNA in the infected tissue, and lack of infectious virion production. In *Alphaherpervirinae*, this usually occurs in sensory nerve ganglia (HILL et al., 1996; OSTERRIEDER; WALLASCHEK; KAUFER, 2014), e.g., the root ganglia of the trigeminal nerve (ASSIS-CASAGRANDE, 2014),

and for some herpesviral species also in lymphocites (e.g., in horses infected by *Equiid HV 1* and *4* (WELCH et al., 1992). In gammaherpesviruses, latency is frequently established in infected lymphoid tissues, resulting in lymphoproliferative malignancies (WIDEN et al., 2012; PELLET; ROIZMAN, 2013; OSTERRIEDER; WALLASCHEK; KAUFER, 2014). The latency mechanism of betaherpesviruses is poorly understood (OSTERRIEDER; WALLASCHEK; KAUFER, 2014), and can occur in different types of organs and tissues, such as secretory glands, kidneys and lymphoreticular cells (PELLET; ROIZMAN, 2013). Most HVs maintain their genomes as circular episomes during the quiescent stage of infection; however, a few HVs (e.g., *Gallid HV 2* and *Human HV* 6) integrate their genome into the host telomeres (OSTERRIEDER; WALLASCHEK; KAUFER, 2014). Immunosuppression, as a result of concurrent disease, stress, and reproductive effort, among other challenges, may reactivate latent HV (YAMAMOTO et al., 2010; WIDEN et al. 2012; ASSIS-CASAGRANDE, 2014), leading to the production of infectious virions and destruction of the infected cell (PELLET; ROIZMAN, 2013).

A broad range of lesions, presenting great pathological differences have been described in *Herperviridae* infections, varying according with the herpesviral subfamily, HV species and host species susceptibility. In mammals, the *Alphaherpesvirinae* subfamily has been macroscopically associated with erosive, ulcerative and vesicular lesions in the oral cavity, skin, tongue, mucocutaneous junctions, conjunctiva and genital mucosa; and microscopically with epithelial infections on the skin and genitalia, commonly accompanied by necrosis, ulceration, intranuclear inclusion bodies (INIBs) – some of them described as Cowdry A type (eosinophilic INIBs with perinuclear chromatin surrounded by a clear halo), either amphophilic, or basophilic – syncytial cells, ballooning degeneration, mixed inflammatory infiltrate, and occasional epithelial hyperplasia and dysplasia (MANIRE et al., 2006; GINN; MANSELL; RAKICH, 2007; VAN ELK et al., 2009; ASSIS-CASAGRANDE, 2014; BELLEHUMEUR et al., 2015). *Alphaherpervirinae* has also been associated with central nervous system lesions, macroscopically characterized by hyperemic and edematous tissue, and microscopically described as acute necrotic meningoencephalitis (mostly mononuclear), with presence of gliosis and INIBs, sometimes accompanied by perivascular cuffing, affecting mainly the grey matter. This subfamily is also able to affect several other organs, such as spleen, lungs and lymphoid tissue (MCADAM; SHARPE, 2010; ASSIS-CASAGRANDE, 2014). The

pathogeny of *Betaherpesvirinae* infections is poorly described, suggesting some apparently non-pathogenic viral species, whereas others are known to cause disease, e.g., fetal death in pigs, as well as rhinitis and occasionally generalized disease in piglets. Microscopically, these pathogenic viruses cause enlargement of the cell nucleus and cytoplasm of the affected cells, commonly containing amphophilic intranuclear and, at times, small basophilic cytoplasmic inclusions. Finally, among other lesions, *Gammaherpesvirinae* has been associated with generalized exanthema, meningeal edema, neoplasm (e.g., T and B lymphomas), genital proliferative lesions, and oral leucoplasia in the esophagus, skin and tongue. Microscopic findings vary from epithelial hyperplasia to sarcomas, lymphocytolysis, perivascular accumulation of mainly mononuclear cells, and fibrinoid necrotizing vasculitis (VAN ELK et al., 2009; ASSIS-CASAGRANDE, 2014; MACLACHLAN; DUBOVI, 2016).

To this date, nine distinct human HVs have been described: three alphaherpesviruses (*Human HVs 1*/*Human simplex HV*-1 [HHV-1/HSV-1], HHV-2/HSV-2, HHV-3*/Varicella-zoster virus/*[VZV]), three betaherpesviruses ([HHV-5]/Human cytomegalovirus-5, HHV-6A, HHV-6B, and HHV-7), and two gammaherpesviruses (HHV-4/Epstein-Barr virus [EBV] and HHV-8/Kaposi's sarcoma-associated HV) (PELLET; ROIZMAN, 2013). Two of these viruses have been associated with neoplasia: HHV-4/Epstein-Barr virus with Burkitt's lymphoma or nasopharyngeal carcinoma, and HHV-8 with Kaposi's sarcoma (COHEN, 2000; SCHULZ, 2005).

Herpesvirus cause relevant diseases in domestic animals, such as Marek's diseases in poultry (*Gallid HV 2* and *3*), Aujeszky disease (also known as pseudorabies) in pigs (the natural host), dogs, cats, horses, sheep, goats and cows (*Suid HV type 1*), and infectious bovine and feline rhinotracheitis in ruminants (*Bovine HV 1*), and cats (*Feline HV 1*), respectively (POWER et al., 1990; PELLET; ROIZMAN, 2013; MACLACHLAN; DUBOVI, 2016).

Additionally, some HVs may pose significant wildlife impact, as observed in fibropapillomatosis, a neoplastic disease of sea turtles caused by the alphaherpesvirus *Chelonid HV 5*; acute and often fatal disease (known as Pachecho's disease) and internal papillomatosis in parrots, caused by different Psittacid herpesviruses; and urogenital carcinoma in pinnipeds, especially in California sea lions (*Zalophus californianus*), but also described in South American

fur seals (*Arctocephalus australis*), by the gammaherpesvirus *Otarine HV 1* (JOHNE et al., 2002; KING et al., 2002; TOMASZEWSKI; KALETA, PHALEN, 2003; DAGLEISH et al., 2013; WORK et al., 2015). Herpesvirus infections are also a threat for captive animals, either undergoing rehabilitation, e.g., Avian HV-like associated to respiratory disease in African penguins (*Spheniscus demersus*) (PARSONS et al., 2015) and Avian HV causing respiratory lesions in Magellanic penguins (*Spheniscus magellanicus*) (NIEMEYER et al., 2017), or part of zoological collections, e.g., *Elephant HV/Elephant endotheliotropic HV* (*EEHV*), e.g., *EEHV 1*, leading to fatal hemorrhagic disease in Asian elephants (*Elephas maximus*), especially of calves (KENDALL et al., 2016; LONG; LATIMER; HAYWARD, 2016), *HHV 1* and *2* in Neotropical primates, and *Saimiriine herpesvirus 1* in Neotropical primates aside from squirrel monkeys (*Saimiri* spp.), its natural host, promoting potentially fatal disease (ASSIS-CASAGRANDE, 2014), and *Equine HV 1* and *9,* able to cause disease in a broad range of host species, e.g., polar bear (*Ursus maritimus*), Persian onager (*E. hemionus onager*), Thomson's gazelle (*Gazella thomsoni*), and giraffe (*Giraffa camelopardalis*) (SCHRENZEL et al., 2008; GREENWOOD et al., 2012).

Alpha-, beta-, and gammaherpesviruses have been described in Cetartiodactyla, an order comprising the Artiodactyla (e.g., suborders Suina [pigs and boars], Tylopoda [camels], and Ruminantia [deers and cows]), and the unranked Cetacea taxon (formed by the unranked Mysticeti and Odontoceti taxa) (EHLERS; LOWDEN, 2004; DAS NEVES et al., 2010; MANESS et al., 2011; COMMITTEE ON TAXONOMY, 2016). The Cetartiodactyla are natural hosts of important herpesviruses: *Ovine HV 2* (sheep), *Alcelaphine HV 1* and *2* (Alcelaphinae subfamily, e.g., wildebeest, *Connochaetes* sp.) and *Suid HV type 1* (Suidae family). The *Ovine HV 2* and *Alcelaphine HV 1* and *2* are the etiological agents of malignant catarrhal fever, a potentially fatal disease in ruminants, especially for members of the Bovinae and Cervidae families. *Suid HV type 1* causes morbidity and mortality in ungulates, lagomorphs, rodents and carnivores, e.g., Iberian lynxs (*Lynx padinus*), wolves (C*anis lupus*), and brown bears (*Ursus arctos*) (ZANIN et al., 1997; WIDEN et al., 2012; VERPOEST et al., 2014; MASOT et al., 2017). Only alpha- and gammaherpesvirus are known to occur in cetaceans, sometimes related to asymptomatic infections (BELLIÈRE et al., 2010; MANESS et al., 2011; VAN ELK et al., 2016). Nevertheless, alphaherpesvirus infections have been associated with cutaneous disease and sometimes fatal localized or systemic infections

(SMOLAREK-BENSON et al., 2006; ARBELO et al., 2010; BELLEHUMEUR et al., 2015; VAN ELK et al., 2016), whereas gammaherpesvirus are usually observed in association with genital mucosal lesions (VAN ELK et al., 2009), and might impact reproduction of the affected populations (LECIS et al., 2014).

2.2.2 A brief introduction on poxvirus

The etymology of “poxvirus” originates from the Anglo-Saxon word “pokkes”, which means “pouch”, in reference to its characteristic vesicular lesions (pustules). The Latin term “variola” means “spotted”, whereas the term “smallpox”, one of the most important poxviral diseases, was introduced to distinguish it from the “great pox”, currently known as “syphilis”, caused by the bacteria *Treponema pallidum* (COLLIER; OXFORD; KELLAM, 2016).

The *Poxviridae* family is formed by large, linear, double stranded DNA viruses, ranging from 135 to more than 300 kb, able to replicate in the cytoplasm of their host cells. The central genome core is formed by a total of 89 well-conserved genes in all sequenced species, while the termini region varies among genera. The virions are pleomorphic, usually brick-shapped or ovoid, ranging between 200 - 400 nm in length, composed of a dumbbell-shaped core, lateral bodies and a lipoproteic outer membrane displaying tubular or globular units, or regular spiral filament (BOURNE; DUFF; VIKØREN, 2012; ICTV, 2017). Two subfamilies are recognized: *Entomopoxvirinae* (invertebrate infecting) and *Chordopoxvirinae* (vertebrate infecting). The family *Chordopoxvirinae* is comprised of at least eleven genera: *Avipoxvirus*, *Capripoxvirus*, *Centapoxvirus*, *Cervidpoxvirus*, *Crocodylidpoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, *Suipoxvirus*, *Yatapoxvirus* and one unassigned genus (the latter containing the *Pteropox virus* and the *Squirrelpox virus*) (ICTV, 2017). Novel genera of *Chordopoxvirinae* have also been proposed, e.g., *Cetaceanpoxvirus* (BRACHT et al., 2006; BOURNE; DUFF; VIKØREN, 2012). These genera are classified according with their infecting animal species, virion morphology, genome size, gene content and antigenic cross-reactivity (GUBSER et al., 2004; BOURNE; DUFF; VIKØREN, 2012; ICTV, 2017). Poxvirus transmission routes may vary among different genera and influence disease

virulence, with direct contact (including the entry through cutaneous abrasions), mechanical (through arthropod vectors and fomites), and respiratory (aerosols) routes being the most commonly recognized, the latter considered the most concerning for viruses such as the *Variola virus* (STANFORD et al., 2007; DAMON, 2013).

After entering the host, poxviruses could cause localized (generally associated to benign skin and/or mucous membrane lesions) or systemic infections (often fatal) (SMITH; KOTWAL, 2002; MACLACHLAN; DUBOVI, 2010; DAMON, 2013). The host range of poxviruses varies according with the viral species, and could be narrow, e.g., *Variola virus*, the etiological agent of smallpox, infecting only humans, or broad, e.g., *Cowpox virus*, able to infect a large number of mammal species, e.g., ruminants, rodents and primates (SMITH; KOTWAL, 2002; MARTINA et al., 2006; DAMON, 2013; HALLER et al., 2014).

Poxviruses are associated with skin and mucosal lesions, with most species presenting marked tropism for keratinocytes. Different macroscopic presentations are described: erythematous macules, papules, vesicles, pustules, scabby cutaneous lesions, proliferative skin lesions, mucosal ulcers, blefaritis and conjunctivitis. Microscopic findings are often observed in the skin, including intercellular edema (spongiosis) and ballooning degeneration (mostly observed in the mid to upper epidermis, caused by poxvirus replication and vascular damage), which could evolve to vesicles depending on the poxviral species (e.g., *Variola virus*), and even ulcers (e.g., *Lumpy skin disease virus*). The dermis may present capillary dilation and endothelial cell swelling (associated with vascular damage due to virus replication). Inflammatory infiltrate, usually composed of neutrophils and monocytes, likely in response to secondary bacterial infections, may be found in the dermis and epidermis. In addition, some poxvirus lead to epidermal hyperplasia and hyperthrophy, e.g., *Avipoxvirus* (by viral proteins similar to epidermal growth factors). In mucosal membranes, the epithelial viral replication may create ulcers. Intracytoplasmatic inclusion bodies, which vary according with the poxvirus genus, can be eosinophilic (A-type inclusion bodies, not formed by all *Chordopoxvirinae*), amphophilic or basophilic (B-type inclusion bodies) when stained with Hematoxylin-Eosin. Some poxvirus species have been associated with mucoid subcutaneous swelling (e.g., *Myxoma virus*), bronchopneumonia (e.g., *Myxoma virus*, *Cowpox virus*) or pneumocyte type II, and bronchiolar epithelium hyperplasia. Furthermore,

lymphoid depletion and necrosis have also been described (GIDDENS et al., 1971; GINN; MANSELL; RAKICH, 2007; MACLACHLAN; DUBOVI, 2010; BOURNE; DUFF; VIKØREN, 2012; DAMON, 2013). Due to its large size and complexity, poxviruses can be easily recognized by the host's immune system (STANFORD et al., 2007). However, these viruses have different mechanisms capable of subverting and evading the host's immune response, e. g., incorporation of host genes into their genomes (SMITH; KOTWAL, 2002; ODOM; HENDRICKSON; LEFKOWITZ, 2009).

Two poxvirus species are known to exclusively infect humans: *Molluscum contagiosum virus* and *Variola virus* (from the *Molluscovirus* and *Orthopoxvirus* genera, respectively) (MOSS, 2013). The *Molluscum contagiosum virus* usually causes localized infections (mostly proliferative skin lesions) (HANSON; DIVEN, 2003). On the other hand, *Variola virus*, the etiological agent of smallpox, was a significant selective factor on human populations, killing millions of people; as observed after its introduction by Europeans in immunologically naïve Native American and Aboriginal Australian human populations (DE SAHAGÚN, 1577; CAMPBELL, 1985; PATTERSON; RUNGE, 2002; GALVANI; SLATKIN, 2003). Smallpox was eradicated in 1977, as a result of vaccination campaigns started in the XIX century (MOSS, 2013). Other poxviruses classified into the genera *Orthopoxvirus* (*Cowpox virus*, *Monkeypox virus*, *Vaccinia virus*), *Parapoxvirus* (*Bovine popular stomatitis virus*, *Orf virus*, *Pseudocowpox virus*, *Seal parapoxvirus*) and *Yatapoxvirus* (*Tanapox virus* and *Yaba monkey tumor virus*) have reportedly infected humans, (DAMON, 2013), occasionally leading to fatal disease (e.g., *Monkey pox*) (ESSBAUER; PFEFFER; MEYER, 2010; ICTV, 2017).

Poxviruses cause relevant diseases in wild and domestic animals (DAMON, 2013; BOURNE; DUFF; VIKØREN, 2006). In domestic animals, *Cowpox virus* has been associated with ulcerated nodules, occasional oral lesions, and even pneumonia in cattle and cats, although the natural hosts are likely different rodent species (DAMON, 2013). Members of the genus *Capripoxvirus* infect ruminants: *Lumpy skin disease virus* cause raised skin nodules in cattle, and *Sheeppox virus* and *Goatpox virus* increase morbidity (e.g., skin lesions) and mortality in mostly young or epidemiologically naïve goats and sheep (MACLACHLAN; DUBOVI, 2010). In addition, goats and sheep are also susceptible to *Orf virus*, the etiological agent of contagious ecthyma, which causes, among other lesions, proliferative scabby lesions on the skin and the lips. Examples of other poxviruses affecting domestic animals

include *Swinepox* (*Suipoxvirus*), *Vaccinia virus* and *Camelpox virus* (both *Orthopoxvirus*) (GUBSER, SMITH, 2002; GINN; MANSELL; RAKICH, 2007; MEGID et al., 2012; RIYESH et al., 2016), and the apparently eradicated *Horsepox virus* (*Orthopoxvirus*) (ESPARZA, 2013). Some poxvirus can infect both domestic and wild mammals, e.g., *Orf virus* in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) (WILSON; MCFARLANE, 2012).

In wildlife, significant poxvirus infections have been described in fish, e. g., in gill lesions of Atlantic salmon (*Salmo salar*) (GJESSING et al., 2015), and identified in reptiles, e.g., in skin lesions observed in Nile crocodiles (*Crocodylus niloticus*) and caimans (*Caiman crocodilus*) (RAMOS et al., 2002; MARSCHANG, 2011). Several species of *Avipoxvirus* have been described in wild birds, such as *Canarypox virus*, *Fowlpox virus*, *Psittacinepox virus* and *Sparrowpox virus,* with variable host ranges (from narrow to broad) depending on the viral species (BOOSINGER et al., 1982; BOURNE; DUFF; VIKØREN, 2012; ICTV, 2017), and associated with warty skin lesions (cutaneous form) and/or grey to brown necrotic diphtheritic lesions on the oral, pharyngeal, laryngeal, tracheal, esophageal and crop mucosa, and less frequently on bronchial epithelium (diphtheritic form) (FRIEND; FRANSON, 1999; BOURNE; DUFF; VIKØREN, 2012). A third presentation of the disease, rarely observed in wildlife, include unspecific symptoms such as depression, anorexia, and cyanosis (BOURNE; DUFF; VIKØREN, 2012). Avian poxviruses can impact the health of wild free-ranging birds, especially those living in isolated islands, e.g., endemic species from Hawaii (United States), Galapagos (Ecuador) and the Canary Islands (Spain), possibly because these species have not coevolved with the virus (WARNER, 1968; SMITS et al., 2005; KLEINDORFER; DUDANIEC, 2006; PAXTON et al., 2016). Furthermore, the infection may elevate morbidity and mortality rates of captive and rehabilitating wild bird species, e.g., Andean condor (*Vultur gryphus*) and Magellanic penguins (*Spheniscus magellanicus*) (KIM et al., 2003; NIEMEYER et al., 2013).

Poxviruses have caused serious impact on wild mammal populations. *Myxoma virus* (*Leporipoxvirus*), the etiological agent of myxomatosis, only causes mild disease (localized fibromas) in its natural American host species – the South American rabbit (*Sylvilagus brasiliensis*) and the brush rabbit (*Sylvilagus bachmani*) - but lead to mass mortalities in European rabbits (*Oryctolagus cuniculus*), and occasionally affected European brown hares (*Lepus europaeus*) and mountain hares

(*Lepus timidus*). Two main forms of myxomatosis have been described: myxomatous (causing blepharoconjunctivitis, skin lesions, mucoid subcutaneous swelling) and amyxomatous (respiratory form) (MARSHALL; REGNERY, 1960; MACLACHLAN; DUBOVI, 2010; BOURNE; DUFF; VIKØREN, 2012). In the Iberian Peninsula, its epidemic had catastrophic ecological effects, once rabbits are key prey species for predators of the Iberian Mediterranean ecosystem and perform important ecological services, e.g., scrubland growth mediated by selective grazing (DELIBES-MATEOS et al., 2008). The massive rabbit morbidity and mortality driven by myxomatosis, along with the rabbit hemorrhagic disease virus (a calicivirus), severely impacted rabbits-specialist predator species, such as the eagle owl (*Bubo bubo*), the Iberian imperial eagle (*Aquila adalberti*), and the Iberian linx (*Lynx pardinus*) (PENTERIANI, GALLARDO; ROCHE, 2002; RODRÍGUEZ; DELIBES, 2002; MORENO et al., 2008; BOURNE; DUFF; VIKØREN, 2012). Another example of poxvirus in lagomorphs is fibromatosis, caused by *Hare fibroma virus* and *Rabbit fibroma virus* (the latter also known as *Shope fibroma virus*) (KERR et al., 2015). Another poxvirus responsible for wild mammal declines is *Squirrelpox virus*, which caused the decline of red squirrels (*Sciurus vulgaris*) in the United Kingdom and Ireland, infected through their contact with infected specimens of the invasive American grey squirrel (*S. carolinensis*), likely a reservoir of the virus (SAINSBURY et al., 2008; MCINNES et al., 2013). This disease is characterized by exudative erythematous dermatitis, leading to severe ulceration and hemorrhagic scabs around the nose, mouth and eyes (MCINNES et al., 2009; MCINNES et al., 2015). Other poxviral diseases infecting wild mammals have also been described in the literature, e.g., *Monkeypox virus*, *Skunkpox virus*, *Raccoonpox virus* and *Volepox virus* (GALLARDO-ROMERO et al., 2012; DAMON, 2013; RADONIĆ et al., 2014).

Several poxviruses associated with skin lesions have been reported in marine mammals, a diverse group that comprises pinnipeds (seals, sea lions and walruses), cetaceans (whales, porpoises and dolphins), sirenians (manatees and dugongs), sea otters (*Enhydra Lutris*) and polar bears (*Ursus maritimus*). In sea otters, poxvirus infections by a possible novel genus - which could be named *Lutrinipoxvirus*, based on ICTV recommendations - have been reported (TUOMI et al., 2014). Pinnipeds are mostly affected by species of the *Parapoxvirus* genus (TRYLAND et al., 2005; ROESS et al., 2011), but sequences with 83% amino acid identity to poxvirus from cetaceans were identified in Steller sea lions (*Eumetopias jubatus*), leading to the

suggestion of a new poxvirus genus (*Pinnipedpoxvirus*) (BRACHT et al., 2006). In addition, a sequence highly similar to cetacean poxviruses has been identified in a Pacific walrus (*Odobenus rosmarus divergens*) (MELERO et al., 2014). The parapoxvíruses identified in pinnipeds have zoonotic potential (ROESS et al., 2011); however, no zoonotic transmission has been reported for cetacean poxviruses. Bracht et al. (2006) and Blacklaws et al. (2013) suggested that the poxviruses detected in cetaceans should be grouped in a novel genus, tentatively named *Cetaceanpoxvirus*. To the author's knowledge, cetacean skin lesions consistent with poxvirus infection have only been tested by molecular techniques in six previous studies (BARNETT et al., 2015; BRACHT et al., 2006; BLACKLAWS et al., 2013; BURDETT-HART et al. 2012; FIORITO et al., 2015; MCALOOSE et al., 2016). Successful methods targeted two highly conserved genes among the *Chordopoxvirinae*: DNA polymerase and DNA topoisomerase I (BUREK et al., 2005). Summarized information on the confirmed molecular identification of *Cetaceanpoxvirus* is listed in Box 2. Further discussion on *Cetaceanpoxvirus* is provided in the article "First molecular identification of cetacean poxvirus in odontocetes from South America: implications on *Cetaceanpoxvirus* taxonomy".

Box 2 - Species of cetaceans and pinnipeds in which gene fragments of *Cetaceanpoxviruses* have been amplified by DNA polymerase (DNA pol) and DNA topoisomerase I (DNA topo) PCR techniques, classified by country, number of positive specimens and study.

	Species	Origin	Positive specimens to: DNA pol	DNA topo	Author
Cetacean	Indo-Pacific bottlenose dolphin *Tursiops aduncus*	Hong Kong (China)	4	1	Bracht et al., 2006
	Atlantic bottlenose dolphin *Tursiops truncatus*	USA	1	1	Bracht et al., 2006
	Rough-toothed dolphin *Steno bredanensis*	USA	2	2	Bracht et al., 2006
	Striped dolphin *Stenella coeruleoalba*	USA	2	2	Bracht et al., 2006
		UK	1	-	Barnett et al., 2015
			1	1	Blacklaws et al.,2013
	Short-beaked common dolphin *Delphinus delphis*	UK	1	1	Blacklaws et al.,2013
		UK	4	-	Barnett et al., 2015
	Harbor porpoise *Phocoena phocoena*	UK	9	9	Blacklaws et al.,2013
			3	-	Barnett et al., 2015
	Bowhead whale *Balaena mysticetus*	USA	1	1	Bracht et al., 2006
	Southern right whale *Eubalaena australis*	Argentina	1	1	Fiorito et al., 2015
Pinnipeds	Pacific walrus (*Odobenus rosmarus divergens*)	Spain	1	-	Melero et al., 2014

2.2.3 A brief introduction on cetacean fungal diseases with special emphasis on paracoccidioidomycosis ceti

The Fungi kingdom comprehends nine eukaryote phyla: Microsporidia, Rozellomycota, Chytridiomycota, Blastocladiomycota, Zoopagomycota, Mucormycota, Glomeromycota, Basidiomycota and Ascomycota. It is believed that only 5% of the species classified into this highly diverse and broad taxon, with an overall estimated number of 1 to 5 million species, have been described (CROUS et al., 2016); including some able to cause disease in plants and animals (FISHER et al., 2012).

Infections caused by fungal agents have been described either as superficial colonization, or epidermal, subcutaneous and deep mycosis, mostly associated with secondary infections (e.g., related to viral immunosuppression, treatment with immunosuppressive agents, pollutants, stress), although some fungal species are also able to act as primary pathogens (RICHARDSON, 1991; SHOTTS et al., 1990; ROTSTEIN et al., 2010).

Fungal pathogens can be classified as environmental (with an environmental saprobe infectious phase) or obligatory (presenting host to host transmission). Most of these infectious agents replicate saprophytically in the abiotic environment (e.g., soil, water, excreta) from where they are transmissible, without the need of a host, which is the case of several human sapronoses (e.g., aspergillosis) (HUBÁLEK et al., 2003), lacking certain characteristics present in other infectious diseases, such as a minimal required host-density to promote transmission or history of coevolution and adaptation to the host (KURIS; LAFFERTY; SOKOLOW, 2014). Some fungal agents are also transmitted through direct contact with infected individuals (MARTEL et al., 2014).

Fungal infections have been described as emerging pathogens across diverse taxa, causing widespread population declines and conservation crises, more so any other pathogen group, in some cases even leading to extinctions and contributing to the biodiversity loss observed in the Anthropocene (FISHER et al., 2012). Examples are observed in: (1) invertebrates: aspergillosis (*Aspergillus sydowii* [Ascomycota]) in soft corals (KIM; HARVELL, 2004) and nosemosis (*Nosema* sp. [Microsporidia]) in bees – this latter a component of the colony collapse disorder (CAMERON et al.,

2011); (2) amphibians: chytridiomycosis (*Batrachochytrium dendrobatidis* [Chytridiomycota]), implicated in the extinction of over 200 frog species worldwide (STUART et al., 2004; LIPS et al., 2006; SKERRATT et al., 2007), and more recently, *B. salamandrivorans* (Chytridiomycota) in European salamanders (MARTEL et al., 2014); (3) reptiles: *Fusarium solani* (Ascomycota) leading to mass mortality of loggerhead sea turtle (*Caretta caretta*) nests (SARMIENTO-RAMÍREZ et al., 2010) and *Ophidiomyces ophiodiicola* (Ascomycota) in wild snake populations from eastern United States (LORCH et al., 2016); and (4) mammals: mass mortality and local extinction events in bats caused by white-nose syndrome, associated to *Pseudogymnoascus destructans* (formerly *Geomyces destructans*) (Ascomycota), mainly in North America (BLEHERT, 2012).

An increasing number of fungi are being described as part of the normal odontocete (YOUNG et al., 1999; TAKAHASHI et al., 2010; RAVERTY et al., 2017) and mysticete mycobiome (SHOTTS et al., 1990; GUASS et al., 2016). In addition, although not as often as other infectious diseases, mycoses are increasingly being reported in cetaceans (DAGLEISH et al., 2008). Kirkwood et al. (1997) detected fungal infections in 10% (2/20) of the analyzed wild harbor porpoise pneumonia cases in the United Kingdom, while Venn-Watson; Daniels; Smith (2012) observed fungal hyphae in 28.6% (12/42) of the captive Atlantic bottlenose dolphins (*Tursiops truncatus*) from the US presenting pneumonia. However, according with Simeone et al. (2015), marine mammals peer-reviewed publications are not generally reliable in establishing the prevalence of endemic disease over time, due to the limited available editorial and “novelty priority” bias. In addition, most of the reports come from odontocete species, although fungal colonization and disease have also been described in mysticetes (BEST; MCCULLY, 1979; REEB et al., 2010; MCALOOSE et al., 2016).

Reports of superficial mycosis in cetaceans are scarce, mainly in captive animals, illustrated by cases of dermatophytosis caused by *Trichophyton* spp. in a captive Atlantic bottlenose dolphin, characterized by proliferative dermatitis, macroscopically observed as multifocal superficial nodules (HOSHINA; SIGIURA, 1956), superficial mycosis caused by *Fusarium* spp. and *F. solani* in Delphinidae, Kogiidae and Monodontidae odontocete families (FRASCA et al., 1996; BOWENKAMP et al., 2001; NAPLES et al. 2012), associated with necrotizing or proliferative skin lesions, sometimes macroscopically described as nodules. *F. solani*,

also in Delphinidae, has been associated with subcutaneous mycosis, characterized by deep granulomatous dermatitis (TANAKA et al., 2012). Furthermore, *Candida albicans* and *Candida* sp. have been detected in white to yellow plaque skin lesions, mainly in the mucocutaneous junctions (e.g., blowhole and vagina), in ulcers in the esophagus and the stomach, and also associated with systemic, occasionally fatal, mycosis, as observed in Delphinidae, Phocoenidae and Monodontidae odontocete families (DUNN; BUCK; SPOTTE, 1982).

Some of the systemic mycotic agents are opportunistic, reportedly associated with immunosuppressive agents, as observed in *Aspergillus* spp. and cetacean morbillivirus infections (DOMINGO et al., 1992; KENNEDY et al., 1992; LIPSCOMB et al., 1994; LIPSCOMB et al.; 1996; STEPHENS et al., 2014; CASSLE et al., 2016; KEMPER et al., 2016), and *A. fumigatus* and severe parasitic pneumonia (DAGLEISH et al., 2006). Nevertheless, fungal pathogens recognized as primary agents - *Coccidiodes immitis* (REIDARSON et al. 1998a), *Blastomyces dermatitides* (CATES et al., 1986), *Histoplasma capsulatum* (VENN-WATSON; DANIELS; SMITH, 2012), and *Cryptococcus gattii* (ROTSTEIN et al., 2010) - have also been described in cetaceans.

Deep mycoses caused by *A. fumigatus* are usually observed in the lungs (JOSEPH et al., 1986; REIDARSON et al., 1998b; DAGLEISH et al., 2008), occasionally extending to the trachea (LIPSCOMB et al., 1994; DELANEY et al., 2013), and brain (DAGLEISH et al., 2006; BARLEY et al., 2007; DAGLEISH et al., 2008). Some reports described *A. terreus* involvement in middle ear otitis in a stranded harbor porpoise, which could have possibly promoted the stranding event (PRAHL et al., 2011), and disseminated aspergillosis in Atlantic bottlenose dolphins (LIPSCOMB et al., 1994), usually involving the lungs. Other known putative secondary agents causing encephalitis in cetaceans include *Rhizopus* species (WÜNSCHMANN; SIEBERT; WEISS, 1999), *F. oxyporum* (STAGGS et al., 2010); *Cladosporium* sp. (MAZZARIOL et al., 2007), *Chladophialophora bantiana* (REIDARSON et al., 2001) and *Cunninghamella bertholletiae* (ISIDORO-AYZA et al., 2014).

C. neoformans and *C. gattii* are basidiomycete yeasts acquired via inhalation, responsible for cryptococcosis in several species of mammals and birds (MALIK et al., 2003; BYRNES et al., 2010; ROTSTEIN et al., 2010). *C. neoformans* grows well in bird feces (NIELSEN; DE OBALDIA; HEITMAN, 2007), whereas *C. gattii* is

possibly associated with trees, mainly *Eucalyptus* sp. (ELLIS; PFEIFFER, 1992). The first usually infects immunocompromised hosts, while the latter is able to cause disease in immunocompetent animals and humans (BYRNES et al., 2010). In cetaceans, *C. gatti* and *Cryptococcus* sp. have been reported primarily in association with pneumonia and disseminated infections in other tissues, such as skin and the nervous system (GALES; WALLAGE; DICKSON, 1985; ROTSTEIN et al., 2010). Vertical transmission of *C. gattii* has been also reported in this taxon (NORMAN et al., 2011).

Among the reportedly diagnosed cetacean mycosis agents are several members of the order Onygenales, a group that presents genomic adaptations that enable its members to use animal substrates (DESJARDINS et al., 2011). This order includes *B. dermatitidis*, *Coccidioides* spp., and *H. capsulatum*, recognized as pathogenic dimorphic fungi, growing as yeasts at mammalian body temperatures and under elevated CO_2 conditions, and as hyphae in soil (GAUTHIER, 2015). Blastomycosis, histoplasmosis and coccidioidomycosis (also known as Valley fever) are deep mycosis affecting cetaceans, mainly their lungs (CATES et al., 1986; JENSEN et al., 1998, REIDARSON et al., 1998a), likely acquired by inhalation of aerolized infectious particles (BUREK, 2001). Another important Onygenales species affecting cetaceans is *P. brasiliensis*, increasingly associated with subcutaneous fungal infections

One of the most commonly reported subcutaneous fungal infections in cetaceans is paracoccidioidomycosis ceti (formerly known as lobomycosis, lobomycosis-like disease, lacaziosis, lacaziosis-like disease or Jorge Lobo's disease), caused by uncultivable *P. brasiliensis* yeasts. The lesions are macroscopically described as white, proliferative, verrucous and crusty skin lesions, and microscopically as chain-forming yeasts located mainly in the dermis, along with granulomatous dermatitis and panniculitis, and the presence of giant cells (MIGAKI et al., 1971; ROTSTEIN et al., 2009). For a long time, *L. loboi* an uncultivable fungae of the Onygenales order, identified in the host as yeast through histopathology, was presumed as the causative agent of such lesions, therefore naming the disease as lacaziosis, lacaziosis-like disease, Lobomycosis or Lobomycosis-like disease (MIGAKI et al., 1971; SIMÕES-LOPES et al., 1993). However, with the avent of molecular analysis, the true etiological agent was identified as *P. brasiliensis*

(ESPERÓN et al., 2012; MINAKAWA et al., 2016; VILELA et al., 2016), and the disease was then renamed as “paracoccidioidomycosis ceti” (VILELA et al., 2016).

Paracoccidioidomycosis ceti has been molecularly confirmed in two dolphin species: Atlantic bottlenose dolphin and Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) (ROTSTEIN et al., 2009; ESPERÓN et al., 2012; UEDA et al., 2013; MINAKAWA et al., 2016; VILELA et al., 2016). Two of those Atlantic bottlenose dolphins also presented positive immunohistochemistry when antibodies against *P. brasiliensis* were used (UEDA et al., 2013). In addition, histologic compatible lesions have been observed in a Guiana dolphin (*Sotalia guianensis*) from Suriname (DE VRIES et al., 1973), Atlantic bottlenose dolphins from Brazil (SIMÕES-LOPES et al., 1993; MORENO et al., 2008), Indo-Pacific bottlenose dolphins (*T. aduncus*) from South Africa (LANE et al., 2014), and several Atlantic bottlenose dolphins from the Atlantic coast of the United States (COWAN, 1993; DURDEN et al., 2009). Differential diagnosis for *P. brasiliensis* lesions in cetaceans includes fusariosis (TANAKA et al., 2011) and cryptococcosis (ROTSTEIN et al., 2010). Recently, Ueda et al. (2017) cultured *Trichosporon asteroides* from white, multifocal, raised skin lesions of an Atlantic bottlenose dolphin. However, no histological evaluation of the lesion was performed. Other species of the genus *Trichosporon* have been previously reported in healthy and injured cetacean skin, as well as in blowhole swabs, gastric contents and fecal material (SHOTTS et al. 1990; BUCK et al., 2006; MORRIS et al., 2011).

L. loboi infection in humans has only been reported in the Amazon basin and some nearby tributaries, with isolated reports in Greece (PAPADAVID et al., 2012) and South Africa (AL-DARAJI; HUSAIN; ROBSON, 2008). Transmission is closely related to aquatic environments and soil contact, and occurs mainly through traumatic injuries, leading to subcutaneous mycosis (REIF; SCHAEFER; BOSSART, 2013). Differential diagnoses include leprosy, paracoccidioidomycosis, chromoblastomycosis, sporotrichosis, anergic cutaneous leishmaniasis, Kaposi’s sarcoma, keloids, fibromas, dermatofibrosarcoma protuberans, neurofibromas, and metastatic lesions (PANIZ-MONDOLFI et al., 2012).

The current etiological agent of paracoccidioidomycosis ceti, *P. brasiliensis*, and a related species, *P. lutzii,* have also been reported in humans and others terrestrial mammals (e.g., dogs, armadillos, sloths), causing paracoccidioidomycosis, considered the most significant human systemic mycosis in South America, and

characterized by primary pulmonary mycosis and systemic secondary granulomatous lesions in oral mucosa, skin and other tissues of humans and other mammals (NAIFF et al., 1986; RICCI et al., 2004; TREJO-CHÁVEZ et al. 2011; MARQUES, 2012; TEIXEIRA et al., 2014). In addition, *P. brasiliensis* has been detected in marine birds: in the feces of an Adelie penguin (*Pygoscelis adeliae*) from the King George Island, Antarctica (GARCIA et al., 1993). *P. brasiliensis* infections are more frequently diagnosed in the Amazon basin (BAGAGLI et al., 2006; TERÇARIOLI et al., 2007; THEODORO et al., 2012), and transmitted via inhalation of soil-related spores (TABORDA; TABORDA; MICHAEL, 1999; THEODORO et al., 2012).

Theodoro et al. (2012) suggested that the ancestral population of *P. brasiliensis*, *P. lutzii* and *L. loboi* originated in northern South America, approximately 40.6 million years ago. The evolutionary divergence of *L. loboi* and *P. brasiliensis* probably was an adaptation to mutually exclusive environments (BAGAGLI et al., 2006; TERÇARIOLI et al., 2007). Bagagli et al. (2008) hypothesized that *L. loboi* may be going through an evolutionary phase of strong saprobic form reduction and specialization towards parasitism, becoming dependable on its host for growth and dissemination. If confirmed, the large geographic distribution of this pathogen will be associated with host migration rather than water itself.

In order to establish the phylogeny of the LLD agent in dolphins, previous studies amplified the *5S* ribosomal DNA (rDNA) gene from its yeast form, which placed the organism within the Fungi kingdom (HAUBOLD et al., 1998). LLD yeast rDNA was 97% homologous to *P. brasiliensis* (ROTSTEIN et al., 2009), and when a variable length from the *18S* rDNA, *internal transcribed spacer 1* (ITS-1), *5.8S* rDNA, *internal transcribed spacer 2* (ITS-2) to *26S* rDNA was amplified, the novel sequence was more closely related to *P. brasiliensis* (*p*-distance 0.011) than to human *L. loboi* (*p*-distance: 0.180) (ESPERÓN et al. 2012). Amplification of the *gp43* gene showed 94.9% homology to *P. brasiliensis,* 87.7% to *P. lutzii* and 84.1% to *L. loboi* (UEDA et al., 2013). In other study, the yeast identified in a Pacific white-sided dolphin presented a 99% *gp43* gene identity to human *P. brasiliensis* (MINAKAWA et al., 2016). Recently, Vilela et al. (2016) were able to amplify the *Kex* gene in dolphin samples, with 100% homology to six *P. brasiliensis* sequences from humans. However, direct molecular and phylogenetic studies evaluating the yeast-infected dolphins from South America, a region where human cases of paracoccidioidomycosis are present, have never been performed.

3 OBJETIVES

3.1 GENERAL OBJETIVE

The general goal of this study was to identify and characterize selected cutaneous and mucocutaneous pathogens of free-ranging cetaceans from Brazil, further contributing to the understanding of the causative agents affecting this taxon.

3.2 ESPECIFIC OBJETIVES:

- To detect and identify herpesvirus in cetacean cutaneous, oral and genital mucosa samples with the aid of an universal nested degenerated PCR and subsequent sequencing and establishment of the phylogenetic relationships.
- To detect and identifify poxvirus in cetacean cutaneous samples through amplification of DNA polymerase and DNA topoisomerase I genes and subsequent sequencing and establishment of the phylogenetic relationships.
- To detect and characterize the fungus *Onygenales* sp. in cetacean cutaneous samples by light microscopy (histopathology) and immunohistochemistry, whenever macroscopic-compatible lesions were present, identifying the agent (putatively *P. brasiliensis*) with the aid of a universal fungal PCR to amplify the *internal transcribed spacer 1* (*ITS1*), *5.8 rDNA* and *internal transcribed spacer 2* (*ITS2*) genes, and subsequent sequencing and establishment of the phylogenetic relationships.
- To employ histopathology to describe and characterize the cutaneous and mucocutaneous alterations presented by the positive animals.
- To design new molecular methods to a better diagnosis of these agents.

4 HERPESVIRUSES IN RIVERINE AND MARINE CETACEANS FROM SOUTH AMERICA

4.1 ABSTRACT

Herpesvirus (HV) infections in cetaceans are frequently associated with skin and mucosal lesions. Although herpesvirus infections have been reported worldwide, their occurrence in southern Atlantic marine mammals is still poorly understood. DNA extraction was tested with the aid of beta-actin PCR in skin, oral and genital mucosal samples from 115 free-ranging specimens of seven cetacean families (Delphinidae [n=61], Pontoporiidae [n=35], Iniidae [n=3], Kogiidae [n=3], Physeteridae [n=1], Balaenopteridae [n=10], and Balaenidae [n=2]) from Brazil, followed by panherpesvirus PCR on the positive beta actin samples (109), using primers for DNA polymerase gene fragment. Tissues samples of HV positive individuals were histologically evaluated. Four out of 109 specimens (3.7%) were positive: skin samples from one Guiana dolphin (*Sotalia guianensis*), one dwarf sperm whale (*Kogia sima*), one Bolivian river dolphin (*Inia boliviensis*) and lingual samples from one Atlantic spotted dolphin (*Stenella frontalis*). Additionally, other tissues samples from the positive individuals were also tested by PCR, providing new positive samples for the Guiana dolphin, the dwarf sperm whale and the Bolivian river dolphin, including a novel sequence found in the dwarf sperm whale's stomach and mesenteric lymph node. Cutaneous histological findings consisted of: (A) Guiana dolphin: marked, focally extensive, chronic proliferative dermatitis; (B) dwarf sperm whale: marked, multifocal, chronic ulcerative and fibrinosuppurative dermatitis and panniculitis with hemorrhage and thrombosis, and (C) Bolivian river dolphin: marked, focally extensive, chronic proliferative dermatitis. Furthermore, the Guiana dolphin presented multifocal lymphocytic encephalitis. Autolysis precluded histological examination of the Atlantic spotted dolphin. Four novel HV species were found: one in the Guiana dolphin, two in the dwarf sperm whale and one in the Bolivian river dolphin, the former possible a novel gammaherpesvirus genus. To the best of our knowledge, these findings constitute the first molecular identification of herpesvirus in South American cetaceans, and the first in riverine dolphins worldwide.

4.2 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; DAURA-JORGE; SIMÕES-LOPES, 2011). In mammals, herpesviruses (HVs) may cause persistent and latent infections potentially activated by host immunosuppression, with periodic or continuous shedding of infectious virus able to induce cutaneous and mucosal lesions (SIEGAL et al., 1981; ROIZMANN et al., 1992). The *Herpesviridae* family, within the order *Herpesvirales*, is formed by single, linear, double-stranded DNA viruses, currently subdivided into *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* subfamilies, and one unassigned genus - the *Iguanid herpesvirus 2* (ICTV, 2017). HV and their natural hosts are intimately related, usually sharing a common evolutionary history, and generally causing only mild disease (DAVISON, 2002).

Martineau et al. (1988) reported the first HV infection in cetaceans almost 30 years ago, diagnosed 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 genetic sequences in stranded Atlantic bottlenose dolphins (*Tursiops truncatus*). Since then, HV sequences have been identified in at least six odontocete families: Delphinidae, Kogiidae, Ziphiidae (SMOLAREK-BENSON et al., 2006; ARBELO et al., 2010), Physeteridae (MIYOSHI et al., 2011), Monodontidae (BELLEHUMEUR et al., 2015), and Phocoenidae (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 (SMOLAREK-BENSON et al., 2006; MELERO et al., 2015).

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; BELLEHUMEUR et al., 2015). Members of the *Alphaherpesvirinae* subfamily have been previously identified in skin lesions of

Atlantic bottlenose dolphins (MANIRE et al., 2006; SMOLAREK-BENSON et al., 2006; BURDETT-HART et al., 2012), beluga whales (BUREK-HUNTINGTON et al., 2015), the skin and penile mucosal samples of a fin whale (*Balaenoptera physalus*) (MELERO et al., 2015), and on a genital swab of a harbor porpoise (*Phocoena phocoena*) (VAN ELK et al., 2016). In belugas, α-HV sequences were also found in proliferative and ulcerative genital lesions of five individuals, and in an ulcerative stomatitis lesion of another specimen (BELLEHUMEUR et al., 2015). Members of this subfamily have been associated with severe localized and fatal systemic infections in cetaceans (BLANCHARD et al., 2001; ARBELO et al., 2010; ARBELO et al., 2012; SOTO et al., 2012; BUREK-HUNTINGTON et al., 2015), as well as encephalitis and meningoencephalitis in the same taxon (SIERRA et al., 2014; VAN ELK et al., 2016). γ-HV infections in cetaceans have been associated to skin lesions (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; VAN ELK et al., 2009; REHTANZ et al., 2012; CRUZ et al., 2014; SIERRA et al., 2015; VAN ELK et al., 2016). However, their pathogenic significance is at times unclear in reports with no histopathology (LECIS et al., 2014; MELERO et al., 2015).

HVs have been identified in cetaceans worldwide; in the Mediterranean Sea (BELLIÈRE et al., 2010), northwestern (MARTINEAU et al., 1988) and northeastern Atlantic Ocean (ESPERÓN; FERNÁNDEZ; SÁNCHEZ-VIZCAÍNO, 2008), Sea of Japan, and northwestern (MIYOSHI et al., 2011), central (WEST et al., 2013), northeastern (BUREK-HUNTINGTON et al., 2015), and southeastern Pacific Ocean (VAN BRESSEM et al., 1994). 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 are no reports of HV molecular detection in South American cetaceans.

This study evaluated the presence of HV in skin, oral and genital mucosal samples from cetaceans of seven families present in Brazil, including riverine and marine species. Additionally, all other available tissue samples from HV-positive specimens, aside from skin, oral and genital mucosa, were also tested by PCR and histologically evaluated.

4.3 MATERIAL AND METHODS

4.3.1 Samples

We evaluated 166 samples (skin, oral and genital mucosa) from 115 individuals of seven cetacean families (Delphinidae [n=61], Pontoporiidae [n=35], Iniidae [n=3], Kogiidae [n=3], Physeteridae [n=1], Balaenopteridae [n=10], and Balaenidae [n=2]) from Brazil (Table 4.1). 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 (3.09°S, 60.48ºW) and two Bolivian river dolphins (*I. boliviensis*) from Rio Guaporé (12.46°S, 64.29°W and 12.48ºS, 64.13ºW). Necropsies were performed following standard procedures (GERACI; LOUNSBURY, 2005). Selected tissue samples were collected and fixed in 10% formalin or frozen at -20ºC or -80°C. Additional data on the number of evaluated specimens, species, sex, age class, and tissue type are listed in Table 4.1. The age class was determined based on total body length and species, as described by Rosas and Monteiro-Filho (2002a) for Guiana dolphin (*Sotalia guianensis*), Da silva and Martin (2010) for the *Inia* genus, Di Beneditto and Arruda Ramos, (2001) and Rosas and Monteiro-Filho (2002b) for franciscana (*Pontoporia blainvillei*), also determined by the franciscana management area (FMA), according with the region where they were found (FMA I: Espírito Santo and Rio de Janeiro states, and FMA II: São Paulo, Paraná and Santa Catarina states), and Lodi and Borobia (2013) for the remaining studied species. Carcass condition was established according with Geraci and Lounsbury (2005). Nutritional condition (classified into three categories: good, moderate and poor) was visually assessed through the dorso-axial muscle mass next to the dorsal fin, fat deposits caudal to the blowhole, and peduncle shape.

All samples used in this study were collected in full compliance with specific federal permits issued by the Brazilian Ministry of Environment and approved by the Biodiversity Information and Authorization System (SISBIO 13.303-1/6, 14104-1/8, 45656-1, 48279-1, and 49597-1). All procedures were performed according with the Ethical Committee of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (process number: 2951280914).

Table 4.1 - Description of the analyzed individuals, including number of evaluated specimens, species, sex (M = male, F = female, U = unknown), age class (C = calf, J = juvenile, A = adult), and number of skin, oral and genital mucosal samples.

	SPECIES	n	SEX			AGE CLASS			NUMBER OF SAMPLES					
			M	F	U	C	J	A	SKIN [2]*	MUCOSAL				
										ORAL				GENITAL
										Tongue	Palate	Oral ulcer	Oral papilloma	Gingiva	Penile ulcer
odontoceti	Guiana dolphin *Sotalia guianensis*	31	18	13	-	4[1]*	9	18	43 (19)	3	-	-	-	-	-
	Atlantic bottlenose dolphin *Tursiops truncatus*	7	6	1	-	1	1	5	12 (9)	-	-	-	-	-	-
	Melon-headed whale *Peponocephala electra*	6	3	3	-	1	1	4	6	-	-	-	-	-	-
	Short-finned pilot whale *Globicephala macrorhynchus*	2	1	1	-	-	2	-	2	-	-	-	-	-	-
	Rough-toothed dolphin *Steno bredanensis*	1	1	-	-	-	-	1	-	-	-	-	1	-	-
	Atlantic spotted dolphin *Stenella frontalis*	4	2	1	1	-	-	4	13 (11)	1	-	-	-	1	-
	Clymene dolphin *Stenella clymene*	2	2	-	-	-	-	2	2	1	1	-	-	-	-
	Pantropical spotted dolphin *Stenella attenuata*	1	1	-	-	-	-	1	1 (1)	-	-	-	-	-	-
	Striped dolphin *Stenella coeruleoalba*	1	-	1	-	-	-	1	1	-	-	-	-	-	-
	Spinner dolphin *Stenella longirostris*	3	3	-	-	-	1	2	3(1)	-	-	-	-	-	-
	Short-beaked common dolphin *Delphinus delphis*	1	1	-	-	-	-	1	2	-	-	1	-	-	-
	Pygmy killer whale *Feresa attenuata*	1	1	-	-	-	-	1	1	-	-	-	-	-	-
	Killer whale *Orcinus orca*	1	-	1	-	-	1	-	1 (1)	-	-	-	-	-	-

	SPECIES	n	SEX			AGE CLASS			NUMBER OF SAMPLES					
			M	F	U	C	J	A	SKIN [2]*	MUCOSAL				
										ORAL				GENITAL
										Tongue	Palate	Oral ulcer	Oral papilloma	Gingiva	Penile ulcer
	Dwarf sperm whale *Kogia sima*	2	2	-	-	-	1	1	5(2)	-	-	-	-	-	-
	Pygmy sperm whale *Kogia breviceps*	1	1	-	-	1	-	-	1	-	-	-	-	-	-
	Sperm whale *Physeter macrocephalus*	1	1	-	-	1	-	-	1	-	-	-	-	-	-
	Franciscana *Pontoporia blainvillei*	35	18	15	2	17	9	9	42 (13)	3	-		-	-	2
	Bolivian river dolphin *Inia boliviensis*	2	2	-	-	-	1	1	2 (2)	-	-	-	-	-	-
	Amazon river dolphin *Inia geoffrensis*	1	1	-	-	-	1	-	1 (1)	-	-	-	-	-	-
Mysticeti	Southern right whale *Eubalaena australis*	2	-	-	2	2	-	-	2	-	-	-	-	-	-
Mysticeti	Humpback whale *Megaptera novaeangliae*	9	4	4	1	5	3	1	9 (1)	-	-		-		-
Mysticeti	Bryde's whale *Balaenoptera brydei*	1	1	-	-	-	-	1	1 (1)	1	-	-	-	-	-
	TOTAL	115	69	40	6	32	30	53	151 (62)	9	1	1	1	1	2

*Values in parentheses correspond to the number skin lesion samples.

4.3.2 Molecular diagnostics

Total DNA extraction from manually homogenized frozen skin and mucosal samples was performed with the DNeasy Blood & Tissue kit (Qiagen®), following the manufacturer's protocol. In order 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 55ºC. Validated DNA samples (beta-actin positive) were subsequently analyzed by the panherpesvirus protocol described by Vandevanter et al. (1996) for DNA polymerase gene to detect HV. In HV positive cases, additional available tissues aside from skin, and oral or genital mucosal were subsequently evaluated by the above described method. Positive samples were identified through direct sequencing using primers TGVseq and TYG, generating an approximately 200 bp-long fragment, excluding primer sequences. Additionally, sequences more similar to the *Gammaherpesvirinae* subfamily 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 with the aid of a PCR that amplifies a segment of the E1 and F1 papillomavirus-genes (FORSLUND et al., 1999; IFTNER et al., 2003; RECTOR et al., 2005).

Phylogenetic analysis was performed on the obtained sequences, selecting the gene sequences with at least the same length previously detected in cetacean species and available on GenBank. Additionally, α- and γ-HV nucleotide (nt) and amino acid (aa) sequences from several other different animal families were included, and *Salmonid herpesvirus* 3 as outlier. Phylogenetic analysis generated a maximum likelihood tree of 1000 bootstrap replicates. Sequence identities were calculated based on the p-distance. Sequence analyses were performed with MEGA software, version 6.0.

4.3.3 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 (HE).

4.4 RESULTS

4.4.1 Molecular findings

Beta-actin was amplified in 109/115 individuals, corresponding to 96.0% of the evaluated skin samples (145/151) - including 96.7% of the skin lesions (60/62) - and 100% of oral or genital mucosal samples (15/15).

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 specimens: 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 4.2. The geographic locations where the positive cases were found are represented in Figure 4.1.

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 the skin of this specimen, the dwarf sperm whale (coinfected with two different HVs, one found in the bronchi, 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) (Appendix 1). The five different nt DNA polymerase gene fragment sequences ranged from 206 to 207 bp. No glycoprotein B gene or papillomavirus amplification 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 stomach and intestines), the Bolivian river dolphin and the Atlantic spotted dolphin were submitted to GenBank database under accession numbers from MF999151 to MF999155, respectively.

The HV phylogenetic lineages were determined based on a phylogenetic tree (Figure 4.2). The Atlantic spotted dolphin and the Guiana dolphin sequences were classified as α-HVs. The dwarf sperm whale and the Bolivian river dolphin sequences were, respectively, closer to α-HV and 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 skin sample of 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 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 aa identity (67.7%) to *Equid herpesvirus 1* sequences (AHN10514.1 and ADI96155.1). The closest cetacean HVs for both sequences were α-HVs from a Risso's dolphin (*Grampus griseus*) from Spain (ALP00299) and to an Indo-Pacific humpback dolphin (*Sousa chinensis*) from Thailand (AOP12480.1) (66.7% aa id). 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 bat fecal samples in China. The closest cetacean HVs, with amino acid 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.

Figure 4.1 – HV cases in cetaceans from Brazil.

Source: Ferreira Machado and Sacristán (2017)

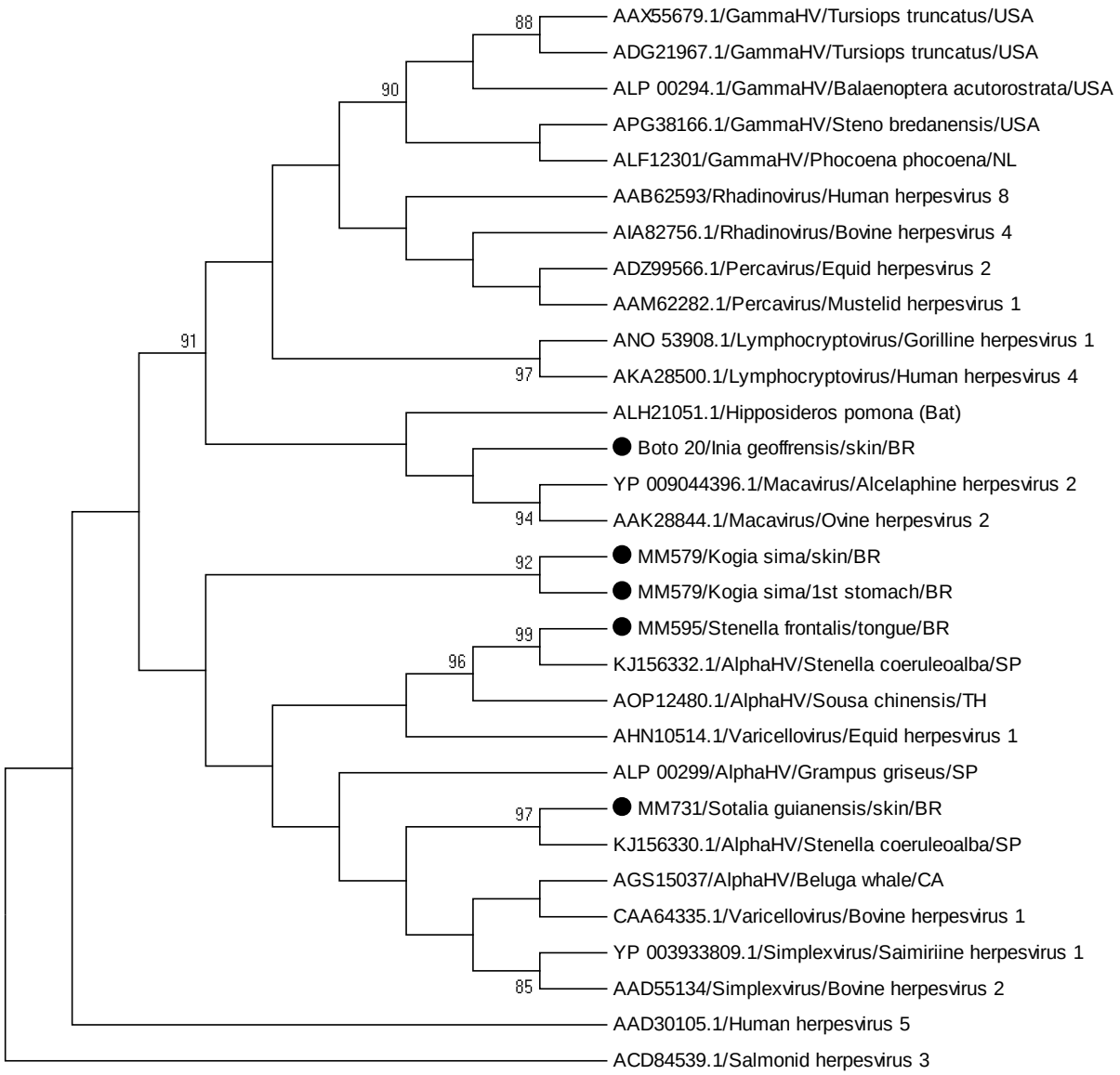
Table 4.2 - Herpesvirus-positive animals: identification (ID), institution of origin, species, age class (C = calf, J = juvenile, A = adult), sex (M= male, F = female, U = unidentified), total body length, nutritional condition (NC), status (captured alive, stranded alive, found dead), tissue condition (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)	NC	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	Good	Captured alive. Code 1	Guaporé river (RO) 12.48ºS, 64.13ºW	27-Sep-15	Nodular verrucous skin lesion (1)	+
MM579	AS	Dwarf sperm whale	M	J	200	Poor	Stranded alive. Code 3	Santos (SP) 23.99ºS, 46.31ºW	04-May-14	Skin lesions (2)	+
										Healthy skin (2)	+
MM595, UNI#353	UNI	Atlantic spotted dolphin	U	A	190	NA*	Found dead. Code 4	São Francisco do Sul (SC) 26.18ºS, 48.53ºW	29-Jun-12	Tongue (1)	+
										Skin (1)	-
MM731 05C1422/335	IBJ	Guiana dolphin	F	C	116	Moderate	Found dead. Code 3	Linhares (ES) 19.25ºS, 39.70ºW	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.

Figure 4.2 – 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.



Source: Sacristán and Esperón (2017).

* BR=Brazil; CA=Canada; NL=The Netherlands; SP=Spain; TH=Thailand; USA=United States of America.

4.4.2 Macroscopic and histological findings

Herein we summarized the macroscopic and histological findings observed in skin and tongue samples of the HV-positive animals, as well as the histologic findings that could possibly be associated with HV infection observed in other tissues samples from these specimens (Figure 4.3). Additional information is available in Appendix 1.

Guiana dolphin. On macroscopy, at least nine well-demarcated, circular or oval white skin lesions, ranging from 1 to 3 cm - one ulcerated and two presenting red ulcerated punctiform centers, were observed in the peduncle. Additionally, the specimen also presented net marks throughout the body. On histopathology, the two HV-positive skin lesions (two white lesions, one of them ulcerated) were characterized by marked, focally extensive, chronic proliferative dermatitis (Figure 4.3a). 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.

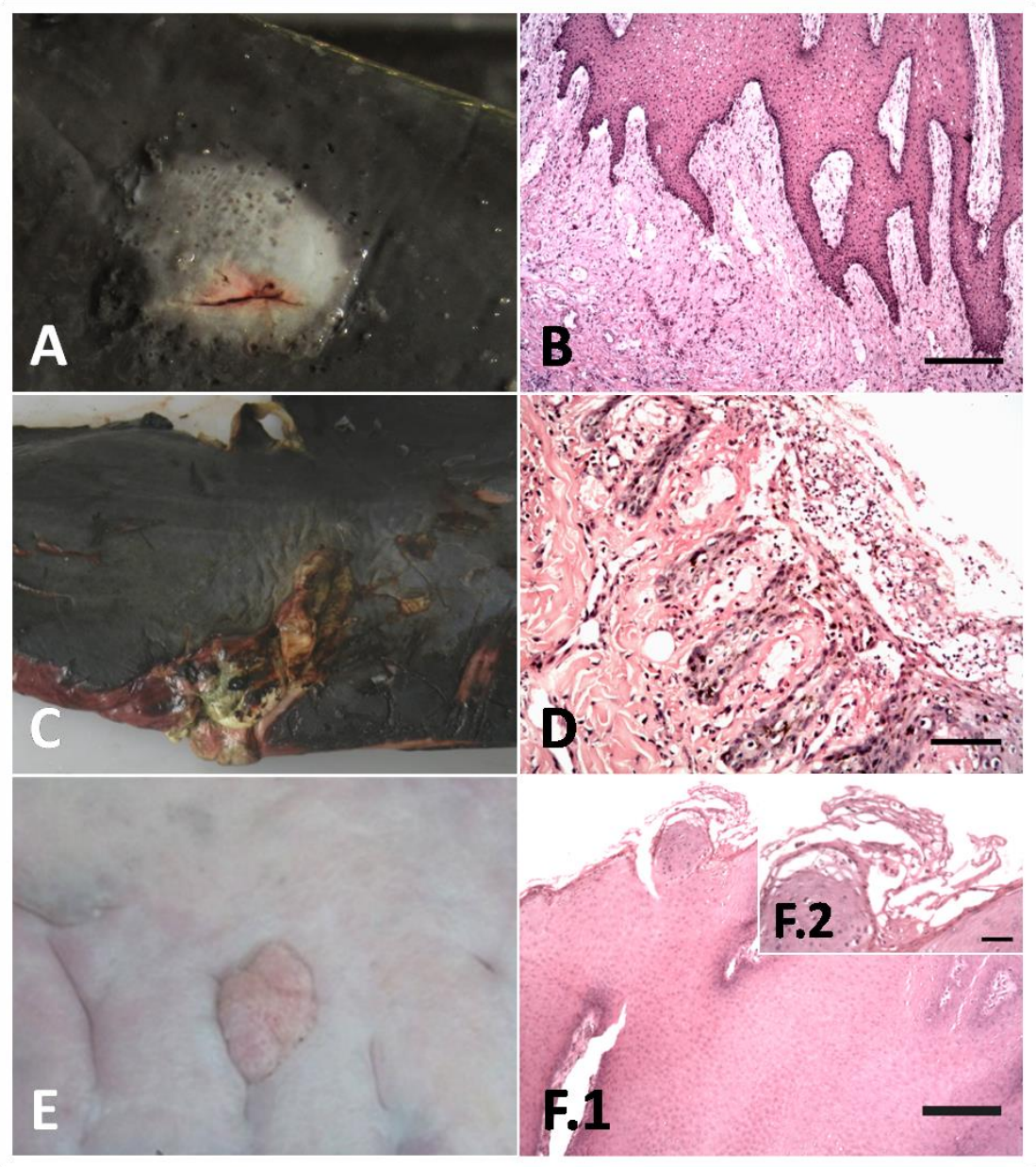
Dwarf sperm whale. Macroscopically, the animal presented multiple (at least 16) parallel *antemortem* cutaneous lacerations throughout the head, dorsum and peduncle, likely caused by interspecific interaction (compatible with bite wounds inflicted by killer whales (*Orcinus orca*) or sharks). Four well-demarcated 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. Additional relevant microscopic findings that could possibly be associated with localized and/or systemic herpesviral infection included: mild, multifocal lymphocytic encephalitis with acute neuronal cortical laminar necrosis and perivascular cuffing and hemorrhage, multicentric lymphoid depletion, mild, multifocal, chronic superficial lymphoplasmacytic balanitis with mild epithelial hydropic and ballooning degeneration and irregular mucosal hyperplasia.

Bolivian river dolphin. Macroscopically, the animal presented an 8 mm in diameter, well-demarcated, raised verrucous skin nodule on the right pectoral fin (Figure 4.3e). Histologically, this nodule was diagnosed as 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 on macroscopy. Advanced autolysis prevented microscopic evaluation.

Figure 4.3 - Macroscopic and microscopic 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, 4X. Bar=200 μm; 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 shark bite lesion, HE, 4X, Bar= 150 μm**;** Bolivian river dolphin: **(E)** Raised verrucous skin nodule; **(F.1)** Chronic proliferative dermatitis, HE, 4X, Bar=200 μm; **(F.2**) detailed view of the inflammatory exudates, HE, 20X, Bar= 50 μm.



Source: Costa Silva and Groch (2014); IBJ (2015); INPA (2015); Sacristán (2017).

4.5 DISCUSSION

We diagnosed HV in 3.7% (4/109) of the evaluated beta actin-positive specimens - 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 (8.3%, 5/60 specimens) from the Netherlands (VAN BEURDEN et al., 2015); α-HV in skin samples from 18 delphinids (11.1%, 2/18 specimens) from Spain (SIERRA et al., 2014); γ-HV in cetacean mucosal lesions (23.1%, 9/32 samples) from the US (SMOLAREK-BENSON et al., 2006); and γ-HV in genital mucosal samples from a captive population of Atlantic bottlenose dolphins (25%, 9/36 specimens) from France and the Netherlands (VAN ELK et al., 2009). However, the occurrence found in this study is slightly higher than the one obtained in odontocete skin lesions from the US, some of them (3.4%, 3/88 samples) infected with α-HV (SMOLAREK-BENSON et al., 2006). One must also remark that the detection of infectious agents is higher in adequately preserved samples (STORCH; WANG, 2013), thus studies on live animals at early stages of the infection are more likely to detect the agent.

Sequence alignment and phylogenetic analysis identified the HV found in the Atlantic spotted dolphin and Guiana dolphin as α-HV, the HV of the dwarf sperm whale was closer to α-HV, whereas the Bolivian river dolphin HV sequence was closer to γ-HV, despite presenting low amino acid identity to the nearest sequences - only 65.1% to three of the γ-HV found in Chinese bats by Zheng et al. (2016). To our knowledge, this is the first report of HVs in Guiana 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, a species with a previous report of γ-HV (AY949830) in a genital slit lesion (SMOLAREK-BENSON et al., 2006).

We identified α-HV in the tongue of an Atlantic spotted dolphin, in skin lesions of a Guiana dolphin, and two sequences more similar to α-HV in healthy and skin lesions of a dwarf sperm whale (Table 4.2). In cetaceans, members of the *Alphaherpesvirinae* subfamily have been previously identified in an oral mucosal lesion (BELLEHUMEUR et al., 2015) and skin lesions (MANIRE et al., 2006;

SMOLAREK-BENSON et al., 2006; BUREK-HUNTINGTON et al., 2015), 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). 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 diagnosed in an Atlantic spotted dolphin is related (98.5% of deduced amino acid identity) to the one found by Sierra et al. (2014) (GenBank sequence nº. 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; HERZING; MARTEN, 2003), and also hybridize (SILVA; SILVA; SAZIMA, 2005; AMARAL et al., 2014), which could enable 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, one requires longer DNA sequences and definition of the molecular clock to promote a comparison between our sequence and the ones found in the Canary Islands.

Despite the amplified fragment's short length (about 200 bp), 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): 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 its identification in genera never previously reported, from different geographical areas. 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 1*.

The HV identified in the Bolivian River dolphin's foci of proliferative dermatitis was more similar to the *Gammaherpesvirinae* subfamily, previously associated with skin lesions in cetaceans (BURDETT-HART et al., 2012; VAN BEURDEN et al., 2015). However, our sequence presented low amino acid identity (40.6%) 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, in the liver of an Indo-Pacific finless porpoise (*Neophocaena phocaenoides*) from a freshwater population of the Yangtze River, China, an ultrastructurally herpes-like virus with only 22% identity to other HVs' DNA polimerase 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 into 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.

The two white skin lesions (one of them centrally ulcerated) collected from the Guiana dolphin and the proliferative verrucous nodule observed on the skin of the Bolivian river dolphin were histologically characterized by proliferative dermatitis with hydropic and ballooning degeneration of lipokeratinocytes. The numerous necrotic ulcerative lesions macroscopically observed in the skin of the dwarf sperm whale were characterized as ulcerative and fibrinosuppurative dermatitis. Despite the absence of viral INIBs suggestive of infection, e.g., HV, the lesions observed in the Bolivian river dolphin and the Guiana dolphin are in accordance with macroscopic 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. In cetaceans, HV is usually considered the etiological agent of a lesion when there is cellular necrosis and typical herpesviral INIB (MARTINEAU et al., 1988; Barr et al.,

1989; SIERRA et al., 2014). Nevertheless, in odontocetes, alpha and gammaherpesviral cutaneous and mucosal lesions have often been characterized by hyperplasia (MANIRE et al., 2006; SALIKI et al., 2006; VAN ELK et al., 2009; VAN BEURDEN et al., 2015; VAN ELK et al., 2016), lipokeratinocyte hydropic change and ballooning degeneration, and presence or absence of INIB (SIERRA et al., 2014; BELLEHUMEUR et al., 2015), sometimes with lymphoplasmacytic inflammatory infiltrates (MANIRE et al., 2006; SALIKI et al., 2006; SIERRA et al., 2014; VAN BEURDEN et al., 2015).

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; WILLIAMS, 2007). The absence of herpesviral INIB on histopathology have been documented in infected pinnipeds (BORST et al., 1986; BODEWES et al., 2015) and cetaceans; in skin lesions of a killer whale with high antibody titer against HV (ABDO et al., 2012), and in a beluga whale with an oral ulcer and five penile lesions positive for HV (BELLEHUMEUR et al., 2015). In spite of the absence of INIBs, the lymphoplasmacytic encephalitis with perivascular cuffing and neuronal necrosis observed in the dwarf sperm whale are in agreement with previous localized central nervous system (CNS) or multisystemic herpesviral infections, sharing characteristics with previous reports of herpesviral-encephalitis in cetaceans (KENNEDY et al., 1992; SIERRA et al., 2014; VAN ELK et al., 2016). However, no HV sequences were obtained from the CNS (Appendix 1).

Papillomavirus is a major differential viral etiology for proliferative lesions in cetaceans, especially in genital mucosal (REHTANZ et al., 2006; GOTTSCHLING et al., 2011; REHTANZ et al., 2012). Concurrent papillomavirus and HV infections have been diagnosed in mucosal lesions of free-ranging Atlantic bottlenose dolphins from Cuba (CRUZ et al., 2014). In the present study, the Bolivian river dolphin's verrucous skin nodule was negative to papillomavirus.

This study reports four 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 specimens from Brazil - the first molecular description of HV infection in cetaceans from South America, and the first evidence of HV infection in riverine dolphins worldwide. Additionally, macroscopic and histologic findings in the Guiana

dolphin and Bolivian river dolphin HV-positive skin lesions and multisystemic herpesviral infection with CNS involvement identified in the dwarf sperm whale were in agreement with previous reports. Four of the detected sequences are possibly novel species, tentatively named *Delphinid herpesvirus 10*, *Kogiid herpesvirus 2*, *Kogiid herpesvirus 3* and *Iniid herpesvirus 1*. The significant differences observed in the HV described in the Bolivian river dolphin could be associated to viral-host coevolution. Further monitoring of Iniidae dolphins is advised in order to clarify this hypothesis.

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Appendix 1. Macroscopic, histologic and molecular findings on the herpesvirus-positive individuals.

ID	Tissue	Gross	Histologic	HV
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.	+
	Tongue	-	Mild, multifocal, chronic lymphoplasmacytic glossitis.	-
	Penis	-	Mild, multifocal, chronic superficial lymphoplasmacytic balanitis with lipokeratinocyte hydropic degeneration and irregular hyperplasia.	NA *
	Eye	Right eye: Focal corneal opacity. Left eye: Focal corneal ulcer	Mild, multifocal, chronic lymphoplasmacytic keratoconjunctivitis; mild fibrinoneutrophilic exudate in the anterior chamber and filtration angle (hypopyon) with focal fibrinocellular thrombosis in *pars plana*.	-
	Cerebrum	Leptomeningeal and neuroparenchymal congestion	Mild, multifocal lymphocytic encephalitis with perivascular cuffing, acute neuronal cortical laminar necrosis and scattered hemorrhage.	-
	Spinal cord, Cerebellum	Congestion	Congestion and scattered hemorrhage.	-
	Lung	Severe pulmonary edema with hemorrhage; Bilateral atelectasia	Marked, diffuse congestion, edema, hemorrhage and fibrin with rare fibrinocellular thrombi; diffuse atelectasia; mild, multifocal	+

ID	Tissue	Gross	Histologic	HV
			lymphoplasmacytic bronchitis and bronchiolitis	
	Pharynx	-	Mild, multifocal, chronic adenitis with lymphoid hyperplasia and lymphocytolysis, and exocytosis with intraductal coccoid bacteria; mild mucosal hyperplasia.	NA
	Trachea	-	Congestion, submucosal edema and serosal hemorrhage	-
	Aorta	-	Moderate, multifocal, acute serosal hemorrhage with focal fibrinocellular thrombus	NA
	Heart	-	Mild, multifocal, acute subendocardial cardiomyocyte degeneration with contraction band necrosis; congestion.	-
	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 globules and 'pink points.'	+
	Spleen	-	Moderate, diffuse lymphoid depletion with histiocytosis; congestion.	NA
	Pancreas	-	Multifocal, acute hemorrhage; diffuse zymogen granule depletion	NA
	Kidney	-	Mild, multifocal, acute tubular degeneration and necrosis with mild tubular ectasia, proteinosis and myoglobin casts; minimal, multifocal, chronic lymphoplasmacytic interstitial nephritis.	-
	Lymph nodes	-	Periaortic: moderate sinus erythrocytosis; mild medullary plasmacytosis. Pancreatic: Moderate sinus erythrocytosis and mild hemosiderosis.	+

ID	Tissue	Gross	Histologic	HV
			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.	
	Esophagus	-	Focal, acute peri-esophageal hemorrhage	-
	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	-	Non-significant findings observed.	+
	Large intestine	-	Moderate, diffuse, transmural edema	
	Skeletal muscle	-	Scattered, acute, segmental myocyte degeneration with hemorrhage	NA
	Thyroid	-	Multifocal, acute pericapsular hemorrhage	NA
	Pituitary	-	Congestion and multifocal, acute hemorrhage	NA

ID	Tissue	Gross	Histologic	HV
	gland			
	Testicle, Epididymis	-	Congestion and interstitial edema	-
Atlantic spotted dolphin	Tongue	-	Advanced autolysis	+
	Skin	Focal subcutaneous hematoma in the left flank; two (5.5x1.5 and 1x0.5 cm) skin lacerations	Advanced autolysis	-
	Intestine	-	Advanced autolysis	-
	Spleen	-	Advanced autolysis	-
	Thymus	-	Advanced autolysis	-
	Hearth	-	Advanced autolysis	-
	Spinal cord	-	Advanced autolysis	-
	Thyroid	-	Advanced autolysis	-
	Esophagus	Focal esophageal ulcer	Advanced autolysis	NA
	Jaw	Multiple dental fracture	NA	NA
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	+

ID	Tissue	Gross	Histologic	HV
			neuthophils, and also rare apoptotic lypokeratinocytes.	
	Kidney	Congestion	Non-significant findings observed.	+
	Muscle	-	Non-significant findings observed.	-
	Liver	-		+
	Blood	-	NA	+
	Brain, meninges	Generalized congestion; hemorrhage	NA	NA
	Lung	Enlarged, severe pulmonary congestion and edema with hemorrhage;focal caseous lesion, 2 cm in diameter, on the left lung;	NA	NA
	Esophagus	Three focal esophageal ulcers	NA	NA
	Jaw	Haematoma	NA	NA

*NA= data not available.

5 FIRST MOLECULAR IDENTIFICATION OF CETACEAN POXVIRUS IN ODONTOCETES FROM SOUTH AMERICA: IMPLICATIONS ON *CETACEANPOXVIRUS* TAXONOMY

5.1 ABSTRACT

The poxviruses identified in cetaceans, temporarily named “cetacean poxvirus” (CePV), belong to the *Chordopoxvirinae* subfamily. We evaluated 151 skin samples from 113 free-ranging specimens from seven cetacean families (Delphinidae [n=60], Pontoporiidae [n=34], Iniidae [n=3], Kogiidae [n=3], Physeteridae [n=1], Balaenopteridae [n=10], and Balaenidae [n=2)]) from Brazil; 3.5% (4/113) presenting typical tattoo skin lesions suggestive of CePV infection and 5.3% (6/113) suspect tattoo skin lesions. In an attempt to detect the virus, we employed PCR amplification of beta-actin to test DNA integrity, subsequently evaluating the poxviral DNA polymerase gene in beta-actin positive samples. The DNA topoisomerase I gene PCR was also amplified in CePV-positive cases, followed by histopathological evaluation of the available tissues. CePV DNA was amplified in 1.9% (2/107) of beta-actin positive specimens: a stranded male Atlantic bottlenose dolphin (*Tursiops truncatus*) from Laguna estuary (28.46º S 48.79º W) (Santa Catarina state) and a Guiana dolphin (*Sotalia guianensis*) from Guanabara Bay (22.82ºS - 43.20º W) (Rio de Janeiro state). Microscopic findings on both positive animals consisted of epidermal ballooning degeneration and keratinocytes containing small, spherical or irregular, homogeneous, pale amphophilic intracytoplasmic inclusions, compatible with poxvirus infection. Viral particles consistent with poxvirus were identified by electron microscopy. We found a relatively high identity between the CePV sequences obtained in the study and those from other *Delphinidae*, despite the geographic distance, suggesting that Delphinids could be infected by a single species of *Cetaceanpoxvirus*. Our findings corroborate with previous identification of this possibly novel genus, tentatively named *Cetaceanpoxvirus.* This is the first study to identify poxvirus in South American odontocetes and CePV specific amino acid motifs in cetaceans.

5.2 INTRODUCTION

The *Poxviridae* family comprises large, linear, double-stranded DNA viruses able to replicate in the cytoplasm of their host (GALVANI; SLATKIN, 2003; MOSS, 2013), and divided into two subfamilies: the invertebrate-infecting *Entomopoxvirinae* and the vertebrate-infecting *Chordopoxvirinae.* The latter includes at least eleven different genera and one unassigned genus (ICTV, 2017). Transmission differs between genera, and may occur via aerosol, fomites, direct contact or arthropods (MCINNES et al., 2006). These viruses cause either (1) localized infection leading to benign skin lesions, or (2) systemic infection, resulting in viral dissemination, and usually death (SMITH; KOTWAL, 2002).

The poxviruses currently identified in cetaceans, temporarily named cetacean poxvirus (CePV) (BRACHT et al., 2006; BLACKLAWS et al., 2013), were first reported in the late 70s in Atlantic bottlenose dolphins (*Tursiops truncatus*) and an Atlantic white-sided dolphin (*Lagenorhynchus acutus*), based on histopathology and electron microscopy techniques (FLOM; HOUK, 1979; GERACI; HICKS; ST AUBIN, 1979). CePV was tentatively classified as a new genus, different from *Orthopoxvirus* and *Parapoxvirus* genera (BLACKLAWS et al., 2013), related to an immediate terrestrial ancestor of the *Orthopoxvirus* genus (BRACHT et al., 2006), and containing at least two groups: CePV-1 in odontocetes, which may contain various sub-groups associated to different odontocete families (BLACKLAWS et al., 2013), and CePV-2 in mysticetes (BRACHT et al., 2006; FIORITO et al., 2015). Both CePV-1 and CePV-2 are considered emerging pathogens in cetaceans (BOSSART, 2007; VAN BRESSEM et al., 2009).

CePV-associated skin lesions have been described by histopathology (GERACI; HICKS; ST AUBIN, 1979; BARNETT et al., 2015; FIORITO et al., 2015), electron microscopy (FLOM; HOUK, 1979; GERACI; HICKS; ST AUBIN, 1979; SMITH et al., 1983; VAN BRESSEM et al., 1993; VAN BRESSEM; VAN WAEREBAEK, 1996; BARNETT et al., 2015; FIORITO et al., 2015), and molecular studies (BRACHT et al., 2006; BLACKLAWS et al., 2013; BARNETT et al., 2015; FIORITO et al., 2015). Serological techniques have been used to detect antibodies against poxvirus in animals presenting pox lesions confirmed by electron microscopy

(SMITH et al., 1983), and in the presence or absence of suggestive pox lesions (VAN BRESSEM; VAN WAEREBEEK; BENNETT, 2006). When identifying the agent was not possible, the condition was characterized based on macroscopic findings and referred to as “Cetacean poxvirus-like lesions” (CePV-like lesions) (FURY; REIF, 2012) or “tattoo-skin disease” (VAN BRESSEM et al., 2009).

Poxvirus skin lesions are known as ‘targets’, ‘watered silk’, ‘rings’, ‘pinholes’, ‘circles’ and ‘tattoo lesions’ (GERACI; HICKS; ST AUBIN, 1979). Early epidermal ring lesions are characterized by circumscribed pale light to dark gray areas, confined to the epidermis, and arranged as single or overlapping circular lesions. Lesions in advanced stages generally present depressed black punctiform centers, sometimes forming a stippled pattern of varying designs, known as tattoo lesions (GERACI; HICKS; ST AUBIN, 1979). These lesions are usually benign; however, one Atlantic bottlenose dolphin died after developing generalized tattoo lesions (SWEENEY; RIDGWAY, 1975). Histological findings include thickened stratum corneum, ballooning degeneration and pale eosinophilic intracytoplasmic inclusions containing typical viral particles within cells of the stratum intermedium (GERACI; HICKS; ST AUBIN, 1979).

CePV has a worldwide distribution, with confirmed reports in several species in the northern (GERACI; HICKS; ST AUBIN, 1979) and southern Atlantic (FIORITO et al., 2015), northern (BRACHT et al., 2006) and southern Pacific (VAN BRESSEM et al., 1993; VAN BRESSEM; VAN WAEREBAEK, 1996), and the North Sea (BLACKLAWS et al., 2013). South American cases of CePV-like lesions were described in several species from the Atlantic and Pacific oceans, based only on visual assessment: in the southeastern Pacific, in short-beaked common dolphin (*Delphinus delphis*), long-beaked common dolphin (*Delphinus capensis*), dusky dolphin (*Lagenorhynchus obscurus*), Chilean dolphin (*Cephalorhynchus eutropia*), Peale's dolphin (*Lagenorhynchus australis*), Burmeister's porpoise (*Phocoena spinipinnis*) and Atlantic bottlenose dolphin (VAN BRESSEM et al., 1993; VAN BRESSEM et al., 2007). In the southwestern Atlantic, the first report of CePV-like lesions was in an Atlantic bottlenose dolphin, in 2002 (SÁNCHEZ et al., 2002), followed by a report in Commerson's dolphin (*Cephalorhynchus commersonii*) and in Guiana dolphin (*Sotalia guianensis*) (VAN BRESSEM et al., 2007; FLACH et al., 2008; ROSAS et al., 2010). Pox-like particles were ultrastructurally identified in

odontocetes: in dusky dolphin, Burmeister's porpoise and long-beaked common dolphin from the southeastern Pacific (VAN BRESSEM et al., 1993; VAN BRESSEM; VAN WAEREBAEK, 1996), and in mysticetes, in a southern right whale (*Eubalaena australis*) from the southwestern Atlantic (FIORITO et al., 2015). CePV infection was also identified by PCR in mysticetes from the southwestern Atlantic and the lesions were characterized by histopathology (FIORITO et al., 2015).

The comparison between CePV sequences identified in animals from different geographic areas could help clarify the phylogeny of this agent. Nevertheless, to our knowledge, no molecular techniques have been used to detect poxvirus-related tattoo lesions in odontocetes from South America. In addition, there is no available information on the specific amino acid motifs of cetacean poxviruses, which could help establishing if they should be classified into a separate new genus. The goals of our study were to identify cetacean poxvirus (CePV) through molecular diagnostics and describe the histopathological characteristics of skin lesions presented by the positive animals.

5.3 METHODS

5.3.1 Samples

We evaluated 151 frozen skin samples from 113 animals from seven different families (Delphinidae [n=60], Pontoporiidae [n=34], Iniidae [n=3], Kogiidae [n=3], Physeteridae [n=1], Balaenopteridae [n=10] and Balaenidae [n=2]) (Table 5.1). Aside from the three free-ranging Iniidae captured in 2015 and released soon after sample collection, the remaining 110 analyzed specimens died either prior or after stranding along the Brazilian coast, between 2005 and 2015.

Table 5.1 - Description of the analyzed individuals, including number of evaluated specimens, species, sex (M = male, F = female, U = unknown), age class (C = calf, J = juvenile, A = Adult), and number of skin samples (tattoo lesions, suspect tattoo lesions and total number).

	SPECIES	n	SEX			AGE CLASS			ANALYZED SKIN SAMPLES [2]*		
			M	F	U	C	J	A	Tattoo	Suspect tattoo	Total
Odontoceti	Guiana dolphin *Sotalia guianensis*	31	18	13	-	4[1]*	9	18	2	8	43
	Atlantic bottlenose dolphin *Tursiops truncatus*	7	6	1	-	1	1	5	3	1	12
	Melon-headed whale *Peponocephala electra*	6	3	3	-	1	1	4	-	-	6
	Short-finned pilot whale *Globicephala macrorhynchus*	2	1	1	-	-	2	-	-	-	2
	Atlantic spotted dolphin *Stenella frontalis*	4	2	1	1	-	-	4	-	-	13
	Clymene dolphin *Stenella clymene*	2	2	-	-	-	-	2	-	-	2
	Pantropical spotted dolphin *Stenella attenuata*	1	1	-	-	-	-	1	-	-	1
	Striped dolphin *Stenella coeruleoalba*	1	-	1	-	-	-	1	-	-	1
	Spinner dolphin *Stenella longirostris*	3	3	-	-	-	1	2	1	-	3
	Short-beaked common dolphin *Delphinus delphis*	1	1	-	-	-	-	1	-	-	2
	Pygmy killer whale *Feresa attenuata*	1	1	-	-	-	-	1	-	-	1
	Killer whale *Orcinus orca*	1	-	1	-	-	1	-	-	-	1
	Dwarf sperm whale *Kogia sima*	2	2	-	-	-	1	1	-	-	5
	Pygmy sperm whale *Kogia breviceps*	1	1	-	-	1	-	-	-	-	1
	Sperm whale *Physeter macrocephalus*	1	1	-	-	1	-	-	-	-	1
	Franciscana *Pontoporia blainvillei*	34	17	15	2	16	9	9	-	1	42
	Bolivian river dolphin *Inia boliviensis*	2	2	-	-	-	1	1	-	-	2
	Amazon river dolphin *Inia geoffrensis*	1	1	-	-	-	1	-	-	1	1
Mysticeti	Southern right whale *Eubalaena australis*	2	-	-	2	2	-	-	-	-	2
	Humpback whale *Megaptera novaeangliae*	9	4	4	1	5	3	1	-	-	9
	Bryde's whale *Balaenoptera brydei*	1	1	-	-	-	-	1	-	-	1
	TOTAL	113	67	40	6	33	29	52	6	11	151

[1]*One of animal included in this group is a fetus. [2]*Values in parentheses correspond to skin lesions.

Necropsies followed standard procedures (GERACI; LOUNSBURY, 2005), adapted to the routines of the involved institutions. The carcasses' preservation status was determined based on established protocols (GERACI; LOUNSBURY, 2005). The nutritional condition was classified into three categories (good, moderate or poor) based on the development of the epaxial musculature and the fat deposit caudal to the blowhole.

At least four of the specimens presented samples of typical tattoo skin lesions (n=6), characterized by well-defined dark margins and a stippled interior, whereas six animals had suspect tattoo skin lesions samples (n=11), presented as dark lesions.

Tissue samples were collected and frozen at -20 or -80°C, or fixed in 10% formalin. Examination of the dental enamel of an extracted tooth (PIERCE; KAJIMURA, 1980; HOHN, 1990) determined the age of specimen MM610 (Atlantic bottlenose dolphin). Total body length was used to establish the age class of the other species: Guiana dolphin (ROSAS; MONTEIRO-FILHO, 2002a), the *Inia* genus (DA SILVA; MARTIN, 2010), franciscanas from franciscana management area (FMA)-I (Espírito Santo and Rio de Janeiro states) (DI BENEDITTO; ARRUDA RAMOs, 2001) and from FMA-II (São Paulo, Paraná and Santa Catarina states) (ROSAS; MONTEIRO-FILHO, 2002b) based on the observed size differences among specimens from both areas, and all remaining species (LODI; BOROBIA, 2013).

All samples used in this study were collected in full compliance with specific federal permits issued by the Brazilian Ministry of Environment and approved by the Biodiversity Information and Authorization System (SISBIO 14104-1/8, 45656-1, 48279-1, and 49597-1). All procedures were in compliance with the Ethical Committee of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (Process number 2951280914).

5.3.2 Molecular diagnostics

Total DNA extraction was performed with the DNeasy Blood & Tissue kit (Qiagen) in blubber-free, transversely trimmed and manually homogenized samples of frozen skin (healthy and affected), according with the manufacturer's protocol, following overnight digestion with 40 µL of proteinase K. The DNA was tested with a housekeeping beta-actin gene PCR assay with a primer set described by Behrens et al. (1998), at a melting temperature of 55ºC. In order to detect the agent, we employed the primers described by Bracht et al. (2006) for DNA polymerase in beta-actin 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 43ºC. In CePV positive cases, all available tissues, aside from skin, were also extracted by the same technique described above and tested by DNA polymerase PCR. Amplified products were read in 1.5% agarose gel stained with Syber® Safe (Invitrogen). Positive samples were identified through direct sequencing using both primers. The identity of the obtained sequences to the most closely related sequences was established based on the p-distance. After ClustalW amino acid (aa) alignment of the CePV sequences obtained in this study and those of similar size available at GenBank, and a representative of all species in the *Chordopoxvirinae* subfamily recognized by the ICTV (International Committee on Virus Taxonomy), a maximum likelihood tree of 1000 bootstrap replicates was generated by phylogenetic analysis. The aa alignment was also employed to detect specific aa motifs. A p-distance analysis was conducted to compare the DNA polymerase and topoisomerase I genes nt and aa identities among the different genera, comprised in the subfamily *Chordopoxvirinae*, and *cetaceanpoxvirus*-1 and dolphin- and porpoise pox sequences. Sequence analyses were performed with MEGA software version 6.0.

5.3.3 Electron microscopy

Transmission electron microscopy (TEM) was performed in two tattoo skin samples from a pair of CePV-conventional PCR positive individuals, initially fixed in formalin and subsequently fixed in Karnovsky solution, containing paraformaldehyde, sodium hydroxide, glutaraldehyde and cacodylate buffer. Samples were washed in 0.1M Cacodyate buffer (CaCo), and post fixed in 1% Osmium tetroxide (in 0.1M CaCo buffer). After a gradient step dehydration using increasing volumes of ethanol, samples were embedded in Epon araldite, which polymerized over 48hr at 60ºC. Ultrathin sections were then obtained. Micrographs were taken in a FEI MORGAGNI 268 Transmission electron microscope and images recorded using a side-mounted Olympus Veleta CCD camera.

5.3.3 Histological examination

Histological evaluation of the positive specimens was performed in formalin-fixed tissues embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin-eosin (HE).

5.4 RESULTS

5.4.1 Molecular findings

We amplified the beta-actin gene in 94.7% (107/113) of the specimens, corresponding to 96.0% (145/151) of the skin samples, including three out of four specimens presenting typical tattoo lesions and all animals with suspected tattoo lesions.

CePV DNA polymerase and DNA topoisomerase I genes were amplified in two out of 107 (1.9%) beta actin positive specimens: an Atlantic bottlenose dolphin (MM610) and a Guiana dolphin (MM672), both presenting typical tattoo skin lesions (three and one, respectively) (Table 5.2). We obtained two 497 nucleotide (nt) sequences of the DNA polymerase gene and two 302 nt sequence fragments of the DNA topoisomerase I gene in the Atlantic bottlenose dolphin and the Guiana dolphin, respectively, not including the respective primers. The new CePV DNA polymerase and DNA topoisomerase I sequences were submitted to GenBank under accession numbers (KU726612) and (KU726611) for the Atlantic bottlenose dolphin and (MF458199) and (MF458200) for the Guiana dolphin, respectively.

The virus was neither amplified on apparently healthy skin samples from the positive Atlantic bottlenose dolphin, nor in other tissues (aside from skin) from the Atlantic bottlenose dolphin (brain, laryngeal tonsil, lung, thymus, spleen, pancreas, liver, kidney, prescapular, pulmonary, mesenteric and rectal lymph nodes) and Guiana dolphin (liver, kidney, muscle).

In the Atlantic bottlenose dolphin, the DNA polymerase gene presented highest nt identity (95.5%) with a sequence from an Indo-Pacific bottlenose dolphin (*T. aduncus*) from Hong Kong (GenBank accession no. AY463006). DNA topoisomerase I gene presented a nt identity of 92.4% with the Guiana dolphin from Brazil reported here. Nevertheless, 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 (*S. bredanensis*) from the United States (US) (GenBank accession numbers: AY463004 and AY952949, respectively), and our Guiana dolphin sequence for the DNA polymerase gene.

All the CePV sequences, including the novel one from Brazil, clustered together, separated from the other analyzed poxvirus genera, in the obtained phylogenetic trees for poxviral DNA polymerase and DNA topoisomerase I genes. Two main groups of CePV were observed: CePV-1 - containing the odontocete sequences - and CePV-2 - comprising the mysticete sequences (Figure 5.1).

The poxvirus DNA polymerase gene fragment obtained from our positive Guiana dolphin presented a 94.1% nt identity to CePVs from a rough-toothed dolphin from the US (AY463004) and an Indo-Pacific bottlenose dolphin from Hong Kong (AY463006). The highest DNA topoisomerase I gene nt identities (93.4%) were with

CePV sequences from short-beaked common dolphin (KC409060) and striped dolphin (*Stenella coeruleoalba*) (KC409051) from the United Kingdom (UK). The CePV identified in the Guiana dolphin showed the highest DNA polymerase and DNA topoisomerase I gene aa identity (98.8% and 95.9, respectively) to sequences from a rough-toothed dolphin from Florida, US (AY463004 and AY952949), and further 98.8% identity for DNA polymerase gene between the Guiana dolphin CePV sequence and the one from the Atlantic bottlenose dolphin (KU726612) from Brazil. The percentage of nucleotide (nt) and amino acid (aa) identities obtained for the DNA polymerase and DNA topoisomerase I gene of each genus are displayed in Table 5.3.

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 three CePV genus specific amino acid sequence motifs across the DNA polymerase catalytic subunit (see Table 5.4). When compared with *Vaccinia virus* reference strain (*Orthopoxvirus* genus), the CePV amino acid sequence motif comprised between residues 524 and 527 presented the insertion of leucine (in CePV-1) or lysine (in CePV-2) between residues 525 and 526 (see Table 5.4).

Table 5.2 - Poxvirus-positive specimens: individual identification, institution of origin, species, sex (M= male, F= female, U= undetermined), age class (AG) (N: newborn, C: calf, J: juvenile, A: adult), total body length, nutritional condition (NC) (G=good, M= moderate, P= poor), degree of tissue preservation, type of tissue sample, and stranding location and date.

ID, and Institution of Origin[1]	Species	Sex	Age Class	Total length (cm)	NC	Condition, Location and Brazilian Federal State[2]	Date of stranding	Analyzed skin sample
MM610 UDESC 33337	*Tursiops truncatus*	M	J	257	G	Stranded dead. Code 3. Laguna estuary (SC) (28.46º S - 48.79º W)	18-01-14	Tattoo skin lesion (3) Healthy skin (1)
MM672 MQ295	*Sotalia guianensis*	M	J	159	G	Found dead. Code 3. Guanabara Bay (RJ) (22.82ºS - 43.20º W)	17-10-09	Tattoo skin lesion (1)

[1]Institution of origin: UDESC: Departamento de Engenharia de Pesca - Universidade do Estado de Santa Catarina, MQ = MAQUA: Laboratório de Mamíferos Aquáticos e Bioindicadores "Prof[a] Izabel M. G. do N. Gurgel".

[2]Brazilian Federal State: SC: Santa Catarina, RJ: Rio de Janeiro.

NA- data not available.

Table 5.3 - Percentage of nucleotide (nt) and amino acid (aa) identity for the DNA polymerase and DNA topoisomerase I genes of each genus.

	DNA polymerase		**DNA topoisomerase**	
	% nt identity	**% aa identity**	**% nt identity**	**% aa identity**
Avipoxvirus	71.3-91.4	76.8-93.8	73.0-90.0	77-95.6
Capripoxvirus	96.3-98.1	96.6-98.9	98.2-99.1	99.1-100
Leporipoxvirus	91.2	93.8	89.7	94.7
Orthopoxvirus	86.5-99.6	96.6-100	88.9-100	93.8-100
Parapoxvirus	90.1-95.3	90.4-94.4	86.2-92.5	89.6-93
Yatapoxvirus	83.1	90.4	84.7	86.7
Cetaceanpoxvirus	82.9-100	87.1-100	84.5-100	83.0-100
Cetaceanpox virus-1	89.9-100	94.9-100	86.5-100	90.2-100
Dolphin pox sequences	91.6-98.1	96.1-98.9	88.3-96.8	92.0-100
Porpoise pox sequences	93.5-100	96.6-100	99.1-100	98.2-100

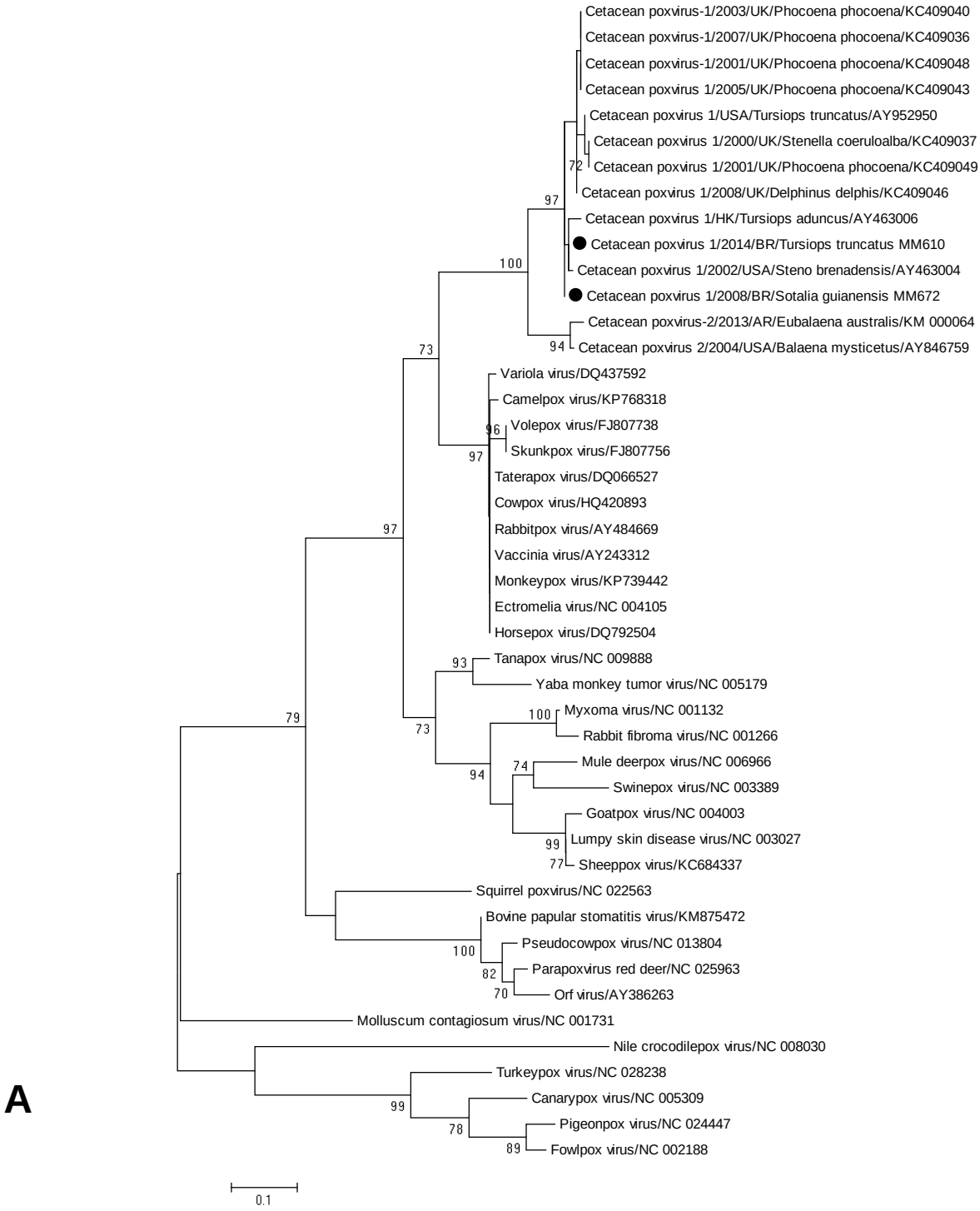
Table 5.4 - Specific amino acid motifs of poxvirus in different genera according with the position 524, 570 and 593 of the *Vaccinia virus* genome.

Genus	**Number 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	n/a*	Q(Q/K)(K/L)LP**	GVVVSNN	PSPRYI(V/I)VHCEPRF***
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

* Not applicable.

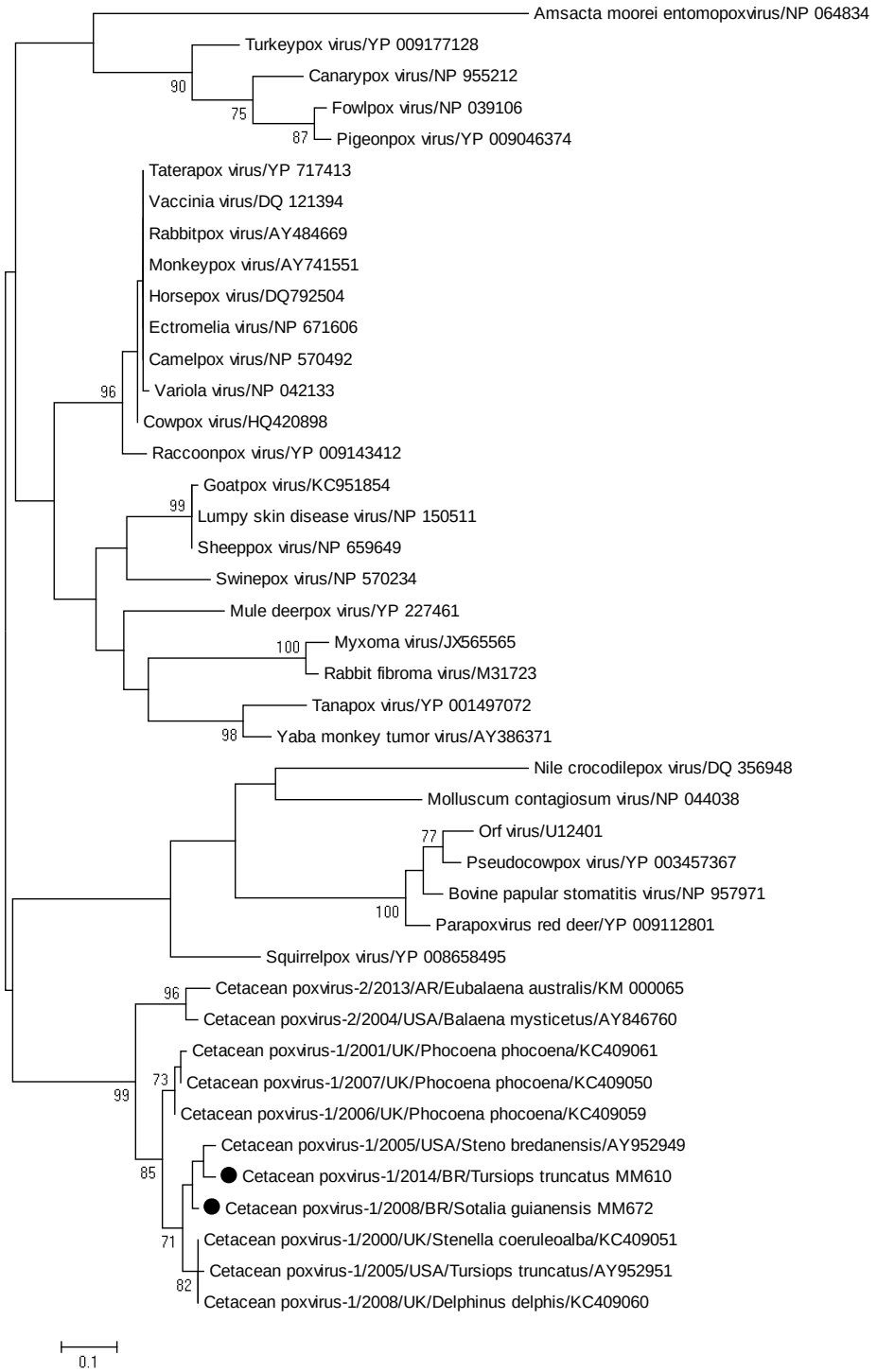
**Cetacean poxvirus 1: QQKLP; cetacean poxvirus 2: QKKLP. ***Cetacean poxvirus 1: PSPRYIVVHCEPRF; cetacean poxvirus 2: PSPRYIIVHCEPRF.

Figure 5.1 - 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 Cetacean poxvirus sequences obtained are expressed as follows: tentative name of the virus; year of detection; place of detection (Ar: Argentina; Br: Brazil; HK: Hong Kong; UK: United Kingdom; USA: United States of America); host species and GenBank Accession Number. The remaining sequences are from recognized poxvirus species and include their GenBank Accession Numbers.



Source: Sacristán and Esperón (2017)

Figure 5.1 - 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 Cetacean poxvirus sequences obtained are expressed as follows: tentative name of the virus; year of detection; place of detection (Ar: Argentina; Br: Brazil; HK: Hong Kong; UK: United Kingdom; US: USA); host species and GenBank Accession Number. The remaining sequences are from recognized poxvirus species, and include their Genbank Accession Numbers.

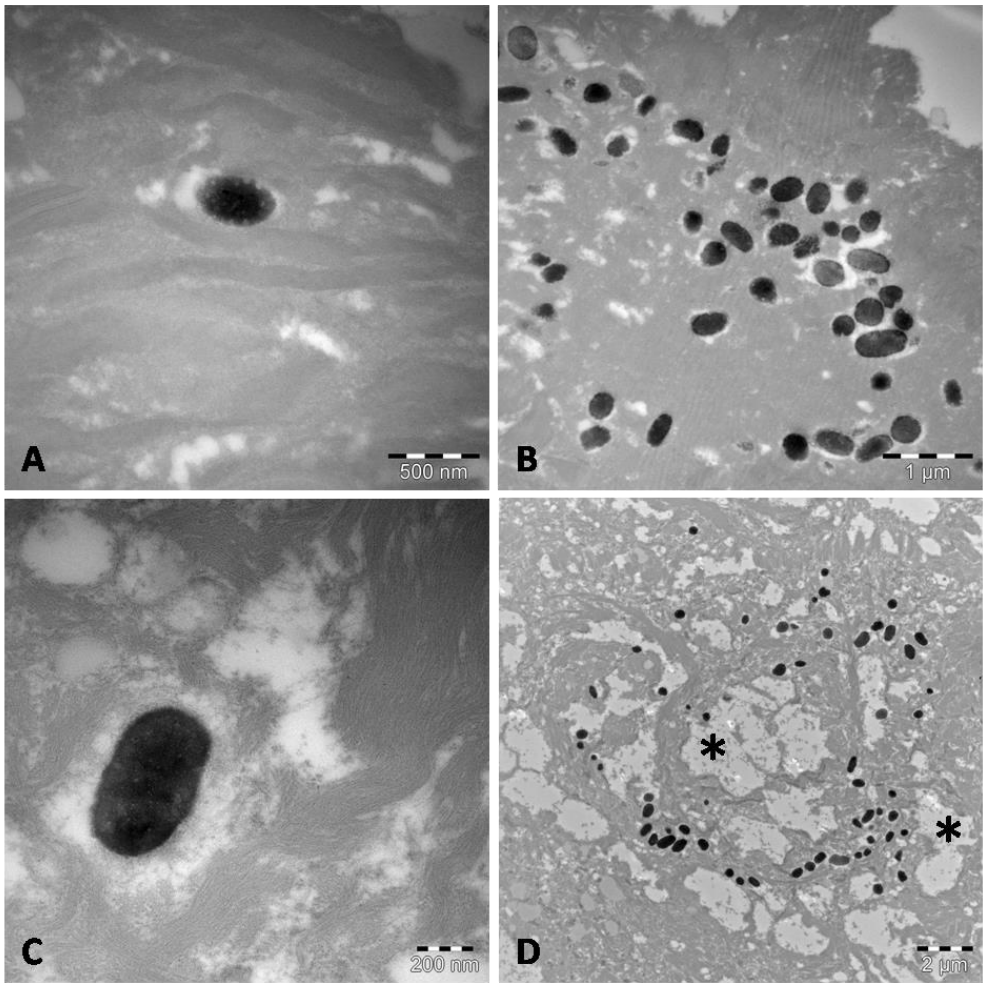


Source: Sacristán and Esperón (2017)

5.4.2 TEM findings

On both CePV positive cases, transmission electron microscopy revealed abundant particles that were in the analysed tissues, and consistent in size and shape with poxvirus. Some keratinocytes containing the viral particles presented irregular clear vacuoles in the cytoplasm (Figure 5.2).

Figure 5.2 – Transmission electron microscopy of tattoo skin lesions in a Guiana dolphin (A), (B) and an Atlantic bottlenose dolphin positive for cetacean poxvirus (C), (D). 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).



Source: Das Neves (2017).

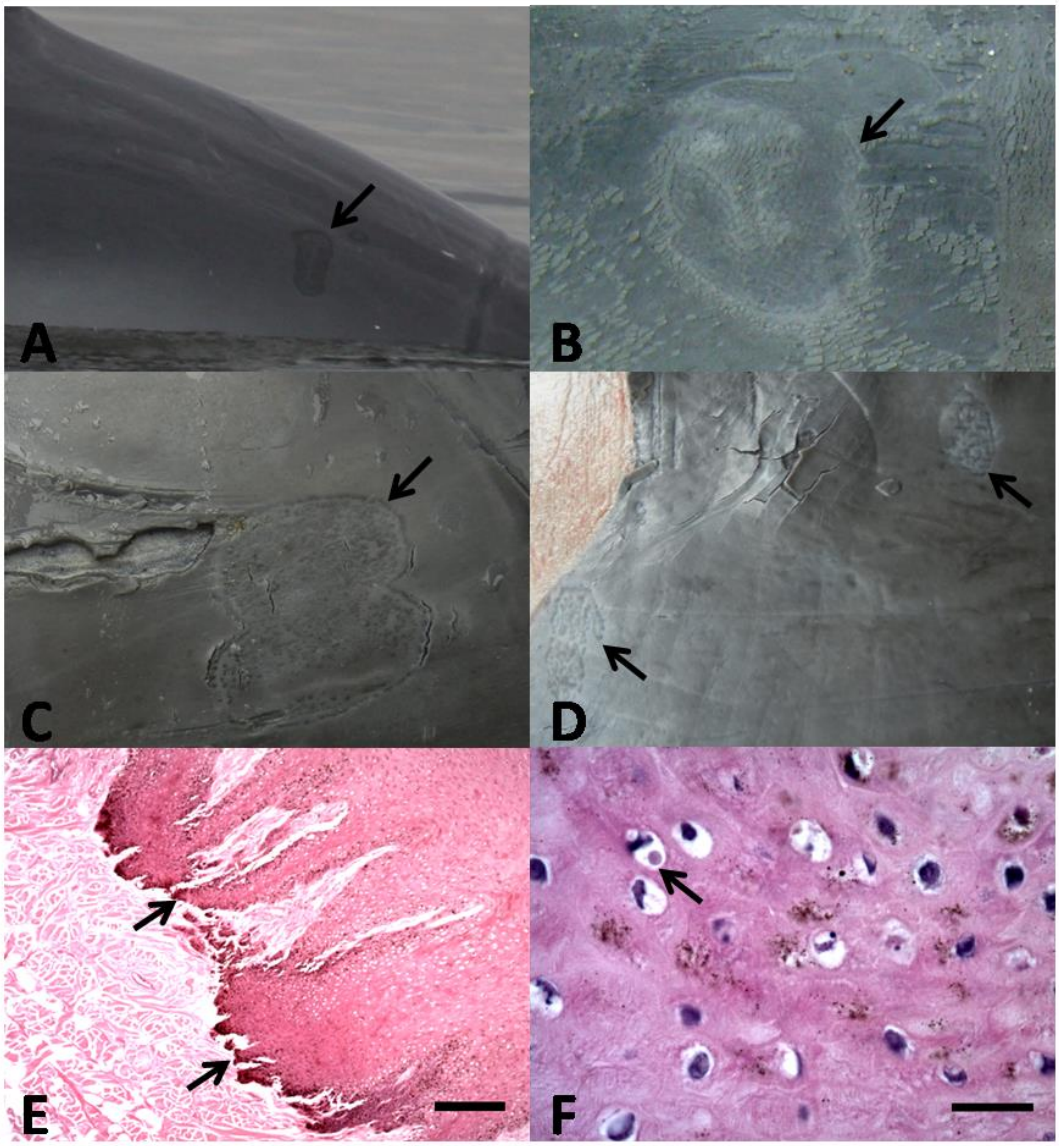
5.4.3 Macroscopic and microscopic findings

Atlantic bottlenose dolphin: a specimen of approximately five years old was found in the Laguna estuary, Santa Catarina state (28.46º S 48.79º W). Linear marks on the left flipper and rostrum were suggestive of interaction with fishing lines. Macroscopically, four round to oval skin lesions presenting a pale light grey stippled interior, with well-defined dark gray irregular margins, ranging between 2-4 cm in diameter, multifocally distributed and consistent with tattoo lesions were observed on the trunk and peduncle (Figure 5.3.C, 5.3.D). Microscopic findings consisted of moderate epidermal hyperplasia, irregular and occasionally fused epidermal rete pegs, epidermal hydropic and ballooning degeneration, mainly in the stratum spinosum, and lipokeratinocytes containing small, spherical or irregular, homogeneous, pale amphophilic intracytoplasmic inclusions with irregularly shaped, compressed and marginalized nuclei. Other significant findings included discrete to moderate mixed bronchitis, mild to moderate bronchopneumonia, edema and multifocal to coalescent moderate verminous suppurative pneumonia caused by unidentified nematodes. Aside from the moderate autolysis, these findings suggest the respiratory process as the cause of death. The nematode *Crassicauda* spp. was found on both pterygoid sinuses.

Guiana dolphin: this juvenile male, an inshore resident of the Guanabara Bay population, was initially identified in July 2008, presenting two small “tattoo” lesions on the right side of its dorsum, 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) (Figure 5.3.A). The individual was monitored until August 2009. In October 2009 the animal was found dead in Ilha do Governador, Rio de Janeiro, Rio de Janeiro state (22.82ºS, 43.20ºW), with fresh net marks and partial fluke amputation. In addition to the two previously observed tattoo lesions, now coalescent and ranging 4x3 cm in size, two new tattoo lesions were identified: on the right flank (2x2 cm in diameter) and next to the blowhole (4x3 cm in diameter). All tattoo lesions presented pale grey stippled interiors, with marked dark gray irregular edges. No other relevant findings were observed during necropsy. The most likely cause of death was interaction with fishing nets. Microscopically, moderate epidermal

hyperplasia, prominent rete pegs, often fused, amphophilic intracytoplasmatic inclusion bodies, and hydropic and ballooning degeneration were observed.

Figure 5.3 - Skin of the specimens affected by cetacean poxvirus. Macroscopic aspect of skin lesions (arrow): Guiana dolphin (A, B) and Atlantic bottlenose dolphin (C, D). Microscopic aspect of a tattoo skin lesion from the Guiana dolphin showing an irregular aspect and hyperplasic epidermal papillae (arrow), Hematoxylin and Eosin (HE), 10X. Bar= 400 μm (E) Presence of amphophilic intracytoplasmic inclusion bodies in the epidermis of the Atlantic bottlenose dolphin (arrow), HE, 40X. Bar= 50 μm (F).



Source: MAQUA (2008), Groch (2014), Sacristán (2014, 2017).

5.5 DISCUSSION

We amplified CePV DNA in two odontocete species – Guiana dolphin and Atlantic bottlenose dolphin - presenting tattoo skin lesions. CePV has been previously amplified in Atlantic bottlenose dolphin from the US (BRACHT et al., 2006). Tattoo skin lesions have been previously observed in Guiana dolphin (VAN BRESSEM et al., 2007; FLACH et al., 2008; ROSAS et al., 2010); however, to the authors' knowledge, this constitutes the first molecular identification of poxvirus in this species. This is also the first report of poxvirus infection in odontocetes from Brazil, from South America, and also from the southern hemisphere. Both specimens presented injuries – the Guiana dolphin concomitant with tattoo skin lesions – that could have potentially served as entry routes for a variety of pathogens (SMITH; SKILLING; RIDGWAY, 1983; SCHULMAN; LIPSCOMB, 1999), including CePV, as described in other members of the *Chordopoxvirinae* subfamily (FENNER, 1992). Both infected specimens were identified as juveniles. This age group seems to be more susceptible to the infection, as previously reported by Barnett et al. (2015). A significant finding in the positive Guiana dolphin is the identification of the virus more than one year after it was first observed. It could be due to persistent infection of the lesion or a reinfection, maybe caused by skin disruption. The Guiana dolphin was a member of the Guanabara Bay population, currently undergoing a marked decline, probably driven by anthropogenic impact, such as bycatch, noise and chemical pollution, boat collision and habitat degradation (AZEVEDO et al., 2017). The Atlantic bottlenose dolphin was part of Laguna estuary population, which inhabits another area greatly impacted by human activities, such as bycatch, noise, chemical and organic pollution (CAVALLI et al., 2008; DAURA-JORGE; SIMÕES-LOPES, 2011). Both species were represented by estuarine inshore animals.

The following differences were noted when the most similar CePV and novel sequences were compared in regards to aa and nt sequences: the Atlantic bottlenose dolphin DNA polymerase gene sequence presented the highest nt identity to Indo-Pacific bottlenose dolphin and rough-toothed dolphin, but the highest aa identity to Guiana dolphin; similarly, the Guiana dolphin presented the highest nt identity for DNA topoisomerase I gene to short-beaked common dolphin, but the

highest aa identity to rough-toothed dolphin. Due to these differences, poxvirus sequences were classified based on amino acid trees (GJESSING et al., 2015), once nucleotide trees were unable to accurately classify the genus, as previously observed by Tuomi et al. (2014). This could possibly be related to the wide variation of GC ratios between different poxvirus genera (HATCHER; WANG; LEFKOWITZ, 2015).

The nt and aa identity of CePV polymerase sequences ranged between 89.9-100% and 94.9-100% in odontocetes, and 82.9-100% and 87.1-100% between odontocetes and mysticetes, respectively. Sequences of poxvirus isolated from Phocoenidae (porpoises) pertaining to the same geographical area (UK), and within a relatively short time frame (2001-2007) were almost identical: 93.5-100% nt and 96.6-100% aa identitities. Nevertheless, sequences obtained from different Delphinidae species, including the two new cases in Atlantic bottlenose dolphin and Guiana dolphin reported in this study, were also highly similar, sharing 91.6-98.1 nt and 96.1-98.9% aa identities, even though detected in different species from very distant areas (Brazil, Hong Kong, UK and US). Despite being more variable than DNA polymerase, this is a common feature among the available sequences of DNA topoisomerase I of poxvirus isolated from cetaceans (Bracht et al. 2006). Odontocete species presented DNA topoisomerase I gene identities between 86.5-100% (nt) and 90.2-100% (aa); 99.1-100% (nt) and 98.2-100% (aa) among porpoises, and 88.3-96.8% (nt) and 92-100% (aa) among Delphinidae. Finally, odontocetes and mysticetes presented similarities up to 84.5% (nt) and 83.0% (aa) between them.

Our data confirm a relative stability between the studied CePV sequences obtained from Delphinidae specimens, despite their geographic distance. The comparison between the CePV aa sequences obtained in this study and those previously described suggests that all sequences could be included into three groups or species, according with the host: mysticetes, and Delphinidae and Phocoenidae odontocete suborders, as observed by Blacklaws et al. (2013). Unfortunately, correct species classification was not possible due to the incomplete information provided by the short genome fragments amplified by the employed PCR methods. This has been observed in DNA polymerase and DNA topoisomerase I gene fragments of the same length in different species within the *Orthopoxvirus* genus (*Chordopoxviridae* subfamily), in which similar DNA polymerase fragments presented 86.5%-99.6% nt and 96.6-100% aa identities and DNA topoisomerase I nt and aa identities varied

between 88.9-100 and 93.8-100%, respectively. These findings show that although belonging to different species, *Orthopoxvirus* sequences could share 100% aa identity; requiring longer sequences to establish the poxviral species. On the other hand, the *Avipoxvirus* genus, also in the *Chordopoxirinae* subfamily, exhibits much more divergence for both DNA polymerase nt (71.3%-91.4%) and aa identity (76.8-93.8%) and DNA topoisomerase I (nt (73.0-90.0%) and aa identity (77.0-95.6%)) than *Orthopoxvirus* (Table 5.3). Our results show that it is not possible to determine the variability within this putative new genus based on the currently available data on cetacean-infecting poxvirus sequences, and consequently, if both PCRs are informative enough to distinguish viral species within this genus. Further molecular studies are are still required to clarify these questions.

The insertion of leucine (in CePV-1) or lysine (in CePV-2) between residues 525 and 526 of the *Vaccinia virus* reference strain is unique to CePV, and is consistent with the clustering of all poxviruses of cetacean origin into a new and unique genus, in accordance with our phylogenetic trees (Figure 5.2) and previous studies (BRACHT et al., 2006; BLACKLAWS et al., 2013; BARNETT et al., 2015; FIORITO et al., 2015). However, in spite of the general consensus on the existence of this putative new genus (tentatively named *Cetaceanpoxvirus*), there is some controversy regarding its number of viral species or subgroups - ranging from two to six - depending on the author's nomenclature (BLACKLAWS et al., 2013; BARNETT et al., 2015).

The observed viral particles presented a similar morphology to that described by Barnett et al. (2015) for cetacean poxvirus. The size variation observed in the viral particles may be in part explained by different maturation stages (mature and immature) (GERACI et al., 1979; VAN BRESSEM et al., 1993; MOSS, 2015). Unfortunately, it was not possible to perform negative contrast, which prevented evaluation of internal features that are characteristic of poxviruses: dumbbell-shaped core, lateral bodies and outer enclosing membrane reported in other poxvirus infections (ICTV, 2017).

The macroscopic aspect of the lesions, characterized by a clear internal area, a stippled aspect and surrounded by a dark margin, are compatible with an advanced tattoo lesion stage, as observed by Geraci; Hicks and St. Aubin (1979) and Smith et al. (1983). Histopathological findings, such as epidermal hyperplasia and

intracytoplasmatic inclusion bodies, along with peripheral chromatin marginalization, specially marked in the stratum basal, hydropic and ballooning degeneration, mainly in the central area of the stratum intermedium, are consistent with previous histopathological descriptions of CePV lesions in cetaceans (GERACI; HICKS; ST AUBIN, 1979; FIORITO et al., 2015). However, the intracytoplasmatic inclusion bodies we observed are amphophilic, and not eosinophilic, as described by Geraci; Hicks and St Aubin (1979) and Fiorito et al. (2015). Marked eosinophilic inclusions are classified as A-type and only produced by certain poxvirus species (FENNER, 1992), whereas amphophilic or basophilic inclusions could be classified as B-type inclusions, which are associated with viral replication and usually found in all poxvirus-infected cells (FENNER, 1992; ROBINSON; KERR, 2008). The epidermal proliferation and irregular aspect of the rete pegs, elongated and often fused, could be related to viral growth factors, as observed in cutaneous poxviral infections by *Orf virus* (FLEMING; WISE; MERCER, 2015).

In this study, we identified two new cases of CePV infection in Atlantic bottlenose dolphin and Guiana dolphin, the first in odontocetes from South America, and also described specific CePV amino acid motifs, confirming that this group belongs to a novel genus. Nevertheless, the evolution of *Poxviridae* is still poorly understood (TUOMI et al., 2014). New approaches are necessary to clarify the taxonomy and evolutionary history of this putative genus tentatively named *Cetaceanpoxvirus*. Complete genome sequencing of selected CePV members would allow further characterization of these viruses' taxonomy and some essential features, such as host specificity and strain virulence. Finally, analyses of other sequences of cetacean poxviruses are also required in order to achieve these goals.

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6 NOVEL AND HIGHLY SENSITIVE SYBR® GREEN REAL-TIME PCR FOR POXVIRUS DETECTION IN ODONTOCETES

6.1 ABSTRACT

Poxviruses are considered emerging pathogens in cetaceans, temporarily named “cetacean poxvirus” (CePV, family *Poxviridae*). These viruses are further classified into two main lineages: CePV-1 in odontocetes and CePV-2 in mysticetes. These viruses have been associated to benign cutaneous lesions known as “tattoos” or “pinholes”. Only a few studies performed the molecular detection of CePVs via DNA polymerase gene (DNA-pol) and/or DNA topoisomerase I gene (DNA-topo) amplification. Herein we describe a new real-time PCR assay based on SYBR® Green and a new primer set to detect a 150 bp fragment of CePV DNA-pol gene also effective for conventional PCR diagnosis. To assess the efficacy of our methods, the relative sensitivity of both novel and current techniques was evaluated through the comparison of the results obtained from 10-fold dilution series of one CePV-positive tattoo skin lesion sample identified from an Atlantic bottlenose dolphin (*T. truncatus*) from Brazil. Additionally, tattoo skin lesions from two Guiana dolphins (*Sotalia guianensis*) from Brazil were also tested by the novel and the currently available methods. The detection limit of our real-time technique was also established. Both novel PCR methods were 1000 to 100,000-fold more sensitive than those previously described in the literature. Skin samples from the two Guiana dolphins were positive to both novel rt- and conventional PCR methods, including a sample previously negative to current conventional DNA-pol and DNA-topo PCR methods. The novel real-time PCR was able to detect from 5 to $5x10^6$ copies per reaction of a cloned positive control. To the authors’ knowledge, this is the first report of a *Cetaceanpoxvirus* real-time PCR detection method, a much more sensitive tool in the diagnosis of CePV-1 infections, able to potentially contribute to the diagnosis of subclinical poxvirus infections, especially in cases of low number of viral copies and compromised DNA integrity (e.g., autolysis, formaldehyde-fixed samples, etc).

6.2 INTRODUCTION

Poxviruses are considered emerging pathogens in cetaceans (BOSSART, 2007). These DNA viruses have large, linear double-stranded genome, and replicate in the cell cytoplasm. The poxviruses infecting cetaceans have been tentatively named cetacean poxvirus (CePV) (BRACHT et al., 2006; BLACKLAWS et al., 2013), and could be further classified in two main lineages: CePV-1 in odontocetes (toothed cetaceans) and CePV-2 in mysticetes (toothless cetaceans) (BRACHT et al., 2006).

CePV infections in cetaceans have been reported since the late 70s (GERACI; HICKS; AUBIN, 1979; FLOM; HOUK, 1979; SMITH; SKILLING, 1979), mainly in skin lesions, known as “tattoos” or “pinholes”, usually characterized by an almost pathognomonic presentation, with a flat or in some advanced stages slightly depressed center, with a stippled interior and delimited by dark margins (GERACI; HICKS; AUBIN, 1979).

Poxvirus lesions have been described in many cetacean species, in animals sustaining an array of physical and health conditions, with individual variations regarding the number of lesions and their developmental stages (BRACHT et al., 2006; BLACKLAWS et al., 2013; FIORITO et al., 2015), however, a possible connection between poxvirus lesions and death was only suggested once (SWEENEY; RIDWAY, 1975). Numerous studies have diagnosed poxvirus-like infections in cetaceans based solely on the visualization of “tattoo-like” lesions (VAN BRESSEM et al., 2007; BEARZI et al., 2009; FURY; REIF, 2012). Presumptive diagnosis is performed by histology, based on the visualization of intracytoplasmic inclusion bodies (FLOM; HOUK, 1979; GERACI; HICKS; AUBIN, 1979; SMITH et al., 1983; BAKER et al., 1992, FIORITO et al., 2015), whereas the identification of viral particles by transmission electron microscopy, and/or poxvirus DNA by PCR are considered the definitive diagnosis (GERACI; HICKS; AUBIN, 1979; FLOM; HOUK, 1979; SMITH et al., 1983; VAN BRESSEM et al., 1993; BRACHT et al., 2006; BARNETT et al., 2015; FIORITO et al., 2015). Viral identification and phylogenetic characterization can only be accomplished with the aid of molecular diagnostics. However, there are very few molecular studies available on CePV detection (BRACHT et al., 2006; BLACKLAWS et al., 2013; BARNETT et al., 2015; FIORITO et

al., 2015). The only two PCR techniques - for DNA polymerase and DNA topoisomerase I genes - to successfully detect poxvirus in cetaceans were described over ten years ago (BRACHT et al., 2006). Barnett et al. (2015) detected poxvirus DNA in all evaluated tattoo lesions, while Blacklaws et al. (2013) obtained mixed results. PCR-negative tattoo lesions could result from a low number or absence of CePV genetic material (e.g., healed lesions), or because such lesions were caused by another etiological agent(s). To establish if the cause is the low number of viral copies or the absence of the virus in the lesions, one requires much more sensitive techniques than those currently available. This study describes a sensitive novel real-time PCR based on SYBR® Green to detect and differentiate poxvirus infections in odontocetes.

6.3 MATERIAL AND METHODS

6.3.1 Samples

The CePV-1 positive control was a tattoo skin lesion sample from a bottlenose dolphin (*Tursiops truncatus*) (MM610) that stranded in Laguna, Santa Catarina State, Brazil, previously diagnosed positive by the conventional DNA polymerase and DNA topoisomerase I PCR methods described by Bracht et al. (2006).

In addition, in order to assess the efficacy of the newly designed primer set, we tested two typical tattoo skin lesions (macroscopically characterized by dark margins and a stippled interior, and microscopically by amphophilic intracytoplasmatic inclusion bodies and ballooning degeneration, mainly in the intermedium stratum) from two Guiana dolphins (*Sotalia guianensis*) – identified as MM499 and MM672 – by conventional and real-time PCR: one of them positive and the other negative for the DNA pol and DNA topo I genes PCRs described by Bracht et al. (2006).

6.3.2 Molecular assays

The assay targets the highly conserved poxvirus DNA polymerase gene and it is guided by a set of degenerate primers (Odonpox-F: 5'-CARGAAATMAAAAAGAARTTTCCATC -3', and Odonpox-R: 5'-ACGTTCTGTTAARAAYCGTCTTAGTA -3'). These primers were designed with the aid of the HYDEN program (LINHART; SHAMIR, 2002), based on the limited data of conserved areas of DNA polymerase gene fragments obtained from odontocetes (porpoises and delphinids) available on GenBank.

Two different PCR protocols were tested with this set of primers, a conventional PCR and a real-time PCR based on SYBR® Green dye. DNA extraction of the control and evaluated samples was performed with the aid of the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's instructions.

The conventional PCR protocol was performed as follows: a final volume of 25 µl contained 2.5 µl of 10X buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer, 1.25 U Fast Start Polymerase® (Roche Applied Science, Penzberg, Upper Bavaria, Germany) and 4 µl DNA template. The thermocycler program was set at 95ºC for 5 min, followed by 40 amplification cycles of 95ºC, 30 sec; 55ºC, 30 sec; 72ºC, 30 sec. The final extension step was performed at 72ºC for 7 min. PCR products were read on a 2% agarose gel electrophoresis, providing a 150 bp sized amplicon.

This conventional PCR protocol was modified into a real-time PCR technique. The reaction (total volume of 20 µl) contained 10 µl 2X KAPA MASTER MIX SYBR® (Kapa Biosystems, Cambridge, Massachusetts, USA), 0.3 µM of each primer, and 4 µl of DNA template. The temperature profile was the same as described above, with the addition of a melting curve step at the end of the reaction. The real-time protocol was performed in a 48-well StepOne™ real-Time PCR System termocycler (Applied Biosystems, Foster City, CA, USA).

Four different PCR protocols were then compared: the conventional and real-time PCRs employing the newly designed primers to amplify a 150-bp fragment of the DNA polymerase gene, and the conventional PCR methods to amplify a 543-bp

fragment from the DNA polymerase gene and a 344-bp fragment from the DNA topoisomerase I gene (BRACHT et al., 2006). The sensitivity of all tested methods was determined by 10-fold serial dilutions analysis of the positive control.

In order to establish the sensitivity and quantification dynamic range of the novel real-time PCR technique, the positive control was cloned as described by Bellière; Esperón; Sánchez-Vizcaíno (2011), and subsequently diluted into ten fold serial dilutions up to less than a copy per reaction, as *per* Sacristán et al. (2016). The standard curve was measured in triplicate.

6.4 RESULTS

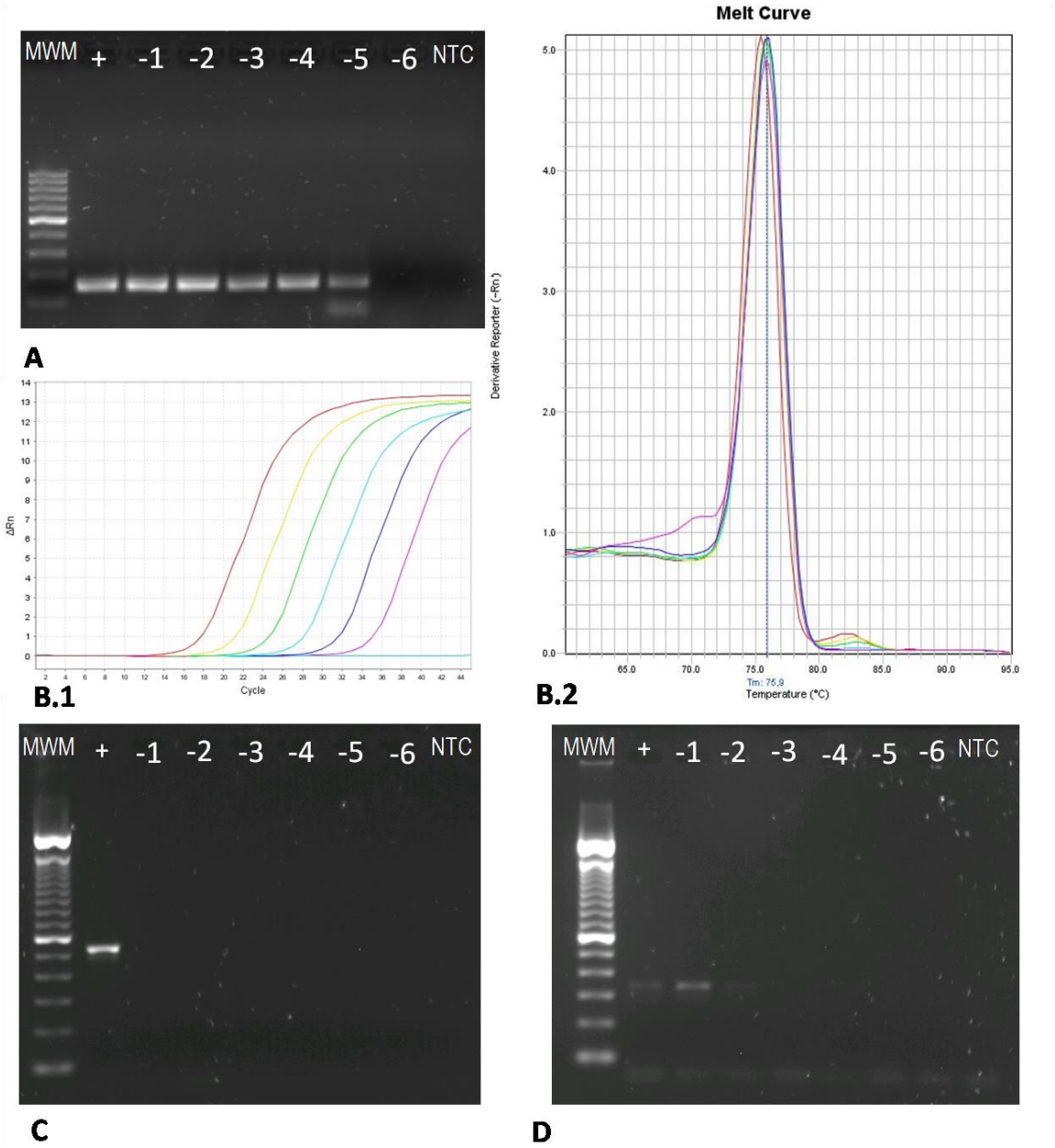
The newly designed methods (real-time and conventional PCR employing primers Odonpox-F and Odonpox-R) detected up to the 10^{-5} dilution of the positive PCR control. Conversely, the conventional PCR for DNA polymerase gene employing FP-DNApol and RP-DNApol primers only detected the undiluted positive control, and the conventional PCR for DNA topoisomerase I gene detected up to 10^{-2} dilution of the positive PCR control (Figure 6.1). Therefore, the novel techniques are 1000 to 100,000-fold more sensitive than those previously described in the literature.

We were able to detect poxvirus DNA by employing novel primers in a conventional and a real-time PCR in two Guiana dolphins presenting typical tattoo lesions, one of the specimens (MM499) previously negative when evaluated by PCR techniques for DNA polymerase and DNA topoisomerase I genes (BRACHT et al., 2006). In addition, we were able to sequence these samples using the real-time PCR product. The sequence from the previously negative Guiana dolphin was: 5' – TCCTAGATACATAGTTGTACATTGTGAGCCACGTTTTAAGAATCTAATTTCTGAAA TATCCATATTCGATAGAGAAATAGAAGGCACTATACCTAGAA – 3'.

The sensitivity results of the real-time PCR carried on ten-fold dilutions of a cloned control showed a dynamic quantification range of 50-$5x10^6$ viral genome copies per reaction, with a R^2 of 0.995 and an efficiency of 99.7%. Moreover, two of the three replicates of 5 viral genome copies per reaction were detected (Figure 6.2). In addition, all replicates of $5x10^6$ copies per reaction were also detected, but linearity was not adequate for their quantification. In conclusion, the novel real-time PCR is

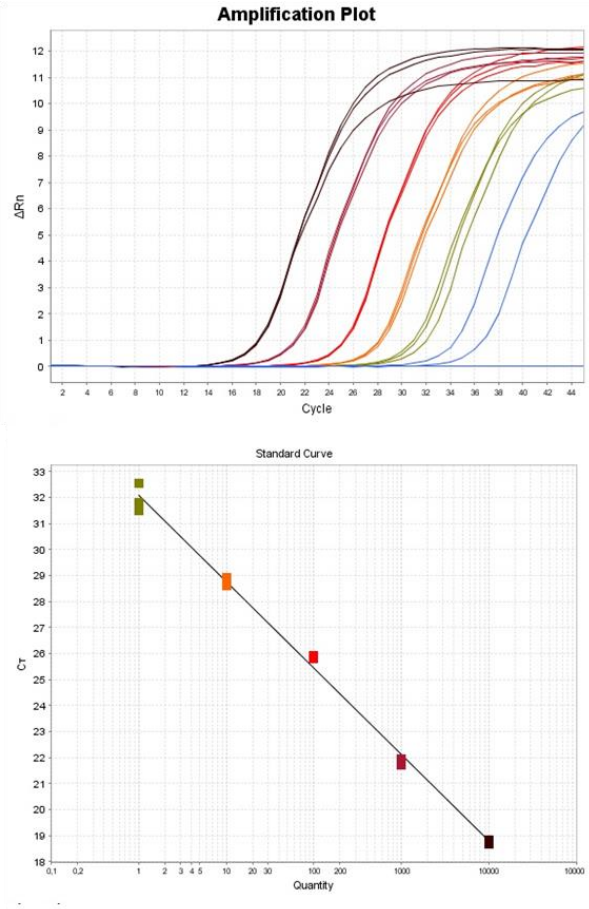
able to detect from 5 to 5x10^6 copies per reaction, and correctly quantifies from 50 to 5x10^6 copies per reaction, being a highly accurate and sensitive technique.

Figure 6.1 Comparison of the novel conventional PCR (A) and real-time PCR targeting the DNA polymerase gene: amplification plot (B.1); and melt curve, (B.2) with the DNA polymerase (C) and DNA topoisomerase I (D) conventional PCR techniques previously described by Bracht et al. (2006) with the MWM: custom molecular weight marker; +: undiluted CePV positive control; −1 to −6: 10x dilution factor of the positive control; NTC: no template control.



Source: Sacristán and Esperón (2017).

Figure 6.2. CePV detection using 10-fold dilutions of a cloned DNA positive control, by triplicate: amplification plot (A); and standard curve (B). For detection (Amplification plot), the concentration of DNA ranged from 5 (two replicates -light blue-) to $5x10^{5*}$copies/reaction (dark purple). Dynamic range of quantification (standard curve) ranges from 50 to $5x10^{5}$copies/reaction, with a R^2= 0.995, and an efficiency = 99.7%.



*All the triplicates of $5x10^{6}$ copies/ reaction were also detected, although they are not included in the amplification plot.

Source: Sacristán and Esperón (2017).

6.5 DISCUSSION

Wildlife diagnosis is particularly challenging, due to our still limited understanding of the diversity of infectious agents affecting these animals. The study of cetaceans infectious diseases could be even more so, once research studies in live animals are challenging and limited, and many times based solely on visual diagnosis. Cetacean strandings represent a great learning opportunity to perform clinical examination and sample collection on live animals, and full *postmortem* examinations on those that died after stranding or that stranded dead, even though in the latter case, one may usually find varying stages of autolysis and predation. Therefore, the detection of short fragments of preserved genes by universal PCR techniques is one of the most successful approaches in wildlife diagnosis, particularly in cetaceans (VANDEVANTER et al., 1996; BELLIÈRE; ESPERÓN, SÁNCHEZ-VIZCAÍNO, 2011; SACRISTÁN et al., 2016).

The novel pair of primers designed in this study can be used in conventional PCR, which reduces its cost, and in real-time-PCR method - this latter a faster technique that allows the user to quantify the viral charge (KLEIN, 2002; MACKAY; ARDEN; NITSCHE, 2002; ESPY et al., 2006). All these characteristics enable the employment of these novel methods by a broader range of laboratories. Both new techniques are markedly more sensitive - able to detect poxvirus DNA up to the 10^{-5} dilution of the positive control - than the two conventional PCRs, respectively amplifying DNA polymerase and DNA topoisomerase I genes, described for cetacean poxvirus by Bratch et al. (2006), and capable of detecting and identifying a novel CePV sample in a previously negative specimen. In addition, the real-time PCR presented the lowest detection limit, below one copy/µL.

Although the novel PCR methods were tested with only a few samples, *in silico* testing employing the described set of primers fully aligned with all odontocetes CePV sequences available, including species from different geographical areas (e.g., from Hong Kong to Brazil) and taxonomically distant families within the odontocete clade (e.g., Phocoenidae and Delphinidae). Therefore, we believe this primer set could be successfully used for CePV detection in other odontocete samples, regardless of species and origin.

To date, several real-time methods have been designed to create sensitive and fast techniques to quantify the expression of certain host genes in order to asses toxicological hazards (SPINSANTI et al., 2006) and detect different infectious agents in cetaceans, such as *Brucella* spp. (SIDOR et al., 2013; WU et al., 2014) and *Morbillivirus* (RUBIO-GUERRI et al., 2013; SACRISTÁN et al., 2016; CENTELLEGHE et al., 2016; YANG et al., 2016). Herein we describe the first real-time method to detect CePV-1 in odontocetes, apparently the most affected cetacean clade. SYBR® Green-based real-time PCR methods are less expensive than Taq-Man-based methods, more sensitive than conventional PCR techniques, and allow phylogenetic studies through PCR product sequencing. In addition, implementing the novel primer set does not imply on further costs, because the same primers used on SYBR® Green-based techniques can be successfully adapted to a conventional PCR method, making it affordable and user friendly. Our novel conventional PCR and real-time PCR - this latter technique with the further advantage of also being faster - are much more sensitive to diagnose CePV-1 infections than the previously described methods, and could potentially contribute to the diagnosis of subclinical poxvirus infections or in any other scenario where the number of viral copies is reduced or the DNA integrity is compromised (e.g., autolysis, formaldehyde fixed samples, etc). Nevertheless, further studies in other tissues, aside from skin, and CePV cases affecting different species and from other geographic areas are needed to assess the accuracy and performance of these novel methods.

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7 LACAZIOSIS-LIKE DISEASE IN TURSIOPS TRUNCATUS FROM BRAZIL: A HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL APPROACH.

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Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach

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ABSTRACT: Cetacean lacaziosis-like disease or lobomycosis-like disease (LLD) is a chronic skin condition caused by a non-cultivable yeast of the order Onygenales, which also includes *Lacazia loboi*, as well as *Paracoccidioides brasiliensis* and *P. lutzii*, respectively responsible for lacaziosis and paracoccidioidomycosis in humans. Complete identification and phylogenetic classification of the LLD etiological agent still needs to be elucidated, but preliminary phylogenetic analyses have shown a closer relationship of the LLD agent to *Paracoccidioides* spp. than to *L. loboi*. Cases of LLD in South American cetaceans based on photographic identification have been reported; however, to date, only 3 histologically confirmed cases of LLD have been described. We evaluated multiple tissue samples from 4 *Tursiops truncatus* stranded in the states of Santa Catarina (n = 3) and Rio Grande do Sul (n = 1), southern Brazil. Macroscopically, all animals presented lesions consistent with LLD. Hematoxylin-eosin, periodic acid-Schiff, Grocott's methenamine silver, and Mayer's mucicarmin stains were used for histological evaluation. Microscopically, numerous refractile yeasts (4–9 µm in diameter) were observed in skin samples (4/4), and for the first time in dolphins, also in a skeletal muscle abscess (1/4). Immunohistochemistry using anti-*P. brasiliensis* glycoprotein gp43 as a primary antibody, which is known to cross-react with *L. loboi* and the LLD agent, was performed and results were positive in all 4 cases. We describe 3 new cases of LLD in cetaceans based on histopathology and immunohistochemistry. This is the first report of LLD in the muscle of cetaceans.

KEY WORDS: Lobomycosis · *Paracoccidioides brasiliensis* · Cetacean · Yeast · Immunohistochemistry · *Tursiops* · Bottlenose dolphin

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INTRODUCTION

Lacaziosis-like disease or lobomycosis-like disease (LLD), an emerging disease in cetaceans (Bossart 2007, Paniz-Mondolfi et al. 2012), is caused by non-cultivable yeast of the order Onygenales. This order also includes *Lacazia loboi*, a non-cultivable fungus responsible for chronic cutaneous and subcutaneous mycoses in humans (Taborda et al. 1999, Herr et al. 2001), as well as other fungal species, such as *Paracoccidioides brasiliensis*, *P. lutzii*, *Coccidioides immitis*, *Blastomyces dermatitides*, and *Histoplasma capsulatum*, all dimorphic pathogenic fungi involved in systemic mycoses in humans (Al-Daraji et al. 2008, Teixeira et al. 2014). Lacaziosis was first described by Jorge de Oliveira Lobo in 1930, in a 52 yr old male worker of an Amazon rubber-tree plantation in Brazil (De Brito & Quaresma 2007).

In cetaceans, LLD causes chronic skin lesions, mainly located on the head, dorsal and pectoral fins, peduncle, and fluke (Reif et al. 2006). Macroscopically, lesions are well demarcated, proliferative, ulcerative or verrucous, varying from whitish to grayish colored, and even light pink (Migaki et al. 1971). Microscopically, granulomas containing occasional giant cells, as well as numerous periodic acid-Schiff (PAS) and Grocott's methenamine silver (GMS) positive, chain-forming yeasts (Rotstein et al. 2009), ranging from 4.1 to 13 µm in diameter (Haubold et al. 2000), are observed.

LLD has a worldwide distribution, with confirmed cases in the northwestern Atlantic, along the Gulf of Mexico coast (Migaki et al. 1971, Reif et al. 2006, Durden et al. 2009), northeastern Atlantic, Biscay Bay (Symmers 1983), Caribbean Sea (De Vries & Laarman 1973, Esperón et al. 2012), southwestern Atlantic (Simões-Lopes et al. 1993), Pacific coast of Japan (Ueda et al. 2013), and the Indian Ocean (Lane et al. 2014).

The first histological description of LLD in cetaceans was reported in common bottlenose dolphin *Tursiops truncatus* by Migaki et al. (1971), followed by reports in Guiana dolphin *Sotalia guianensis* (De Vries & Laarman 1973) and Indian Ocean humpback dolphin *Sousa plumbea* (Lane et al. 2014). LLD has not been observed or diagnosed in South American river dolphins such as the Amazon river dolphin *Inia geoffrensis* or the tucuxi *Sotalia fluviatilis* from the Amazon and Orinoco River basins, where human lacaziosis is endemic (Paniz-Mondolfi & Sander-Hoffmann 2009).

The first histological diagnosis of LLD in Brazil was reported in a transient *T. truncatus* from Laguna, Santa Catarina State, in 1993 (Simões-Lopes et al. 1993). The animal was part of a threatened population, known for engaging in cooperative fishing with local fishermen (Simões-Lopes et al. 1998). A second case was histologically diagnosed in another *T. truncatus* from the Tramandaí estuary, Rio Grande do Sul State, in 2008 (Moreno et al. 2008). Two individuals, 5% of the photo-identified animals, were reported to transit between the Laguna and Tramandaí areas (Simões-Lopes & Fabian 1999). One of these individuals was diagnosed with LLD in Tramandaí 2 yr later (Moreno et al. 2008). Suggestive macroscopic lesions were observed in a *T. truncatus* from the Mampituba River (Moreno et al. 2008) inhabiting the North Bay (Flores et al. 2005), in *S. guianensis* from the Paraná estuary (Van Bressem et al. 2009), and in several *T. truncatus* from the same Laguna population, 21 yr later (Daura-Jorge & Simões-Lopes 2011). Photographic visual assessment has been a useful tool to detect and monitor LLD (Van Bressem et al. 2007, Murdoch et al. 2008, Daura-Jorge & Simões-Lopes 2011). However, Tajima et al. (2015) recently reported a case of suggestive LLD lesions in *T. aduncus* presenting no histological or immunohistochemical evidence of yeast, showing that diagnoses based on photographic visual assessment should be considered carefully.

It is believed that infection in cetaceans occurs through injured skin, as observed in shark bites that became granulomatous (Murdoch et al. 2008, Van Bressem et al. 2009, Paniz-Mondolfi et al. 2012, Ueda et al. 2013). At least 2 cases of mother and calf with suggestive LLD lesions have been described (Kiszka et al. 2009, Van Bressem et al. 2009); however, horizontal or vertical transmission has not been established. To date, only a single case of dolphin-to-human transmission based solely on histopathology has been reported (Symmers 1983) and until recently, it was believed that the same agent species (*L. loboi*) was involved, although this hypothesis was partially discredited by Esperón et al. (2012) through molecular analysis. Interestingly, serological cross-reactivity has been demonstrated among serum from human patients with lacaziosis, and *P. brasiliensis* (Camargo et al. 1998), and positive immune-staining with *P. brasiliensis* antisera has previously been reported in *T. truncatus* with LLD (Ueda et al. 2013). Nevertheless, all 3 agents present significant differences in size (Haubold et al. 2000), potential differences in host/organism interaction (Haubold et al. 2000), and phylogeny. Molecular studies show a greater homology between the LLD agent and *P. brasiliensis* than *L.*

Table 1. Identification, date and place of stranding in Brazil, and size of the studied adult male *Tursiops truncatus*. SC: Santa Catarina, RS: Rio Grande do Sul

Identification	Date	Location	Size (cm)
MM625	21 Oct 2014	Baía Sul, Florianópolis, SC 27.61°S, 48.62°W	317
MM626	30 Oct 2013	Laguna estuary, SC 28.39°S, 48.88°W	301
MM509	04 Oct 2011	Florianópolis, SC 27.57°S, 48.42°W	320
Lobisomem (GEMARS 1259)	03 Nov 2005[a]	Tramandaí estuary, RS 29.98°S, 50.13°W	339

[a]Specimen previously diagnosed with lacaziosis-like disease (Moreno et al. 2008)

loboi (Rotstein et al. 2009, Esperón et al. 2012, Ueda et al. 2013). Our goal is to further characterize the agent and pathogenesis of 4 cases of LLD in dolphins from southern Brazil with the aid of histological and immunohistochemical techniques.

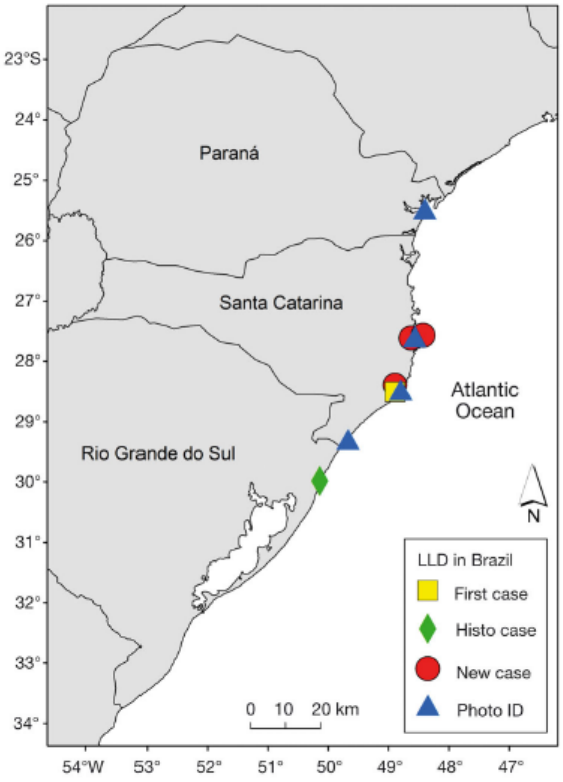

Fig. 1. Lacaziosis-like disease (LLD) cases in adult male *Tursiops truncatus* in Brazil. Symbols represent first case reported, photo-identified cases, old case confirmed by histo-pathology, and new cases confirmed by histo-pathology (2011–2014)

MATERIALS AND METHODS

Samples

We evaluated 4 adult male *Tursiops truncatus* found dead along the southern coast of Brazil, in the states of Santa Catarina (n = 3) and Rio Grande do Sul (n = 1) (Table 1, Fig. 1). The specimen from Rio Grande do Sul had been previously diagnosed with LLD based on histological evaluation by Moreno et al. (2008). Necropsies were performed following the standard procedures established by the institution of origin. Animal MM509 was necropsied according to the protocol described by Geraci & Lounsbury (2005), while only skin samples were collected from animals MM625, MM626 and Lobisomem. Tissue samples were collected and fixed in 10% neutral buffered formalin or 70% alcohol, and frozen samples were stored at –80°C.

Histological examination

Histological evaluation was performed on formalin-fixed tissues embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin-eosin (HE), PAS, GMS, and Mayer's mucicarmin stains.

Immunohistochemistry

Immunohistochemistry (IHC) was performed in 3–4 µm formalin-fixed paraffin sections of suspected lesions incubated with a rabbit polyclonal antibody against *Paracoccidioides brasiliensis* glycoprotein gp43 diluted at 1:50 000, modified from Ueda et al. (2013). The polyclonal antibody used in this study was kindly provided by the Laboratory of Clinical Mycology, College of Pharmaceutical Sciences, São Paulo State University, campi Araraquara (Brazil), and synthesized by inoculation of 1.0 mg ml^{-1} of *P. brasiliensis* 14-3-3 protein in a rabbit. IHC antigen retrieval was achieved with 10 mM of citric acid solution, cooked under pressure, and a horseradish peroxidase polymer system with 3,3′-diaminobenzidine chromogen (HRP/DAB) for signal detection and amplification. Positive human *P. brasiliensis* slides were used as controls.

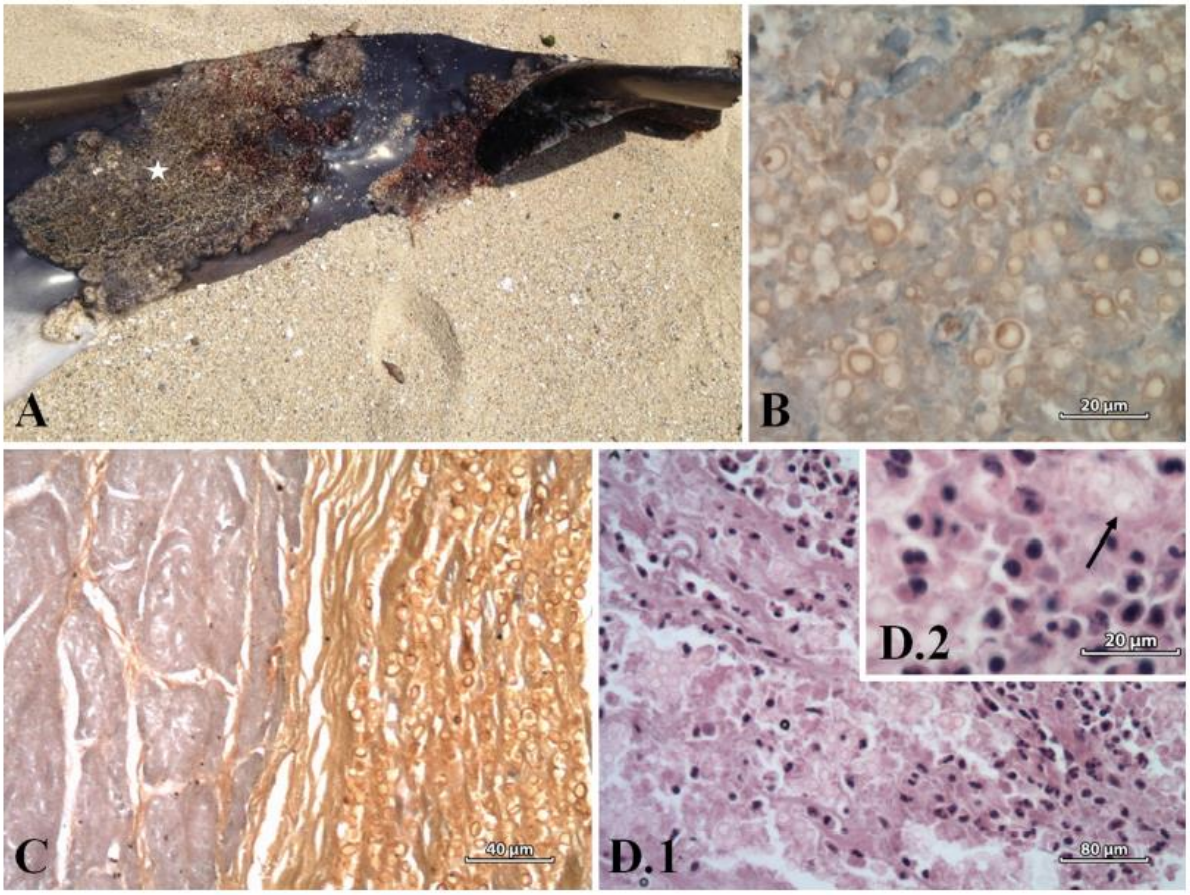

Fig. 2. *Tursiops truncatus* affected by lacaziosis-like disease (LLD). (A) Specimen MM625. Macroscopic cutaneous LLD lesions on the peduncle (star). (B) MM625, skin. Spherical yeast cells stained immunohistochemically. (C) MM509, muscle: presence of yeast in the muscle abscess, stained with Grocott's methenamine silver. (D.1) Center of the abscess shown in (C), hematoxylin-eosin stain. Note the presence of yeast and moderate number of necrotic poly-morphonuclear cells. (D.2) Detailed view of yeasts (arrow) and suppurative response

RESULTS

The affected specimens of *Tursiops truncatus* were found in 3 different locations along the southern coast of Brazil: Tramandaí estuary (n = 1), Laguna estuary (n = 1), and Florianopolis island (n = 2) (Fig. 1).

Macroscopic evaluation showed multiple, elevated, nodular to verrucous, crusty, whitish skin lesions, ranging from 5 to 40 cm in diameter, mainly located on the rostrum, peduncle, and fins, and occasionally distributed throughout the body (Fig. 2). Cut surfaces exhibited irregular circumscribed nodules extending into the deep dermis and epidermis, suggestive of LLD. Microscopically, the dermis exhibited extensive mild granulomatous lesions and necrosis, especially at the dermal–epidermal junction, characterized by the presence of macrophages and multinucleated giant cells occasionally filled with yeast (Table 2). The epidermis was multifocally irregular and hyperplastic. Numerous round, oval, or elliptical yeasts were observed, ranging from 4 to 9 µm in diameter, with doubly contoured, birefringent, and approximately 1 µm thick walls. Yeasts were poorly stained by HE and were negative for Mayer's mucicarmin and positive for GMS and PAS (Fig. 2). Occasionally, yeasts formed chains connected by a tubular isthmus, resembling a string of pearls. Due to the availability of material for an IHC assay, the case diagnosed by Moreno et al. (2008) was also included in our study. All tested samples were positive for IHC performed with antibodies against *Paracoccidioides brasiliensis* gp43 protein in all 4 of our investigated cases.

Table 2. Location and histopathological findings of the new studied cases of lacaziosis-like disease in adult male *Tursiops truncatus* from Brazil

Identification	Location of the lesions	Histopathological description
MM509	Medium dorsal region of the body, peduncle, and fluke	Perivascular granulocytic diffuse dermatitis, acanthosis, focal areas of atrophy of the dermis and multifocal presence of yeast, polymorphonuclear cells and giant cells. Necrotic muscle layer abscess, characterized by a suppurative exudation composed of necrotic polymorphonuclear cells, cellular debris and numerous yeasts, surrounded by a thin fibrous capsule.
MM625	Jaw, beside the pectoral fin, peduncle, and fluke	Perivascular dermatitis, mixed infiltrate, acanthosis. Multifocal presence of yeast and giant cells.
MM626	Medial part of the body	Presence of numerous yeasts, without inflammatory cells. Loss of epidermis.

DISCUSSION

Microscopic findings were consistent with previous histological descriptions of LLD in cetaceans (Migaki et al. 1971, Haubold et al. 2000). In 1 individual, a necrotic muscle layer abscess was observed, filled by numerous yeasts and mixed inflammatory infiltrate, surrounded by a thin fibrous capsule. This finding has not been described in any other LLD cases before, and may be considered an indication of the invasive ability of this subcutaneous mycosis. The presence of the yeast in the muscle could occur through lympho-hematogenous spread, as observed in secondary lesions in other Onygenales members, such as *Paracoccidioides brasiliensis* (Brummer et al. 1993). Nevertheless, given the proximity of the skin lesion, it is more plausible that it was a contiguous infection rather than a systemic infection.

All studied skin and muscle samples were positive for IHC against *P. brasiliensis*. This result was consistent with the IHC findings reported by Ueda et al. (2013) in LLD-affected dolphins in Japan, in which positive immune-staining for *P. brasiliensis* antisera was observed in skin samples from dolphins with LLD. The evidence observed in our study places the LLD yeast into the order Onygenales.

The exact location where the individuals were infected is still unknown, but contact between animals inhabiting the estuaries of Tramandaí and Laguna, which are separated by only 219 km (Simões-Lopes & Fabian 1999), has been reported. These areas are close to large ports and cities and are heavily impacted by human activities, such as direct discharge of untreated wastewater and consequent chemical pollution and biological contamination (Fabricio 1989, SDM 1998, Andrade 2004, Eichler et al. 2012, Moresco et al. 2012). All of these factors may lead to immunosuppression, increasing animals' susceptibility to infections (Wilson et al. 1999, Reif et al. 2006, Van Bressem et al. 2007, 2009, Murdoch et al. 2008).

The individual from the Tramandaí estuary belonged to an inshore population actively involved in local dolphin-human cooperative fishing (Moreno et al. 2008). The social organization in which the other 3 animals lived remains unclear, although some animals in Laguna were also involved in such activities (Simões-Lopes et al. 1998). Despite a recent report of LLD in an offshore *Tursiops truncatus* (Rotstein et al. 2009), cases of LLD (Cowan 1993, Durden et al. 2009) and suggestive LLD (Van Bressem et al. 2007, 2009, Kiszka et al. 2009) are more frequently reported in individuals of inshore populations. This could be related to influx of terrestrial pollutants (Woodward-Clyde Consultants 1994) in ecosystems sustaining enclosed bodies of water with freshwater influx, leading to variations in temperature, tides anaerobic sediment conditions, and salinity levels. Salinity could also be involved in dermal lesions in resident inshore cetaceans (Wilson et al. 1999, Murdoch et al. 2008, Burdett Hart et al. 2011). Another aspect to be considered is the fact that offshore animals are rarely examined in comparison to inshore animals.

Even though some resident dolphins had LLD, there were no cases of human lacaziosis registered in areas of cooperative fishing between dolphins and fishermen studied by Siciliano et al. (2008). Reif et al. (2006) drew a similar conclusion regarding dolphins with LLD from Florida (USA), where LLD is considered endemic and human lacaziosis has never been reported. On the other hand, in the Amazon basin endemic areas of human lacaziosis, river dolphins failed to present any suggestive lesions of infection (Da Silva et al. 2008).

Direct molecular and phylogenetic studies evaluating LLD agents affecting dolphins from different locations have never been performed. The possibility of a remote zoonotic origin or a common ancestor cannot be excluded, and may indicate posterior diverging evolution in order to adapt to different hosts and environments (Paniz-Mondolfi et al. 2012).

The present study describes the histopathology of LLD in *T. truncatus* and the first use of IHC techniques to characterize the agent in 4 cases in the Americas. It is also the first time LLD yeast has been described in a skeletal muscle abscess. Nevertheless, additional genetic research is needed in order to establish the taxonomy of the LLD agent in dolphins.

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8 PARACOCCIDIOIDOMYCOSIS CETI IN *TURSIOPS TRUNCATUS*, BRAZIL

8.1 ABSTRACT

We report paracoccidioidomycosis ceti (former lacaziosis-like disease) in a wild Atlantic bottlenose dolphin (*Tursiops truncatus*) from Brazil, a country with human paracoccidiodomycosis reports. We molecularly identified *Paracoccidioides brasiliensis*, instead of *Lacazia loboi,* as the etiological agent. This is the first identification of the paracoccidiodomycosis etiological agent in South American cetaceans.

8.2 INTRODUCTION

In cetaceans, the etiology of chronic, proliferative, verrucous, granulomatous cutaneous lesions caused by non-cultivable chain-forming yeasts of the order Onygenales (comprising the pathogenic fungi *Lacazia loboi*, *Paracoccidioides brasiliensis* and *P. lutzii*), was only recently solved (ROTSTEIN et al., 2009; ESPERÓN et al., 2012; UEDA et al., 2013; MINAKAWA et al., 2016; VILELA et al., 2016). Some authors initially suggested, based solely on their inability to be cultured, and macroscopic and microscopic similarities, that *L. loboi*, the etiological agent of lacaziosis (previously known as lobomycosis) in humans - a disease characterized by chronic yeast-associated cutaneous and subcutaneous lesions, prevalent mainly in the Amazon basin (TALHARI; TALHARI, 2012), caused similar skin lesions in dolphins (VILELA et al., 2016). Nevertheless, molecular assays identified non-cultivable yeast of the *Paracoccidioides* genus as the etiological agent of this emerging disease (ESPERÓN et al., 2012; UEDA et al., 2013, VILELA et al., 2016), named paracoccidiodomycosis ceti (VILELA et al., 2016). The *Paracoccidioides* genus comprises dimorphic fungi of five phylogenetic species - at least four different cryptic species of *P. brasiliensis* (S1, PS2, PS3 and PS4) and *P. lutzii*. In Brazil,

these agents have been detected in other mammals, mainly in armadillos, but are most notoriously known for causing paracoccidioidomycosis - the most important human systemic mycosis in South America (TEIXEIRA et al., 2014).

Paracoccidioidomycosis ceti has been molecularly diagnosed in originally wild dolphins subsequently kept in captivity - an Atlantic bottlenose dolphin (*T. truncatus*) from Cuba (ESPERÓN et al., 2012), and two *T. truncatus* and one Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) from Japan (UEDA et al., 2013; MINAKAWA et al., 2016), and in seven *T. truncatus* from the eastern coast of the United States: one stranded dead, four released back to the wild, one kept in captivity and one submitted to rehabilitation (ROTSTEIN et al., 2009; VILELA et al., 2016). The agent of similar macroscopic lesions visually identified in South American cetaceans (VAN BRESSEM et al., 2015), was identified through histology and immunohistochemistry as a member of the order Onygenales in four *T. truncatus* specimens from Brazil (SACRISTÁN et al., 2016). However, until now, there were no molecular data available in South American cetaceans, the region in which *L. loboi* and *P. brasiliensis* emerged (TEIXEIRA et al., 2014).

8.3 MATERIAL AND METHODS

In October 2011, an adult male *T. truncatus* (MM509) was found dead in Florianopolis (27.57°S, 48.42°W), southern Brazil, presenting several multifocal, elevated, nodular to verrucous, crusty, whitish skin lesions in the peduncle and the abdominal region (Figure 8) caused by a member of the Onygenales order (SACRISTÁN et al., 2016).

Genomic DNA extraction from frozen skin lesion, liver, spleen and lung samples was performed with the aid of ZR Fungal/Bacterial DNA Miniprep™ kit (Zymo®, Irvine, CA, USA). In order to detect the agent, we employed primers ITS1-Fand ITS-4 (GARDES; BRUNS, 1993) at a melting temperature of 55ºC, to amplify by PCR a 700-bp fragment comprising the *18S* rRNA gene, *internal transcribed spacer*-1 (*ITS-1*), *5.8 S* rRNA gene and *ITS-2*, until the *26S* rRNA gene. Positive samples were identified by direct sequencing (GenBank accession no. MF433034).

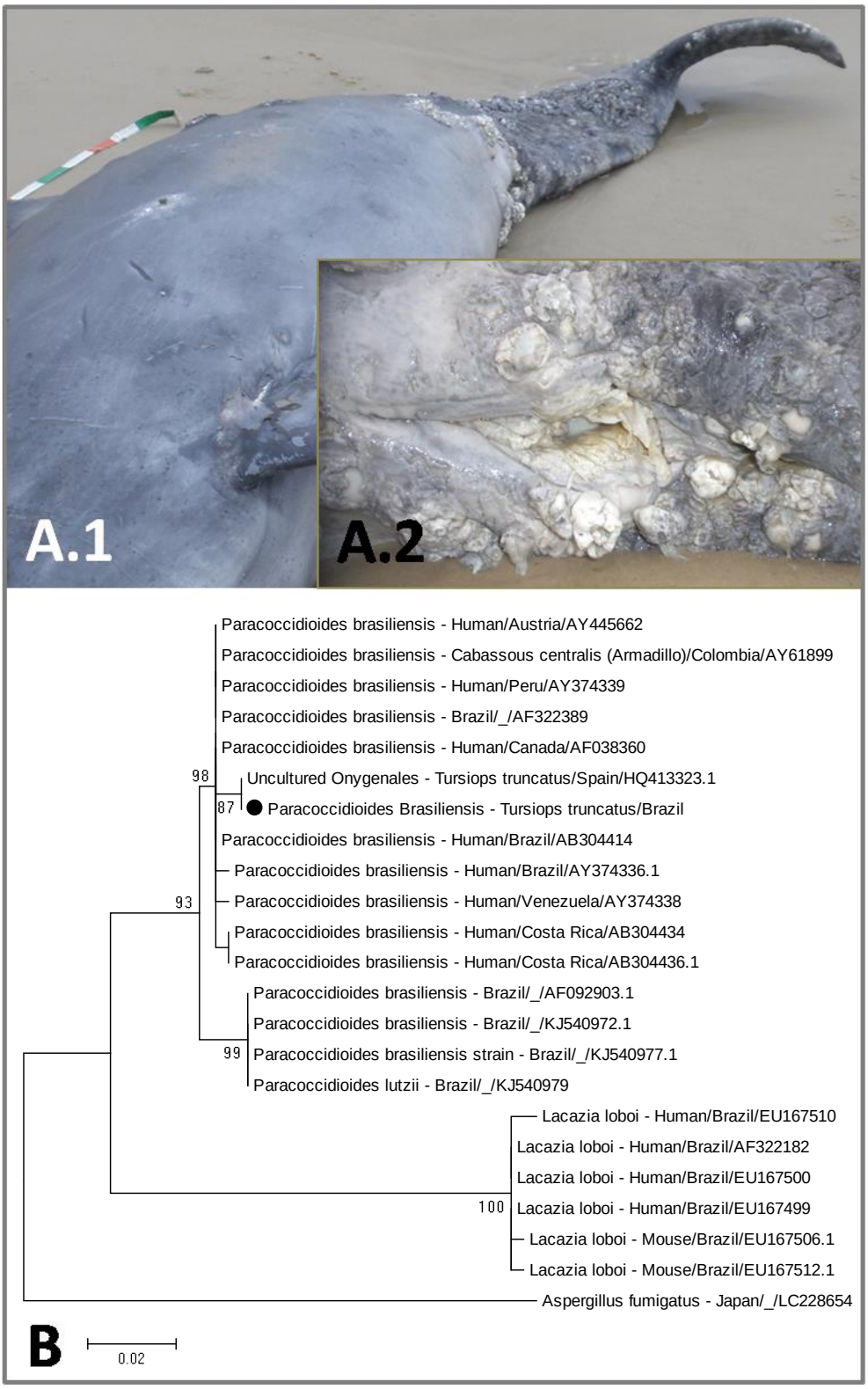
MEGA6 was selected to generate the ClustalW alignment and the 1000 bootstrap maximum likelihood phylogenetic tree (Figure 8). All procedures were performed according with the Ethical Committee of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (Process number 2951280914).

8.4 RESULTS AND DISCUSSION

A 654 bp sequence (excluding primers) was obtained from a skin lesion sample, whereas liver, spleen and lung samples were negative. The subsequent sequence shared a 100% nt identity to the one (HQ413323) obtained from a verrucous cutaneous lesion presenting characteristic intradermic yeast in a *T. truncatus* (ESPERÓN et al., 2012) - to this date the only available *P. brasiliensis* sequence from that genetic region in cetaceans - followed by 98.6% nt identity to *P. brasiliensis* involved in humans cases in Brazil (AB304414, AF322389, AF038360), and 98.2% identity to *P. brasiliensis* identified in a northern naked-tailed armadillo (*Cabassaus centralis*) from Colombia (AY618999), and only 83.1% similarity with *L. loboi* (AF322182). This could be due to different human- and cetacean-infecting *P. brasiliensis* strains, consistent with the current distribution of human *P. brasiliensis* (in the Americas, from Mexico to Argentina) (TEIXEIRA et al., 2014), and dolphin cases (in the Atlantic coast of the United States [ROTSTEIN et al., 2009; VILELA et al., 2016], Cuba [ESPERÓN et al., 2012], Pacific Ocean and Sea of Japan [UEDA et al., 2013; MINAKAWA et al., 2016], and southwestern Atlantic [present report]).

Paracoccidioidomycosis is the most relevant human systemic mycosis in Latin America, reportedly leading to high morbidity/mortality in Brazil (TEIXEIRA et al., 2014). This agent's impact on cetacean health (aside from skin lesions), along with its zoonotic potential, remains unknown. The *P. brasiliensis* we identified, more similar to the one previously identified in a *T. truncatus* from Cuba than those from humans, is the first molecular confirmation of this agent in dolphins, both in Brazil and in South America. Our findings amplify the distribution range of paracoccidioidomycosis ceti, an emerging disease in cetaceans.

Figure 8 –A.1) Macroscopic view of the lesion; A.2) Close-up view of the whitish proliferative, verrucous lesions around the genital slit; B) Maximum likelihood tree of the alignment of the nucleotide sequence found in this study, 21 members of the order Onygenales and *Aspergillus fumigatus.*



Source: R3 Animal (2011); Sacristán (2017)

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9. FINAL COMMENTS

The aim of this study was to determine the infectious etiology of cutaneous and mucocutaneous lesions in cetaceans from Brazil, through the analyses of a decade of sample collections (2005-2015). To the authors' knowledge, this is the first study to molecularly detect and identify selected emerging infectious pathogens – herpesvirus, poxvirus and *Paracoccidioides brasiliensis* – affecting this clade in Brazil, and in the case of herpesvirus and *P. brasiliensis,* also in South America.

The available literature particularly documents the connection between herpesvirus activation and immunosuppression, which could also be caused by the agent itself. Poxvirus is also considered a potentially immunosuppressant agent to other vertebrate species. Therefore, the presence of cutaneous "tattoo" lesions caused by poxvirus, proliferative whitish and verrucous lesions associated with *P. brasiliensis*, and proliferative or whitish lesions potentially related with herpesvirus could be used as health indicators, even though viral immunosuppression is not well established in cetaceans.

Among the novel herpesvirus sequences we described, three of them – both sequences from dwarf sperm whale and especially the one from the Bolivian river dolphin – greatly differ from previously known sequences. The macroscopic and microscopic findings observed in the Guiana dolphin and the Bolivian river dolphin are similar to previously reported herpesvirus skin lesions, although the marked herpesvirus genetic divergence observed in the Bolivian river dolphin requires further research. On the other hand, the herpesvirus detected in dwarf sperm whale's skin lesions could have been an incidental finding, related to systemic infection. Future studies, employing techniques such as electron microscopy, amplification of longer fragments or the complete genome and/or viral culture are necessary to correctly classify these novel herpesviruses, which in the case of the Bolivian river dolphin could possibly lead to a new genus within the *Gammaherpesvirinae.*

We identified characteristic cetacean poxvirus amino acid motifs, providing new evidence to further support its inclusion in a new genus, *Cetaceanpoxvirus.* In order to do so, one requires either cell culture or sequencing of longer fragments - preferably of the complete genome - to establish the type species. Detailed and

comprehensive anatomopathological studies are required to further understand this agent's impact on cetaceans.

The relative stability observed in the odontocete cetacean poxvirus allowed us to design novel real-time and conventional PCR techniques. Unfortunately, it was not possible to design new methods for the other studied agents, due to the high variability between the herpesviruses and limited number of described *P. brasiliensis* sequences. The newly designed techniques are highly sensitive and more efficient in diagnosing these agents in cetaceans when compared to those currently available in the literature.

Upon histopathology and immunohistochemistry, we detected Onygenales yeasts in raised, verrucous and whitish cutaneous lesions of four Atlantic bottlenose dolphins, and in muscular tissue of one specimen; this latter finding indicates this agent's invasive potential. Subsequently, *P. brasiliensis* was identified as the etiological agent of a yeast-associated cutaneous lesion in the latter specimen's, similar to those previously reported in cetaceans from other latitudes. We confirmed the role of *P. brasiliensis* as an etiological agent of this type of lesion, previously attributed, without any molecular or immunohistochemical diagnostic support, to *Lacazia loboi*.

P. brasiliensis is responsible for the most relevant human systemic mycosis in South America, the paracoccidioidomycosis. Comparative studies between human and dolphin paracoccidioidomycosis cases are fundamental to further clarify this agent's cycle and pathology. The zoonotic potential of cetacean-infecting yeasts is still not fully understood.

Finally, we believe that further studies are necessary to provide new sensitive tools to diagnose these agents, understand their cycle and associated pathological processes, their zoonotic potential, and clarify the natural history of these agents and their hosts, the potential impact of cutaneous and mucocutaneous lesions in cetaceans - specially in endangered species and populations - and their role as health indicators of marine and riverine environments.

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ANNEX A

Authorization of the co-authors of the article “Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach” for its inclusion in this Thesis.

São Paulo, 30 de Junho de 2017.

Eu Rodrigo Albergaria Ressio, CPF:26753933808, co-autor do artigo “Lacaziosis-like disease in Tursiops truncatus from Brazil: a histopathological and immunohistochemical approach”, publicado em 2016 pela revista: “Diseases of Aquatic Organisms”, declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada “Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast” da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

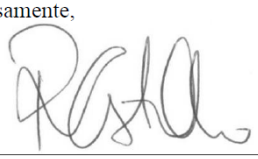
Atenciosamente,

_____

São Paulo, 28 de Junho de 2017.

Eu (PEDRO VOLKMER DE CASTILHO), CPF: 023.276.189-24, co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,



(Assinatura)

São Paulo, 28 de Junho de 2017.

Eu Natália Coelho Couto de Azevedo Fernandes, CPF:.35181590896., co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Natália Coelho Couto de Azevedo Fernandes
(Assinatura)

São Paulo, 28 de Junho de 2017.

Eu Samira Costa da Silva, CPF:100140327-40, co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

(Assinatura)

São Paulo, 28 de Junho de 2017.

Eu Fernando Esperón Fajardo Passaporte: BE169737, co-autor do artigo "Lacaziosis-like disease in *Tursiopstruncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista:"Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada"Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Fernando Esperón Fajardo

São Paulo, 28 de Junho de 2017.

Eu Fábio Gonçalves Daura Jorge, CPF:02625479965, co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Fábio G. Daura Jorge

São Paulo, 28 de Junho de 2017.

DECLARAÇÃO

Eu Kátia Regina Groch, CPF 74975141972, coautora do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

(Assinatura)

São Paulo, 28 de Junho de 2017.

Eu Cristiane Kiyomi Miyaji Kolesnikovas, CPF 176.142.858-67, co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

(Assinatura)

São Paulo, 28 de Junho de 2017.

Eu Juliana Marigo, CPF: 255.015.058-94, co-autora do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Juliana Marigo

(Assinatura)

Torres, 30 de Junho de 2017.

Eu, **PAULO HENRIQUE OTT**, CPF: 577.839.500-00, co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

(Assinatura)

São Paulo, 28 de Junho de 2017.

Eu LARISSA ROSA DE OLIVEIRA, CPF: 884365860-34., co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

_____

(Assinatura)

São Paulo, 28 de Junho de 2017.

Eu Angélica María Sánchez Sarmiento, CPF: 23404036859, co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Angélica M.S. Sarmiento

(Assinatura)

São Paulo, 28 de Junho de 2017.

Eu Paulo César de Azevedo Simões Lopes, CPF 500281620-72, co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

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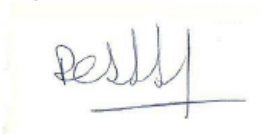

Paulo César de Azevedo Simões Lopes

São Paulo, 28 de Junho de 2017.

Eu José Luiz Catão Dias, CPF 02959788800, co-autor do artigo "Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach", publicado em 2016 pela revista: "Diseases of Aquatic Organisms", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Research and characterization of selected pathogens of cutaneous and mucocutaneos lesions in cetaceans from the Brazilian coast" da autoria de Carlos Sacristán Yagüe, aluno de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

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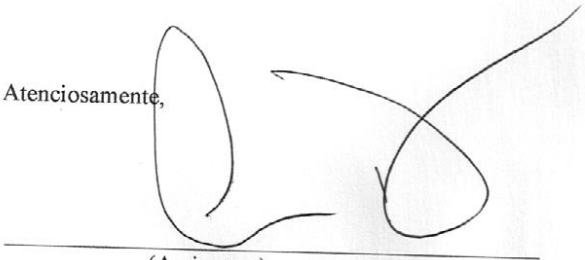

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