ANGELICA MARIA SANCHEZ SARMIENTO

***Brucella* spp. in aquatic mammals of Brazil:** serological, molecular,
histopathological and immunohistochemical investigation

SAO PAULO

2018

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***Brucella* spp. in aquatic mammals of Brazil: serological, molecular,
histopathological and immunohistochemical investigation**

Thesis presented to the Graduate Program in
Experimental and Comparative Pathology of
the School of Veterinary Medicine and Animal
Sciences of the University of São Paulo to
obtain a PhD in Sciences.

Department:

Pathology

Concentration area:

Experimental and Comparative Pathology

Advisor:

Prof. Dr. José Luiz Catão Dias

SAO PAULO

2018

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T. 3617
FMVZ

Sánchez Sarmiento, Angélica María

Brucella spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation / Angélica María Sánchez Sarmiento. – 2018. 171 p. : il.

Título traduzido: *Brucella* spp. em mamíferos aquáticos no Brasil: investigação sorológica, molecular, histopatológica e imunohistoquímica

Tese (Doutorado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Patologia, São Paulo, 2018.

Programa de Pós-Graduação: Patologia Experimental e Comparada.

Área de concentração: Patologia Experimental e Comparada.

Orientador: Prof. Dr. José Luiz Catão Dias.

1. Brucellosis. 2. Cetaceans. 3. Pathology. 4. Pinnipeds 5. South America. I. Título.

São Paulo, 05 de março de 2015

CERTIFICADO

Certificamos que o Projeto intitulado "Brucella spp. em mamíferos marinhos: Investigação imunohistoquímica, histopatológica, sorológica e molecular", protocolado sob o CEUA nº 3926161213, utilizando 416 Espécies silvestres brasileiras (416 machos), sob a responsabilidade de José Luiz Catão-dias, foi aprovado na reunião de 16/01/2014, e está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo.

We certify that the Research "título em inglês", protocol number CEUA 3926161213, utilizing 416 Brazilian wild species (416 males), under the responsibility José Luiz Catão-dias, was approved in the meeting of day 01/16/2014, and agree with Ethical Principles in Animal Research adopted by Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo.

Atenciosamente,

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

São Paulo, 04 de janeiro de 2018
CEUA N 3926161213

Ilmo(a). Sr(a).

Responsável: José Luiz Catão Dias

Área: 0

Prof. Dr. José Luiz Catão-dias (orientador)

Título da proposta: "Brucella spp. em mamíferos aquáticos no Brasil: Investigação sorológica, molecular, histopatológica e imunohistoquímica."

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** a Alteração do cadastro (versão de 19/dezembro/2017) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Ola, gostaria de solicitar por gentileza, alteração do título da pesquisa na CEUA No. 3926161213, com caráter de urgência; uma vez que o depósito da tese será feito nos próximos dias e para isto precisaremos que alicença da CEUA, tenha o mesmo título. ".

Comentário da CEUA: "Alteração de título aprovada."

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais

Faculdade de Medicina Veterinária e Zootecnia da Universidade
de São Paulo

Roseli da Costa Gomes
Secretária Executiva

Faculdade de Medicina Veterinária e Zootecnia da Universidade
de São Paulo

São Paulo, 04 de janeiro de 2018
CEUA N 3926161213

Ilmo(a). Sr(a).
Responsável: José Luiz Catão Dias
Área: 0
Prof. Dr. José Luiz Catão-dias (orientador)

Título da proposta: "Brucella spp. em mamíferos aquáticos no Brasil: Investigação sorológica, molecular, histopatológica e imunohistoquímica".

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** a Emenda (versão de 19/dezembro/2017) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Solicitamos por gentileza, alteração do título da pesquisa com caráter de urgência; uma vez que o depósito da tese será feito nos próximos dias e para isto precisaremos que a licença da CEUA, tenha o mesmo título. O título correto seria: Brucella spp. em mamíferos aquáticos no Brasil: Investigação sorológica, molecular, histopatológica e imunohistoquímica. Em inglês: Brucella spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation. Gostaríamos ainda, de atualizar o número de animais que foram utilizados no estudo; menor que o inicialmente proposto (n=330) para um total de 221 mamíferos aquáticos; sendo eles 161 cetáceos, 39 pinípedes e 21 peixes-bois, de ambos sexos. "

Comentário da CEUA: "Alteração do "n" aprovada."

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade
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Número: 48279-3	Data da Emissão: 21/06/2017 14:03	Data para Revalidação*: 21/07/2018
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Dados do titular

Nome: KATIA REGINA GROCH	CPF: 739.751.419-72
Título do Projeto: Pesquisa e Caracterização da Morbilitvirose 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	Coleta e transporte de tecidos de mamíferos marinhos do litoral brasileiro	04/2015	12/2019

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Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	José Luiz Catão Dias	Supervisor	029.597.888-00	8914397-B SSP-SP-SP	Brasileira
2	Angélica María Sánchez Sarmiento	Pesquisadora colaboradora	294.040.368-59	V863133-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

1	Amostras biológicas	Sangue, Animal encontrado morto ou partes (carcassa/ossos/pele, Fragmento de tecido/órgão)
2	Método de captura/coleta	Outros métodos de captura/coleta, Captura manual

Atividades X Táxons

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

Material e métodos

1	Amostras biológicas (Carnívoros)	Animal encontrado morto ou partes (carcassa/ossos/pele, Fragmento de tecido/órgão, Sangue
2	Método de captura/coleta (Carnívoros)	Outros métodos de captura/coleta/necropsias e amostras em acervo)

Destino do material biológico coletado

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

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EVALUATION FORM

Author: SÁNCHEZ SARMIENTO, Angélica María

Title: *Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation.

Thesis submitted to the Postgraduate Program in Experimental and Comparative Pathology of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain a PhD degree in Sciences

Date: ____/____/____

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Prof. Dr. _____

Institution: _____ Trial: _____

Dedico este trabajo a mí mamá Luz Amparo,

A mí abuelita Lilia,

Y a mis amores Jonas y Violeta,

Con quienes fui bendecida por Dios

ACKNOWLEDGMENTS

A Deus meu infinito amor e agradecimento pela vida, pelas oportunidades, pelas conquistas que tenho alcançado e por ter colocado no meu caminho todas as pessoas que tem contribuído na realização deste grande objetivo.

Agradeço a minha família, em especial a minha mãe Luz Amparo pelo amor e por ter se sacrificado tanto pelos seus filhos, por ser um exemplo de dedicação, honestidade e determinação no trabalho. Com ela aprendi a força da fé e do pensamento positivo. Aos meus irmãos Santiago e Paula Andrea e a minha sobrinha Angelica Sofia, pela saudade que me deu forças e por saber que podia contar com eles para toda vida mesmo na distância. A minha avó Lilia pelas suas orações e porque ainda em seus 90 anos continua sendo esse exemplo de luta, generosidade, bondade e amor. Ao meu Pai, e os meus irmãos Juanfe e Luigi que sempre estarão nos meus pensamentos.

Ao meu amor, Jonas, obrigada por ser tão companheiro, me aconselhar, apoiar e incentivar a correr atrás dos meus objetivos. Eu admiro muito sua inteligência, curiosidade e dedicação no trabalho! Você é o amor da minha vida e tenho grande orgulho de te-lo ao meu lado. A Violeta nossa “fofahermosa” pela fidelidade, por estar sempre do meu lado tao radiante, jovial e carinhosa. Agradeço também a Maridalva e Nivaldo (meus *suegritos*), a Yana (*cuñadita*), o Alexandre, a Neném, o tio Luiz, a tia Ivete, a Mariana Sayuri, o Luizinho e a Stelinha pelo carinho e pela fortuna que tenho de serem minha família Brasileira. Super obrigada Luizinho pela ajuda com a revisão do inglês!

Agradeço muitíssimo aos Professores José Luiz Catão Dias e Eliana Reiko Matushima, meus orientadores no Brasil por terem me aceitado como parte do LAPCOM e com isto aberto as portas ao mundo da pesquisa em animais selvagens. Meu sonho sempre foi trabalhar com patologia em animais selvagens, em particular os animais marinhos e vocês me permitiram alcança-lo, dando todo o apoio e amizade. *Muchas gracias* Catão pela paciência, confiança, conselhos e por todo o aprendizado durante meu doutorado!

Aos Professores Lara Keid e Rodrigo Soares pela ajuda para cumprir tarefas críticas do projeto, perseverando e me dando apoio até o ultimo momento. Também a Jaque Diniz e ao Alex pela gentileza e colaboração em Pirassununga;

Ao pessoal do IUSA, Espanha, em especial a Eva Sierra, Daniele Zucca, Oscar Quesada, Manuel Arbelo e Antonio Fernandes, pelos ensinamentos e ajuda durante o estágio sanduíche e pela amizade;

Ao pessoal do Instituto Adolfo Lutz, Natalia Fernandes, Juliana Guerra, Rodrigo Ressio e Cintia Cirqueira pela parceria para o desenvolvimento das várias ideias e pela amizade;

Agradeço a Natalia Fernandes, Juliana Guerra, Pedro Navas e Josué Díaz Delgado pela assessoria e ajuda com as leituras histopatológicas e de imunohistoquímica de mamíferos marinhos.

Ao Professor Jose Soares, Narleia da Silva, Nicolle Queiroz, Gisele Oliveira e Antonio de Sousa (VPS), pelo apoio na realização dos testes sorológicos.

Aos demais professores, funcionários e colegas da FMVZ e do LAPCOM; Pedro Navas, Sandy Pulecio, Pablo Cruz, Silmara Rossi, Samira Costa, Nicolas Céspedes, David Salas, Carlos Sacristán, Carol Ewbank, Eduardo Machado, Natalia Silvestre, Jorge Oyakawa, Sândara Sguário, Juliana Marigo, Kátia Groch, Josué Díaz Delgado, Laura Reisfeld, Arícia Duarte Benvenuto, Camila Molina, Cíntia Favero, Danny Fuentes, Fabíola Prioste, Gilbert Alvarado, Jilma Aleman, Gislaine Dalazen, Marcelo Carvalho, Marco Gattamorta, Ralph Vanstreels, Roberta Ramblas, Valéria Ruoppolo, Mayara Caiaffa, Marina Pellegrino e todos os alunos e estagiários que passaram pelo LAPCOM. Obrigada a aqueles que me brindaram assessoria científica e/ou ajuda nas respectivas etapas do meu projeto e também a aqueles que fizeram os meus dias na USP mais felizes pelo companheirismo, pelas conversas, conselhos, risadas, bandejões e cafezinhos!

Um agradecimento especial a Sandy e Pedro pela nossa amizade ou melhor que isso “irmandade”. Tenho certeza que com sua dedicação, integridade e nobreza de espírito o seu futuro será muito brilhante! Queridos amigos, saibam que têm um lugar muito especial no meu coração!

A todos os colaboradores do BTMM, Associação R3 Animal, Aquário de Santos, Associação de Pesquisa e Preservação de Ecossistemas Aquáticos, Centro de Mamíferos Aquáticos do Instituto Chico Mendes de Conservação da Biodiversidade, Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul, Instituto Baleia Jubarte, Departamento de Engenharia de Pesca da Universidade do Estado de Santa Catarina; Laboratório de Mamíferos Aquáticos - Departamento de Ecologia e Zoologia, Universidade Federal de Santa Catarina, Laboratório de

Mamíferos Aquáticos – Instituto Nacional de Pesquisas da Amazônia, Laboratório de Mamíferos Aquáticos e Bioindicadores “Prof^a Izabel M. G. do N. Gurgel”, Projeto Baleia Franca, Projeto Biopesca, Projeto Toninhas - Universidade da Região de Joinville, Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul, Universidade Estadual do Rio Grande do Sul, e Universidade do Vale do Rio dos Sinos – UNISINOS. Sua parceria foi fundamental para realização deste trabalho. Agradecimentos especiais ao Vitor Luz e Ana Meirelles, pela confiança e pelo excelente trabalho feito na AQUASIS;

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela bolsa de doutorado e ao Banco Santander pelo patrocínio recebido para realização do estágio sanduíche no exterior;

A Society for Marine Mammalogy-SMM, pelo apoio financeiro recebido por meio da Small Grant in Aid of Research (2015 e 2016);

A Cetacean Society International-CSI, pelo apoio financeiro recebido para participação na Bienal dos mamíferos marinhos (2015 e 2017);

Após 8 no Brasil, se conclui uma etapa muito importante de minha vida. Eu devo muito a este país: a realização dos meus objetivos profissionais, amor, família e amigos que levarei por toda a vida. Muito obrigada! *Muchas gracias!*

ABSTRACT

SANCHEZ SARMIENTO, A. ***Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation. [*Brucella* spp. em mamíferos aquáticos no Brasil: investigação sorológica, molecular, histopatológica e imunohistoquímica]**. 2018. 171 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

Aquatic mammals are sentinels of zoonotic pathogens that may be a threat to species conservation, to humans and domestic animals. *Brucella* genus is responsible for brucellosis, one of the main widespread zoonosis. In aquatic mammals, *Brucella* spp. was firstly described in 1994 and currently there are two recognized species infecting that group: *B. ceti* and *B. pinnipedialis*. In cetaceans, *Brucella* spp. has been linked to asymptomatic infection, acute or chronic disease and several pathological processes which may lead to stranding and/or death. In pinnipeds, pathogenicity of *Brucella* spp. is still unclear. Data about *Brucella* spp. in aquatic mammals in South Atlantic Ocean particularly in Brazil is limited, with a significant knowledge gap despite the rich diversity of this group. In this research, we investigated exposure to and/or infection by *Brucella* spp. by serological, molecular, histopathological, immunohistochemical and microbiological methods in samples of aquatic mammals of Brazil kept at the Marine Mammal Tissue Bank, Department of Pathology, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Brazil. Sera from 63 cetaceans [families Iniidae (n=37), Delphinidae and Kogiidae (n=26)], 35 pinnipeds (Southern elephant Seals, *Mirounga leonina*), and 21 West Indian manatees (*Trichechus manatus*) were tested via the Rose Bengal Test, a commercial competitive enzyme-linked immunosorbent assay (c-ELISA) and the serum agglutination test (SAT; selected c-ELISA positives) to detect *Brucella* spp. antibodies. In addition, DNA from selected samples of 124 cetaceans, 4 pinnipeds and 1 manatee were analyzed by conventional PCR and/or real-time PCR (qPCR) targeting the genus *Brucella* spp. Culture, histopathology and immunohistochemistry (IHC) were performed in samples from exposed or infected individuals (serology and/or conventional PCR/qPCR positives; n=13). Antibodies against *Brucella* spp. were detected in 3/35 (8.6%) of the *M. leonina* tested while Iniidae and Kogiidae cetaceans and manatees were negative. *Brucella* spp. DNA was detected in 4/124 (3.2%) of the cetaceans tested by conventional PCR/qPCR: one Clymene dolphin

(*Stenella clymene*) with acute brucellosis, positive by serology and IHC; and three other individuals without available sera: one asymptomatic Franciscana (*Pontoporia blainvillei*), positive by qPCR and IHC; one case of chronic brucellosis in a Spinner dolphin (*Stenella longirostris*) presenting fibrinosuppurative atlanto-occipital osteoarthritis with abscess formation, positive by qPCR and by IHC; one Guiana dolphin (*Sotalia guianensis*), positive by conventional PCR/qPCR and by IHC and previously diagnosed with cetacean morbillivirus. Despite negative PCR amplification, *Brucella*-type lesions were found in one *S. clymene* and one pigmy killer whale (*Feresa attenuata*), both positive by serology and by IHC. Positive serology and/or IHC suggested exposure and/or *Brucella*-infection in three short-finned pilot whale (*Globicephala macrorhynchus*), two melon-headed whale (*Peponocephala electra*), a bottlenose dolphin (*Tursiops truncatus*) and a *S. clymene*. Main part of the *Brucella* spp. exposed or infected cetaceans came from State of Ceará, Northeastern Brazil. Culture was not successful, hampering strains classification. All the pinnipeds and the manatees tested by PCR were negative. Opportunistic screening of *Leptospira* spp. exposure via the microscopic agglutination test on all sera samples used for the *Brucella* spp. screening gave negative results. In conclusion, we found *Brucella* spp. exposure, asymptomatic infection, as well as cases of acute and chronic brucellosis and coinfection by *Brucella* spp. and other pathogens. To our knowledge, this research is the first long-term survey of *Brucella* spp. in aquatic mammals in South America, widening the hosts and the geographic range of this agent and contributing to the understanding of pathological and epidemiological aspects of its infection. Further research is needed to fully characterize the strains involved, evaluate their public health relevance and the possible occurrence and impacts in other species of aquatic mammals in Brazil.

Keywords: brucellosis, cetaceans, pathology, pinnipeds, South America.

RESUMO

SANCHEZ SARMIENTO, A. ***Brucella* spp. em mamíferos aquáticos no Brasil: investigação sorológica, molecular, histopatológica e imunohistoquímica. [*Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation]**. 2018. 171 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

Os mamíferos aquáticos são sentinelas de patógenos zoonóticos, os quais podem representar uma ameaça a sua conservação, aos humanos e aos animais domésticos. O gênero *Brucella* é responsável pela brucelose, uma das zoonoses mais comuns. Nos mamíferos aquáticos *Brucella* spp. foi descrita pela primeira vez em 1994 e atualmente duas espécies são reconhecidas nesse grupo: *B. ceti* e *B. pinnipedialis*. Nos cetáceos, *Brucella* spp. tem sido associada a infecção assintomática, doença aguda ou crônica e diversos processos patológicos que podem levar ao encalhe e/ou morte. Nos pinípedes, a patogenicidade de *Brucella* spp. ainda não é clara. Dados sobre *Brucella* spp. em mamíferos aquáticos no Oceano Atlântico Sul, particularmente no Brasil, são limitados com uma significativa lacuna de conhecimento mesmo com a rica diversidade. Nesta pesquisa, investigou-se exposição a e/ou infecção por *Brucella* spp. com métodos sorológicos, moleculares, histopatológicos, imunohistoquímicos e microbiológicos em amostras de mamíferos aquáticos do Brasil armazenadas no banco de tecidos de mamíferos marinhos, Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo. Soro de 63 cetáceos [famílias Iniidae (n=37), Delphinidae e Kogiidae (n=26)], 35 pinípedes (Elefante-marinho-do-sul, *Mirounga leonina*) e 21 peixe-bois marinhos (*Trichechus manatus*) foi testado pelo Rosa de Bengala, um kit comercial de ensaio imunossorvente ligado a enzima de competição (c-ELISA) e a prova de soroaglutinação lenta em tubo (SAT; c-ELISA positivos selecionados) para detecção de anticorpos anti-*Brucella* spp. Em adição, DNA de amostras selecionadas de 124 cetáceos, 4 pinípedes e 1 peixe-boi marinho foram analisadas pelo PCR convencional e/ou em tempo real (qPCR) tendo como alvo o gênero *Brucella* spp. Realizou-se cultivo, histopatologia e imunohistoquímica (IHQ) nas amostras dos indivíduos expostos ou infectados (positivos na sorologia e/ou PCR convencional/qPCR; n=13). Anticorpos anti-*Brucella* spp. foram detectados em 3/35 (8.6%) dos *M. leonina* testados enquanto cetáceos das famílias Iniidae e

Kogiidae e os peixe-bois foram negativos. DNA de *Brucella* spp. foi detectado em 4/124 (3.2%) dos cetáceos analisados por PCR convencional/qPCR: um golfinho-climene (*Stenella clymene*) com brucelose aguda, positivo pela sorologia e IHQ; e três indivíduos adicionais sem soro disponível: uma Franciscana (*Pontoporia blainvillei*), assintomática, positiva pela qPCR e IHQ; um caso de brucelose crônica em um golfinho-rotador (*Stenella longirostris*), apresentando osteoartrite fibrinosupurativa atlantoccipital com formação de abscesso, positivo pela qPCR e IHQ; um boto-cinza (*Sotalia guianensis*), positivo pela PCR convencional/qPCR e IHQ e previamente diagnosticado com morbillivirus dos cetáceos. Apesar da PCR negativa, lesões compatíveis com brucelose foram encontradas em um *S. clymene* e uma orca-pigmeia (*Feresa attenuata*), ambos positivos pela sorologia e IHQ. Exposição e/ou infecção por *Brucella* spp. foi confirmada pela sorologia e/ou IHQ positiva em três baleia-piloto-de-aleta-curta (*Globicephala macrorhynchus*), dois golfinhos cabeça de melão (*Peponocephala electra*), um golfinho nariz de garrafa (*Tursiops truncatus*) e um *S. clymene*. A maior parte dos cetáceos expostos a/ou infectados por *Brucella* spp. foram provenientes do estado de Ceará, nordeste do Brasil. A cultura não foi bem-sucedida, prejudicando a classificação das cepas envolvidas. Todos os pinípedes e peixe-bois testados foram negativos na PCR. Triagem oportunístico para avaliar exposição a *Leptospira* spp. por meio do teste de aglutinação microscópica nos soros testados para *Brucella* spp. foi negativo. Em conclusão, confirmou-se exposição a *Brucella* spp., infecção assintomática, brucelose aguda e crônica e coinfecção por *Brucella* spp. e outros agentes patogênicos. Dentro de nosso conhecimento, esta pesquisa constitui o primeiro trabalho de longo prazo sobre *Brucella* spp. em mamíferos aquáticos na América do Sul, ampliando a sua faixa de hospedeiros e localização geográfica e contribuindo na compreensão dos aspectos patológicos e epidemiológicos de sua infecção. Pesquisas adicionais são necessárias para caracterizar as cepas envolvidas, avaliar a sua relevância na saúde pública, bem como sua possível ocorrência e impactos em outras espécies de mamíferos aquáticos no Brasil.

Palavras chave: brucelose, cetáceos, patologia, pinípedes, América do Sul.

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1 GENERAL INTRODUCTION

Aquatic mammals are considered sentinels of riverine, marine and coastal waters; they have an important role as indicators of the environmental health for sharing susceptibility to certain toxins, chemicals and pathogens with men (MARTINEAU et al., 2002; WELLS et al., 2004; MOORE, 2008; WISE et al., 2009; BOSSART, 2011; GIBSON et al., 2011). Consequences of long-term climate change effects and environmental degradation are modulating the occurrence of diseases in these animals (BOSSART, 2011). There are some emerging and resurging diseases, result of environmental distress syndrome (BOSSART, 2007), that are threatening the survival of aquatic mammals. Many emerging pathogens have demonstrated a potential to reduce the abundance of threatened and endangered wild animal populations and monitoring and surveillance of these populations may contribute to the knowledge of the epidemiology of the diseases and may prevent their spread (DOBSON; FOUFOPOULOS, 2001; MÖRNER et al., 2002). As long as anthropogenic disturbances continue to drastically alter the natural environment, and rapid human and animal translocations occur, increasing contact between wild and domestic animals, further outbreaks of novel and emergent pathogens in wildlife will follow (DOBSON; FOUFOPOULOS, 2001; MÖRNER et al., 2002). There are at least 30 emerging and re-emerging pathogens infecting cetaceans and pinnipeds (MILLER, D., EWING, R.Y., BOSSART, 2001), responsible for viral, bacterial, fungal and parasitic diseases. Some of them have resulted in large mortality outbreaks or epizooties (VEDROS et al., 1971; LIPSCOMB, 1994; DUIGNAN et al., 1996; GULLAND et al., 1996; MILLER et al., 2004; COLEGROVE et al., 2016).

Along the Brazilian coast the diversity and occurrence of aquatic mammals are high, with at least 46 cetaceans, seven pinnipeds and two species of manatees, respectively (SILVA BARRETO, 2010; ROCHA-CAMPOS et al., 2011; CYPRIANO-SOUZA et al., 2017). Due to their biological characteristics, those species are more vulnerable compared to other taxonomic groups adding the fact that they face serious threats due to anthropic activities (BARRETO et al., 2011; ROCHA-CAMPOS et al., 2011). To monitor the causes of mortality with special emphasis on the infectious diseases, is included in the list of priority actions of the National Action Plans for the conservation of Aquatic mammals in Brazil (BARRETO et al., 2011), with several of the species mentioned in the National List of Brazilian Fauna

threatened of extinction (IN MMA nº 3, 26/05/2003). In the last years, evidence of the occurrence of infectious diseases already reported in aquatic mammals in other areas of the world was described in aquatic mammals in Brazil, reinforcing the relevance of monitoring the health of this group of animals and highlighting areas of knowledge gaps.

Firstly, in 1993, and then in 2011, verrucous proliferative lesions similar to those caused by *Paracoccidioidomycosis ceti* were visually identified in cetaceans in Southern Brazil (SIMOES-LOPES et al., 1993; DAURA-JORGE; SIMOES-LOPES, 2011; VAN BRESSEM et al., 2015). Clues about the etiology of the disease were obtained through a histological and immunohistochemical study, which identified yeasts of the order Onygenales as the etiological agent of such lesions in Atlantic bottlenose dolphins (*Tursiops truncatus*) (SACRISTÁN et al., 2016). Recently the causative agent for those lesions in Brazil was confirmed through molecular methods as *P. ceti* (SACRISTÁN et al., 2017b).

Toxoplasmosis was described in 2013 in a Guiana dolphin (*Sotalia guianensis*) in Paranaguá, Brazil (GONZALES-VIERA et al., 2013) and a following comprehensive immunohistochemical and histopathological study revealed two more species susceptible to toxoplasmosis, one *T. truncatus* stranded in Rio de Janeiro State and one Killer whale (*Orcinus orca*) from captivity in Sao Paulo State (COSTA-SILVA, 2016). The presence of *Cryptosporidium* spp. and *Giardia* sp. have been detected in various species of aquatic mammals in the northern and northeastern region of Brazil (BORGES et al., 2017).

Some years later the first case of morbillivirus, one of the most important viral immunosuppressive agents in cetaceans and pinnipeds in the world, was confirmed in one *S. guianensis* stranded in Espirito Santo State (GROCH et al., 2014). Herpesvirus (Gammaherpesvirinae subfamily) was detected in one individual of the same species from the northern Brazilian coast (SEADE et al., 2017). In addition, an extensive histopathological and molecular research revealed herpesviruses and poxvirus in 3/70 (4.3%) and 2/70 (2.9%) of the cetaceans analyzed, respectively. On that study, Alphaherpesvirus were identified in a *Stenella* sp. and a dwarf sperm whale (*Kogia sima*), while Gammaherpesvirus was identified in a Bolivian river dolphin (*Inia boliviensis*). Poxviruses were identified in “tattoo” skin lesions of a *T. truncatus* and a *S. guianensis* (SACRISTÁN et al., 2017a).

Some advances regarding the detection of bacterial pathogens have been accomplished; however, the information is still limited. Anti-*Leptospira* spp. antibodies were found in captive manatees (*Trichechus inunguis*) from the Brazilian Amazon at elevated rates; 23/74 (31.1%) (MATHEWS et al., 2012) and in captive West indian manatees (*Trichechus manatus*) from northeast Brazil; 5/54 (9.2%) (ATTADEMO, 2014). Tuberculosis caused by *Mycobacterium pinnipedii* was detected in a stranded South American Sea Lion (*Otaria byronia*) in Rio Grande do Sul State (DE AMORIM et al., 2014) and *M. fortuitum* and *M. abscessus* were identified in an Amazonian manatee under human care (REISFELD et al., 2017). Some previous research attempted to investigate exposure to/and or infection by *Brucella* spp. in manatees and riverine dolphins in Brazil. A serological study of 67 *I. geoffrensis* from Tefé, Amazon State did not detect anti-*Brucella* antibodies (ROCCA, 2014). In captive *T. manatus* of the northeastern Brazil, 6/58 (10.4%) were found positive on initial screening (Rose Bengal Test), but negative at the confirmatory test (Complement Fixation Test) (ATTADEMO, 2014).

The genus *Brucella* spp. is responsible for the brucellosis, considered one of the most important and widespread zoonotic diseases in the world, according FAO, WHO and the OIE. This genus is comprised by 12 species, two of them infecting aquatic mammals: *B. ceti* (cetaceans) and *B. pinnipedialis* (pinnipeds). *Brucella* spp. in marine mammals was first reported approximately two decades ago, when it was isolated from an aborted fetus bottlenose dolphin (EWALT et al., 1994) and in the same year from carcasses of four common seals (*Phoca vitulina*), two harbor porpoises (*Phocoena phocoena*) and one common dolphin (*Delphinus delphis*) (ROSS et al., 1994). The potential hosts of *Brucella* spp. in aquatic environments include 130 aquatic mammals, and *Brucella* spp. have detected or exposure have been proved in several species or subspecies of cetaceans, pinnipeds, marine otter, freshwater otter and the polar bear (FOSTER et al., 2002; NYMO; TRYLAND; GODFROID, 2011; GUZMÁN-VERRI et al., 2012; HERNÁNDEZ-MORA; PALACIOS-ALFARO; GONZÁLEZ-BARRIENTOS, 2013).

In cetaceans, *Brucella* spp. has been linked to asymptomatic infection, acute or chronic disease as well as several pathological processes including non-suppurative meningoencephalitis, placentitis and orchitis which may lead to infertility, stranding and/or death (FOSTER et al., 2002; HERNÁNDEZ-MORA et al., 2008; GONZÁLEZ-BARRIENTOS et al., 2010; NYMO; TRYLAND; GODFROID, 2011;

GUZMÁN-VERRI et al., 2012). In pinnipeds, the pathogenicity of *Brucella* spp. is still unclear; seropositivity and isolation are reported without signs of disease or associated lesions, suggesting host adaptation or low pathogenicity (NYMO; TRYLAND; GODFROID, 2011; NYMO et al., 2013). Impacts on individual and population levels of *Brucella* spp. in marine mammals, especially their effects on fertility and population dynamics, are poorly understood (TRYLAND et al., 2001; DAGLEISH et al., 2008; NYMO; TRYLAND; GODFROID, 2011). Aquatic *Brucella* strains can cause infection and abortion in domestic animals as a result of experimental infection (RHYAN et al., 2001). Furthermore, natural cases of human brucellosis associated with aquatic mammals *Brucella* species have been reported in people without previous contact with marine mammals (SOHN et al., 2003; MCDONALD et al., 2006), as well as a case of laboratory acquired infection (BREW et al., 1999). On the other hand, the presence of *Brucella* spp. of terrestrial origin in California sea lions (*Zalophus californianus*) have been reported (AVALOS-TÉLLEZ et al., 2014). While the literature indicated *Brucella* spp. is present in several aquatic mammal species at different geographic locations around the world, the information in South America is still limited.

Brucellosis in aquatic mammals is considered of great interest by the specialists in the area (VENN-WATSON, S., STAMPER, 2010) and increasing the knowledge about *Brucella* spp. in the aquatic environment and the possible implications in the human health is an important topic within the "One Health" perspective (LARSEN et al., 2013a). Diseases with epizootic potential, zoonotic implications and complex pathogenesis involving other cofactors such as pollution, genetics and immunological dysfunction (BOSSART, 2011), should be studied through a multidisciplinary approach, making use of tools applied to conservation medicine (AGUIRRE et al., 2002).

In Brazil, brucellosis caused by the species *B. abortus*, *B. suis*, *B. canis* and *B. ovis* is of mandatory report (BRASIL, 2013). *B. abortus* is the most prevalent species resulting in considerable economic losses (POESTER; GONÇALVES; LAGE, 2002). Since 2001, the National Program for the control and Eradication of Brucellosis and Tuberculosis (PNCEBT) is being implemented in the country and based on compulsory vaccination, control of animal movement and certification of brucellosis-free herds (BRASIL, 2006).

Therefore, taking into account the importance of the disease to the human health and to the aquatic environment and the potential threat it may represent to aquatic mammal species conservation, in front of the diversity of those species inhabiting the Brazilian territory, we aimed to contribute to the knowledge of *Brucella* spp., its occurrence, pathological and epidemiological aspects in aquatic mammals in the country. Opportunistically, we also investigated and obtained negative results for the presence of antibodies anti-*Leptospira* spp. another relevant zoonosis on sera samples used for the *Brucella* screening.

1.1 PRESENTATION OF THE CHAPTERS

This thesis is organized in 7 chapters, including this introductory one. Chapter 3 is a previously published manuscript, while chapters 4 to 6 are written and formatted following selection of peer reviewed, international journals for publication.

The chapter 1, introduces this thesis with a brief review of the state of the art of infectious diseases in aquatic mammals in Brazil, with special emphasis in brucellosis.

The chapter 2 presents a general review about the genus *Brucella* while focusing in pathological processes that have been associated with *Brucella*-infection in aquatic mammals in different locations around the world. The objective is not to offer a complete review about the actual status of this genus, but instead to compile available data in the literature about *Brucella*-related lesions in aquatic mammals in order to facilitate compared pathology exercise.

The chapter 3 constitutes the first report of brucellosis in the Brazilian Coast, published in 2017 as a “letter to the editor” in the journal Transboundary and Emerging Diseases, coauthored by Vitor L. Carvalho, Carlos Sacristán, Kátia R. Groch, Rodrigo A. Ressio, Natalia C. C. A. Fernandes, Juliana M. Guerra, Samira Costa-Silva, Josué Díaz-Delgado, Cintia Favero, Nairléia dos Santos Silva, José Soares Ferreira Neto, Ana C.O. Meirelles and José L. Catão-Dias.

The chapter 4 surveys *Brucella* spp. and *Leptospira* spp. antibodies in riverine dolphins from the Amazon basin and cetaceans and manatees from the Atlantic Ocean, Brazilian coast, using different serological approaches and thus, concerning the detection of several *Brucella*-seropositive cetacean species, appeals to the

relevance of gaining knowledge of *Brucella*-infection in marine mammals in the Brazilian coast. This chapter was prepared to submit to the journal Diseases of Aquatic Organisms and is coauthored by Vitor L. Carvalho, Ana C.O. Meirelles, Waleska Gravena, Juliana Marigo, Carlos Sacristán, Samira Costa-Silva, Kátia R. Groch, Nairléia dos Santos Silva, José Soares Ferreira Neto and José L. Catão-Dias.

The chapter 5 presents a serological screening for *Brucella* spp. and *Leptospira* spp. antibodies in Southern Elephant Seals (*Mirounga leonina*) caught at Elephant Island, Antarctica, discussing the implications for the Antarctic region. This chapter was prepared to submit to the journal Diseases of Aquatic Organisms and is coauthored by Valeria Ruoppolo, Monica Muelbert, José Soares Ferreira Neto and José L. Catão-Dias.

The Chapter 6 describes a comprehensive investigation of *Brucella* in marine mammals of Brazil, by combining molecular, serological, pathological, immunohistochemical and microbiological techniques. This chapter will be submitted to the journal Frontiers in Veterinary Science and is coauthored by Vitor L. Carvalho, Josué Díaz-Delgado, Rodrigo A. Ressio, Natália C. C. A. Fernandes, Juliana M. Guerra, Carlos Sacristán, Kátia R. Groch, Natalia Silvestre-Perez, Eduardo Ferreira-Machado, Samira Costa-Silva, Pedro Navas-Suárez, Ana C.O. Meirelles, Cintia Favero, Juliana Marigo, Carolina P. Bertozzi, Adriana C. Colosio, Milton C.C. Marcondes, Marta Cremer, Nairléia dos Santos Silva, Jose Soares Ferreira Neto, Lara B. Keid, Rodrigo Soares, Eva Sierra, Antonio Fernandez and José L. Catão-Dias.

The chapter 7 closes this thesis with a list of final considerations, briefly mentioning the conclusion of the results presented in the previous chapters while emphasizing the key points of interest that deserves further investigation.

2 LITERATURE REVIEW

2.1 GENUS *BRUCELLA* AND BRUCELLOSIS

Brucellae are Gram-negative, non-motile and non-sporing coccobacilli (short rods) measuring 0.6 to 1.5µm by 0.5-0.7µm. They lack capsule and their outer cell membrane resembles that of other Gram-negative bacilli with a dominant lipopolysaccharide (LPS) component and three main group of proteins. They are aerobes, but some species requires CO₂ to growth, with an ideal temperature of 37°C and their metabolism is mainly oxidative. Brucellae are divided into smooth strains and rough or mucoid variants, which lack the O chains of the LPS -natural in *Brucella canis* and *Brucella ovis*- (ALTON; FORSYTH, 1996).

The genus *Brucella* spp. currently comprises 12 species; the “classical” strains: *B. abortus* (bovines), *B. melitensis* (sheep and goats), *B. suis* (swine, cattle, rodents, wild ungulates), *B. ovis* (sheep), *B. canis* (canines), *B. neotomae* (rodents), *B. papionis* [baboons (*Papio* spp.)], *B. vulpis* (red foxes (*Vulpes vulpes*)), *B. ceti* (cetaceans: dolphins, whales and porpoises), and *B. pinnipedialis* (pinnipeds: seals, sea lions and walruses), and the “atypical” strains: *B. microti* (common voles (*Microtus arvalis*), red foxes (*V. vulpes*) and soil) and *B. inopinata* (a single isolate from a breast implant) (OSTERMAN; MORIYÓN, 2006; FOSTER et al., 2007; HUBÁLEK et al., 2007; SCHOLZ, 2008; SCHOLZ et al., 2009, 2010, 2016; KAZMIERCZAK, 2012; WHATMORE et al., 2014). For detailed species description see <http://www.bacterio.net/brucella.html>. The knowledge about the genus *Brucella* showed to be expanding over time. Recent studies demonstrated *Brucella* strains presence in a new group of hosts; amphibians. Interestingly, phenotypic and genetic features suggest those strains represent an evolutionary link between a soil-associated ancestor and the mammalian host-adapted pathogenic *Brucella* species (AL DAHOUK et al., 2017).

Brucellae are responsible for one of the most widespread and important zoonosis in the world, the brucellosis. This disease is responsible for relevant morbidity, considerable loss of productivity in animal husbandry in the developing world and has become an imported disease in the developed world as result of intentional tourism (PAPPAS et al., 2005). Formerly known as Mediterranean fever, Malta fever or undulant fever, *B. melitensis* the aetiologic agent of this disease, was

firstly discovered by Sir David Bruce, a military physician after his arrival to Malta Island in 1884 (TAN; MD; DAVIS, 2011). *B. melitensis* followed by *B. suis*, *B. abortus* and *B. canis*, are responsible for disease in men (CORBEL, 2006). *B. melitensis*, the most virulent *Brucella* strain for man is associated with infection in sheep and goats but other species such as dogs, cattle and camels can be infected as well (CORBEL, 2006). *B. abortus* -commonly carried by cattle and in less extent bison, buffalo, camels, dogs and yaks- is the most widespread cause of infection for man, but is often subclinical and less pathogenic to humans compared to *B. melitensis* and *B. suis* (CORBEL, 2006). *B. suis* has a restricted occurrence but can be a local source of human infection and in dependence of the biovar (subtype) can be as pathogenic as *B. melitensis*. *B. suis* has been associated with pigs (biovars 1,2,3), hares (biovar 2), caribou and reindeer (biovar 4). *B. canis* infection is widespread in some countries, but less frequently associated with human disease that when present tends to be mild (CORBEL, 2006).

An important issue, regarding wildlife terrestrial brucellosis is to distinguish between a spillover infection from domestic animals and a sustainable infection in wild species (GODFROID, 2002). Brucellae in wildlife are rarely implicated in human disease. However, “Marine” *Brucella* strains have been associated to zoonotic infection (BREW et al., 1999; SOHN et al., 2003; MCDONALD et al., 2006).

2.1.1 Epidemiology and transmission

Brucellosis in humans and domestic animals remains as an important disease and have a widespread distribution, occurring in Africa, Asia and the Middle East, North America, South America, Europe and Australia with variable prevalence influenced by multiple factors (CORBEL, 2006; BAMAIYI, 2016). There are only few countries in the world that are considered officially free of the disease although cases still occur in people returning from endemic countries (CORBEL, 2006).

In humans, *Brucella* spp. infection may occur by inhalation, conjunctival or skin contamination (this may occur via cuts or abrasions), ingestion, and accidental self-inoculation with live vaccines is also a possibility. The possible ways of transmission in men include: person to person (extremely rare, due to close personal or sexual contact, blood donation or tissue transplantation), contaminated environments (e.g., water sources, inhalation of contaminated dust, or additional materials, contact

infection of skin or conjunctivae from soiled surfaces) occupational exposure (farmers, veterinarians, laboratory workers, etc.) and via foodborne (CORBEL, 2006). Although underestimated, the disease is endemic in several areas with high prevalence (PAPPAS et al., 2005). In some countries like the United States, a relationship between the disease and the socioeconomic status have been exemplified comparing disease distribution and increase in case reports related to Hispanic population and the illegal importation of unpasteurized dairy products (FOSGATE et al., 2002).

In domestic animals, transmission occurs by direct contact with infected animals or environment contaminated by fetuses and materials from abortion via ingestion, inhalation, conjunctival inoculation, skin contamination or inoculation from infected milking cups. Sexual transmission and artificial insemination can also be sources of infection (CORBEL, 2006). In cattle, sheep, goats and swine, susceptibility is the greatest in sexually mature animals and young animals are often resistant (CORBEL, 2006). Interestingly, breed may affect susceptibility in sheep and some polymorphism genes have been implicated in a substantial susceptibility in cattle and pigs (CORBEL, 2006).

2.1.2 Pathogenicity and associated pathology

The pathogenicity of *Brucella* spp. is related to the persistence capacity in mononuclear phagocytes in the reticuloendothelial system (ALTON; FORSYTH, 1996). After entering the human body, brucellae are taken by local tissue lymphocytes and transferred through regional lymph nodes into the circulation and are subsequently seeded throughout the body, with tropism for the reticuloendothelial system (PAPPAS et al., 2005). Molecular determinants and mechanisms for cell internalization include the Cyclic β -1,2-glucan molecule -secreted into the periplasm of *Brucella*- which help intracellular brucellae to avoid fusion of the phagosome with lysosomes and the type IV secretion system that translocate brucellae effector proteins into host cells and is critical for both survival and replication in infected host cells (POESTER; SAMARTINO; SANTOS, 2013). After entrance, a host effective defense depends mainly upon cell-mediated immunity (ALTON; FORSYTH, 1996). The clinical signs of infection vary depending on the organ affected, individual susceptibility and presence of coinfections (THAKUR et al., 2012).

In humans, brucellosis is characterized by undulant fever and nonspecific signs as lymphadenopathy, hepatomegaly, splenomegaly. Almost every organ and system can be affected by the disease. However, osteoarticular disease is oftenly the most common complication (PAPPAS et al., 2005). The distribution pattern of the three existing *Brucella* arthritis forms: monoarthritis, sacroiliitis and spondylitis, is related to differences in the *Brucella* species involved (GONZÁLEZ-GAY; GARCÍA-PORRÚA, 2014) and possible genetic predisposition (BRAVO et al., 2003). The second most affected system is the reproductive system, causing epididymo-orchitis (NAVARRO-MARTÍNEZ et al., 2001) and spontaneous abortion (KHAN; MAH; MEMISH, 2001). Gastrointestinal complications are reported and include, hepatitis, liver granulomas and ascites (CERVANTES et al., 1982; AKRITIDIS; PAPPAS, 2001). The central nervous system involvement is also related and includes meningitis, encephalitis, meningoencephalitis, meningovascular disease, brain abscesses and demyelinating syndrome (SHAKIR et al., 1987). Brucellosis may also cause respiratory complications such as pneumonia and pleural effusion (PAPPAS et al., 2003). Cardiovascular involvement is uncommon but both endocarditis and epicarditis have been described (REGUERA et al., 2003; KAYA; ESKAZAN; ELALDI, 2013). Endocarditis remains as the main cause of mortality to brucellosis (PAPPAS et al., 2005). Cutaneous manifestations of brucellosis are very rare, but include Papulonodular and maculopapular eruptions, erythema nodosum-like lesions and vasculitic lesions (KORKMAZ; KARTA, 2016).

Brucella spp. has marked tropism for the pregnant reproductive tract of domestic animals producing a chronic infection resulting in reproductive failure (POESTER; SAMARTINO; SANTOS, 2013). In females *Brucella* spp. cause placentitis, fetal death and abortion and/or metritis. Most significant pathological processes in males include orchitis, seminal vesiculitis and/or epididymitis, which may lead to partial or permanent infertility. Bacteremia also induces a granulomatous reaction and/or abscesses in lymphoid tissues such as spleen, iliac, mesenteric and supramammary lymph nodes and additional organs (POESTER; SAMARTINO; SANTOS, 2013). Meningitis, pericarditis, pleuritis, pneumonia, splenitis and hepatitis have been also documented in bovines with in-utero *Brucella* spp. infection (HONG et al., 1991; XAVIER et al., 2009). Additional affection of other systems (e.g. musculoskeletal) has been also reported (POESTER; SAMARTINO; SANTOS, 2013).

2.1.3 Control and treatment

Controlling animal brucellosis, causes a corresponding decline in occurrence of the disease in humans (SELEEM; BOYLE; SRIRANGANATHAN, 2010). Eradication and control of brucellosis can be accomplished when involving all the concerned parties: farmers, milk and dairy industry, breeding companies, consumers and politicians (SELEEM; BOYLE; SRIRANGANATHAN, 2010). Although cost effective control measures are known, there is a lack of funds and/or political “know how” to implement the measures in many countries (SELEEM; BOYLE; SRIRANGANATHAN, 2010). The control of this disease in developing countries requires a considerable effort that includes education, proper laboratory facilities and trained personal to collect and test samples, record keeping and active control and surveillance programs which includes vaccination of all susceptible hosts and elimination of infected animals (SELEEM; BOYLE; SRIRANGANATHAN, 2010).

In domestic animals, the most commonly used vaccines are the classical *B. abortus* strain 19 in cattle and Rev 1 in small ruminants. The rough mutant RB51 strain (which does not interfere with serological diagnosis) is increasingly used but the evidence that it is equal or even better than S19 is conflictive (MORIYÓN et al., 2004). Susceptible wildlife may be a source of contagion or spill-over (MICK et al., 2014) and vaccination may be a necessary measure in areas where these animals come in contact with domestic livestock (MORIYÓN et al., 2004). However, wildlife vaccination gave controversial results and caused concern because this could lead to environmental contamination and infection of other wildlife species (OLSEN et al., 1997; ELZER et al., 1998; GODFROID, 2002).

Vaccines have been used for immunization of domestic animals with success. However, no satisfactory vaccine against human brucellosis is available (PAPPAS et al., 2005). Medical treatment of brucellosis should involve antibiotics that can penetrate macrophages and can act in the acidic intracellular environment. Generally, there is a need of combined treatment which includes doxycycline, streptomycin, rifampin, gentamicin, trimethoprim-sulfa-methoxazole, ofloxacin and ciprofloxacin (PAPPAS et al., 2005).

2.2 “MARINE” *BRUCELLA* STRAINS

Brucella spp. in marine mammals was firstly detected in 1994, in dolphins of the Delphinidae and Phocoenidae families and seals from the Pochidae family (EWALT et al., 1994; ROSS et al., 1994). After that, several reports expanded the knowledge of *Brucella* genus in aquatic mammals. The potential hosts of *Brucella* spp. in aquatic environments include up to 130 species and interestingly, in almost half of them seropositivity have been demonstrated (HERNÁNDEZ-MORA; PALACIOS-ALFARO; GONZÁLEZ-BARRIENTOS, 2013). *B. ceti* has already been identified (by molecular or microbiological methods) and/or anti-*Brucella* antibodies have been detected in several species/subspecies of cetaceans, pinnipeds, otters and the polar bear, while no evidence of *Brucella* exists in riverine dolphins, manatees or dugongs (GUZMÁN-VERRI et al., 2012). Comprehensive reviews about prevalence of infected/seropositive species are available in the literature (NYMO; TRYLAND; GODFROID, 2011; GUZMÁN-VERRI et al., 2012; HERNÁNDEZ-MORA; PALACIOS-ALFARO; GONZÁLEZ-BARRIENTOS, 2013). These data ratify *B. ceti* and *B. pinnipedialis* as widespread pathogens, present in several marine mammal species and locations around the world.

The divergence of different species of the genus *Brucella* may have taken place 60 million years ago, concomitant with the radiation of their mammalian hosts (Artiodactyla) from other mammalian hosts (BOURG; O'CALLAGHAN; BOSCHIROLI, 2007). Although *Brucella* species are recognized as having a preferred host, it has been suggested that transmission from cetaceans to pinnipeds may occur (MAQUART et al., 2009), as it has been reported for terrestrial *Brucella* strains (CORBEL, 2006). Additional studies showed "Marine" *Brucella* strains further subdivision (BOURG; O'CALLAGHAN; BOSCHIROLI, 2007; GROUSSAUD et al., 2007; DAWSON et al., 2008b; MAQUART et al., 2009). A more large-scale global surveillance is required in order to fully understand the distribution, ecology and genetic relatedness of *Brucella* isolates from marine mammals (DAWSON et al., 2008b).

By using a 9 locus MSLSA (BruMLSA₉) approach and more recently, an extended 21 locus scheme (BruMLSA₂₁) (WHATMORE; PERRETT; MACMILLAN, 2007; WHATMORE et al., 2016); distinct sequence types (STs) have been identified in *Brucella* isolated from marine mammals. *B. ceti* forms two distinct clusters most commonly associated with dolphins (Delphinidae), and less extensively from beaked whales (Ziphiidae) (ST26), or harbor porpoises (*Phocoena phocoena*) (ST23)

(GROUSSAUD et al., 2007; DAWSON et al., 2008b). There is one group that corresponds to *B. pinnipedialis* (STs 24 and 25), further subdivided into (STs 53 and 54) that appears specific for hooded seals (*Cystophora cristata*), as previous descriptions (GROUSSAUD et al., 2007; MAQUART et al., 2009). The ST27 remains distinct from the main marine mammal *Brucella* clusters (WHATMORE et al., 2016) and have been associated with naturally acquired infection of humans, seems more pathogenic to man, and might be associated with hosts and intermediaries that favors contact with humans, by consumption of raw sea food (WHATMORE et al., 2008). On the other hand, a mild and uncomplicated laboratory acquired infection was associated with the genotype ST23 (WHATMORE et al., 2008).

2.2.1 Epidemiology and transmission in marine mammals

Little is known about the epidemiology of *Brucella* spp. infection in marine mammals, making difficult to understand the pathways of infection and exposure in cases of naturally acquired zoonotic infection (LAMBOURN et al., 2013). An important aspect of *Brucella* spp. in marine mammals, is to study the ecology of infection -through multidisciplinary research- to clarify whether the infection is sustainable in a population and which factors are crucial for maintenance of infection in a population (GODFROID, 2002).

In marine mammals, potential methods of direct transmission would be social/sexual interaction, vertical (in-utero infection), physical trauma (violent interactions among species) and ingestion (including predation) (FOSTER et al., 2002). However, the transmission cycle of marine *Brucella* strains is not yet elucidated and some of the hosts or vectors are still unknown. The participation of pulmonary parasites in the indirect transmission of the disease has been suggested (DAGLEISH et al., 2008). Classic and molecular typing methods, immunologic and/or electron microscopy immunolabeling techniques, proved *Brucella* spp. presence in *Parafilaroides* lungworms, in a pacific harbor seal (*Phoca vitulina richardsi*) (GARNER et al., 1997) and *Pseudalius inflexus* in *P. phocoena* (PERRETT et al., 2004b; DAWSON et al., 2008a; MAIO et al., 2014).

It has been speculated that the source of the infection through these parasites could be linked to the food sources of marine mammals such as fish. Natural infection by *Brucella melitensis* biovar 3 was described in the fish species *Clarias*

gariepinus on the Nile River, Egypt (EL-TRAS et al., 2010). In that case it was suggested that infection was caused via the skin and ingestion, due to water contamination of inadequate disposal practices of carcasses and contaminated materials (aborted fetuses, fetal membranes, placentas) (EL-TRAS et al., 2010). Recently, experimental infection of Atlantic cod (*Gadus morhua*) with *B. pinnipedialis* hooded seal strain demonstrated that fish are capable of sustain infection for at least 28 days, indicating its potential as reservoirs and potential role in transmission of *B. pinnipedialis* to hooded seals in the wild (NYMO et al., 2016).

2.2.2 *Brucella*-associated pathology in marine mammals

Brucella spp. in marine mammals have been identified from various tissues or organs with or without pathological associated processes (FOSTER et al., 2002; NYMO; TRYLAND; GODFROID, 2011; GUZMÁN-VERRI et al., 2012; HERNÁNDEZ-MORA; PALACIOS-ALFARO; GONZÁLEZ-BARRIENTOS, 2013). In cetaceans, *Brucella*-infection is common, but only a portion of the individuals diagnosed showed clinical or pathological associated signs, which raises the hypothesis that a significant proportion of the infected animals surpass the infection and remain as carriers and potential disseminators (GUZMÁN-VERRI et al., 2012).

Pathological processes/lesions caused by *Brucella* infection in marine mammals are similar to those reported in humans and domestic animals, mainly indicating involvement of the central nervous, reproductive, respiratory, musculoskeletal and integumentary systems. Available literature data about pathological processes/lesions associated to several cetacean species and a sea otter is summarized in Table 1.

Individuals from the Delphinidae family seems to be more susceptible to develop chronic form of the diseases including meningitis/ meningoencephalitis (GONZÁLEZ et al., 2002; DAGLEISH et al., 2007a, 2008; HERNÁNDEZ-MORA et al., 2008; JAUNIAUX et al., 2010). *B. ceti* has the ability to cause chronic infection in multiple organs before crossing the blood-brain barrier (GONZÁLEZ-BARRIENTOS et al., 2010) and can lead to starvation, stranding and can be implicated as the primary cause of death (DAGLEISH et al., 2007b; HERNÁNDEZ-MORA et al., 2008; GONZÁLEZ-BARRIENTOS et al., 2010; GRATTAROLA et al., 2016). Coinfection by *Brucella* spp. and additional pathogens have been described, for example cetacean

morbillivirus (WEST et al., 2014), *Toxoplasma gondii* (ALBA et al., 2013) and a triple coinfection by *Brucella* spp., *Listeria monocytogenes*, and *T. gondii* (GRATTAROLA et al., 2016).

On the other hand, the pathogenicity of *B. pinnipedialis* is still unclear. Seropositivity and isolation are reported without signs of disease or associated lesions, suggesting a host adaptation or low pathogenicity (NYMO; TRYLAND; GODFROID, 2011; NYMO et al., 2013). For example, *Brucella* spp. have been isolated from various tissues from healthy hooded seals where infection is considered endemic (TRYLAND; SØRENSEN; GODFROID, 2005). *Brucella* spp. was detected in placental tissues of *Z. californianus* suffering from abortion and premature parturition on San Miguel Island, California, but the classical *Brucella*-associated histological lesion, placentitis, was not present on those samples (GOLDSTEIN et al., 2009). In Common Seals (*Phoca vitulina*) and Grey Seals (*Halichoerus grypus*) where *B. pinnipedialis* was isolated; most of the individuals were suffering from verminous pneumonia, but a clear link with *Brucella* infection was not established (PRENGER-BERNINGHOFF et al., 2008).

Table 1. Pathological lesions/processes associated to *Brucella* infection in cetaceans and a marine otter

System	<i>Brucella</i> associated lesion/pathological process	Species	Reference
Central nervous system	<ul style="list-style-type: none"> Non-suppurative meningoencephalitis Meningitis 	<i>Phocoena phocoena</i>	(DAGLEISH et al., 2008; JAUNIAUX et al., 2010)
		<i>Tursiops truncatus</i>	(VENN-WATSON et al., 2015)
		<i>Delphinus delphis</i>	(DAVISON et al., 2013)
	<ul style="list-style-type: none"> Meningeal congestion, cerebral edema Chronic, non-suppurative meningoencephalitis, encephalitis, meningitis and choroiditis involving the brainstem, cerebrum, cerebellum, periventricular encephalon and choroid plexus with ependymal loss and marked lymphoplasmacytic perivascular cuffing (more severe in the spinal cord, medulla oblongata, cerebellum, third and fourth ventricle and milder in the cerebral cortices) Occasional hydrocephalus, vascular damage (hemorrhage) and fibrinoid necrosis, fibrosis, microgliosis, degeneration of Purkinje cells, gliosis, perivascular necrotic foci (grey matter), white matter necrosis and laminar necrosis of the cerebro-cortical grey matter 	<i>Lagenorhynchus acutus</i>	(DAGLEISH et al., 2007b)
		<i>Stenella coeruleoalba</i>	(GONZÁLEZ et al., 2002; MUÑOZ et al., 2006; HERNÁNDEZ-MORA et al., 2008; DAVISON et al., 2009; GONZÁLEZ-BARRIENTOS et al., 2010; ALBA et al., 2013; GAROFOLO et al., 2014; ISIDORO-AYZA et al., 2014; GRATTAROLA et al., 2016)
	<ul style="list-style-type: none"> Moderate to severe congestion, non-suppurative meninges, choroiditis, perivascular cuffing (more severe in 	<i>Globicephala melas</i>	(DAVISON et al., 2015)

	para-ventricular areas and hindbrain)		
	• Brain congestion, chronic meningitis	<i>Physeter macrocephalus</i>	(WEST et al., 2014)
Respiratory system	• Fetal distress, interstitial pneumonia	<i>Eubalaena australis</i>	(MCALOOSE et al., 2016)
	• Acute purulent pneumonia, severe chronic interstitial/bronchointerstitial pneumonia	<i>Phocoena phocoena</i>	(DAGLEISH et al., 2008; JAUNIAUX et al., 2010)
	• Lung abscess adhered to pleura, pulmonary and dorsal aorta granulomatous vasculitis, granuloma in lung and pulmonary lymph node, lymphadenomegalia	<i>Balaenoptera acutorostrata</i>	(NIEMEYER et al., 2015)
	• Pneumonia	<i>Physeter macrocephalus</i>	(WEST et al., 2014)
	• Mineralized nodule in a granuloma	<i>Tursiops truncatus</i>	(MILLER et al., 1999; COLEGROVE et al., 2016)
	• Fetal distress and in-utero pneumonia		
	• Chronic pyogranulomatous bronchopneumonia with bronchitis and bronchiolitis	<i>Stenella clymene</i>	(SÁNCHEZ-SARMIENTO et al., 2017)
	• Lung edema and perivascular lymphocytic infiltrates	<i>Stenella coeruleoalba</i>	(MUÑOZ et al., 2006; ALBA et al., 2013)
Reproductive system	• Granulomas, bronchiolar microcalcifications, atelectasis, peribronchiolar leucocytes aggregates		
	• Testicular abscess, with coagulative necrosis and fibrosis	<i>Phocoena phocoena</i>	(DAGLEISH et al., 2008; JAUNIAUX et al., 2010)
	• Endometritis		
	• Testicular granulomas	<i>Balaenoptera acutorostrata</i>	(NIEMEYER et al., 2015)
	• Granulomatous orchitis, caseation and calcification	<i>Balaenoptera acutorostrata</i>	(OHISHI et al., 2003)
	• Subacute and chronic purulent orchitis		
	• Granulomatous lesions in uterus		
	• Ovaritis, nodular lesion with calcification	<i>Balaenoptera edeni</i>	OHISHI et al., 2003)
Musculoskeletal system	• Granulomatous orchitis, caseation and calcification		
	• Severe necrotizing placentitis, metritis, abortion	<i>Tursiops truncatus</i>	(EWALT et al., 1994; MILLER et al., 1999)
		<i>Cephalorhynchus hectori maui</i>	(BUCKLE et al., 2017)
		<i>Stenella coeruleoalba</i>	(GONZÁLEZ-BARRIENTOS et al., 2010)
	• Chronic severe disco-spondylitis with disruption of the adjacent soft tissues	<i>Tursiops truncatus</i>	(ISIDORO-AYZA et al., 2014)
	• Severe remodeling of the occipital condyles and the atlas	<i>Lagenorhynchus acutus</i>	(DAGLEISH et al., 2007b)
	• Fibrino-suppurative and erosive osteoarthritis at the left escapulo humeral joint.	<i>Globicephala melas</i>	(FOSTER et al., 2015)
	• Fibrinopurulent osteoarthritis	<i>Stenella coeruleoalba</i>	(GONZÁLEZ-BARRIENTOS et al., 2010)
Integumentary system	• Atlanto-occipital joint showed chronic, nonsuppurative and suppurative arthritis	<i>Delphinus delphis</i>	(DAVISON et al., 2013)
	• Chronic severe osteoarthritis in proximal interphalangeal joint of the left hind fifth digit	<i>Enhydra lutris nereis</i>	(MILLER et al., 2017)
	• Purulent abscess in blubber	<i>Tursiops truncatus</i>	(DAWSON et al., 2006)
	• Ulcerative dermatitis	<i>Phocoena phocoena</i>	(FOSTER et al., 1996; ROSS et al., 1996; JAUNIAUX et al., 2010)
	• Subcutaneous lesions ^{&}		
	• Subcutaneous steatitis	<i>Monodon monoceros</i>	(RAVERTY; NIELSEN; LING, 2006)
	• Subcutaneous lesions ^{&}	<i>Stenella coeruleoalba</i>	(FOSTER et al., 1996; GRATTAROLA et al., 2016)
	• Pyogranulomatous inflammation/abscess		

	<ul style="list-style-type: none"> • Subcutaneous lesions^{&} 	<i>Delphinus delphis</i>	(FOSTER et al., 1996; ROSS et al., 1996)
Lymphatic system	<ul style="list-style-type: none"> • Splenic petechial hemorrhages • Chronic mesenteric lymphadenitis with moderate eosinophils • Splenic lymphocyte necrosis, follicular hyalinosis, sinusoidal histiocytosis 	<i>Stenella coeruleoalba</i>	(ALBA et al., 2013; GAROFOLO et al., 2014; GRATTAROLA et al., 2016)
	<ul style="list-style-type: none"> • Splenic and lymph nodes lymphoid depletion 	<i>Phocoena phocoena</i>	(JAUNIAUX et al., 2010)
	<ul style="list-style-type: none"> • Lymphadenomegalia and lymphoid depletion 	<i>Physeter macrocephalus</i>	(WEST et al., 2014)
	<ul style="list-style-type: none"> • Pulmonary lymph node, lymphadenomegalia 	<i>Balaenoptera acutorostrata</i>	(NIEMEYER et al., 2015)
	<ul style="list-style-type: none"> • Endocarditis, thickening and vegetative nodule of the mitral valve, scattered necrosis and distrophic calcification, focal myocardial degeneration, pericardial fibrosis 	<i>Stenella coeruleoalba</i>	(GONZÁLEZ-BARRIENTOS et al., 2010)
Gastrointestinal system	<ul style="list-style-type: none"> • Hepatic coagulative necrosis 	<i>Phocoena phocoena</i>	(JAUNIAUX et al., 2010)

[&]lesions were not detailed

2.2.3 Pathogenicity of marine *Brucella* strains

Experimental studies demonstrated variable potential pathogenicity of marine *Brucella* strains across terrestrial hosts. Inoculation of marine *Brucella* strains (M1068/91, M2357/93 and M644/93, respectively from three different groups: porpoise, seal, dolphin) caused fulminant systemic infection in guinea pigs. However, in sheep, seroconversion with low and transient titers were verified, along with abortion and lamb mortality at relatively low rates (within 4 days after infection), and without isolation of microorganisms in comparison to a *B. melitensis* strain, demonstrating lower pathogenicity of the marine *Brucella* strains evaluated (PERRETT et al., 2004a). Pregnant cattle infected via intravenous route with a *Brucella* strain isolated from a pacific harbor seal caused seroconversion and abortion in one animal, and the *Brucella* strain presence was demonstrated in fetuses and dams indicating abortogenic potential. However, this strain was less pathogenic in comparison with *B. abortus* (RHYAN et al., 2001). In the BALB/c mouse model the *B. pinnipedialis* strain, specific for the hooded seal showed to be less pathogenic than *B. suis* 1330 and the exposure to PCB 153 didn't affect the course of infection (NYMO et al., 2014).

In vitro experiments demonstrated three infection patterns of marine *Brucella* strains: (1) strains able to infect human macrophages (THP-1 macrophage-like) to the same extent that virulent strains *B. suis* 1330 or *B. melitensis* 16M; (2) strains able to enter the cells as classical virulent strains but were eliminated after 48 h and (3) strains isolated from hooded seals were not able to enter and infect cells

(MAQUART; ZYGMUNT; CLOECKAERT, 2009). *B. pinnipedialis* strains (reference strain; NTCT 12890 and a hooded seal isolate; B17) are able to enter Human macrophage-like cells (THP-1), two murine macrophage cell lines (RAW264.7 and J774A.1), and a human malignant epithelial cell line (HeLa S3) but are gradually eliminated during the next 72-96 hours (LARSEN et al., 2013b). *B. pinnipedialis* mechanism of entrance in human macrophages involves but seems not limited to lipid-rafts, whereas in murine macrophages nitric oxide is not released and intracellular bacteria does not induce cell death (LARSEN et al., 2013b).

Additional research shows, marine mammal brucellae -*B. pinnipedialis* reference strain (NCTC 12890) from common seal, *B. ceti* reference strain (NCTC 12891) from harbor porpoise and a *B. ceti* Atlantic white-sided dolphin (*Lagenorhynchus acutus*) isolate (M83/07/1)-were able to enter hooded seal alveolar macrophages, however, they did not multiply and were eliminated within 48 hours, on the contrary of a classical *B. suis* pathogenic strain (LARSEN et al., 2013a). This could reflect host specificity rather than lack of multiplication in preferential host specific macrophages (LARSEN et al., 2013a). These findings also suggested that persistent infection is restricted to preferential hosts and also that the hooded seal may not be a reservoir species, but rather a spillover host exposed to those strains in a niche to which no other seals are exposed (LARSEN et al., 2013a).

3 OBJECTIVES

3.1 GENERAL OBJECTIVE

The main goal of this study was to investigate the presence of *Brucella* spp., as well as the pathological and epidemiological aspects in Brazil, using samples from aquatic mammals kept at the Marine Mammal Tissues Bank, School of Veterinary Medicine and Animal Sciences, Department of Pathology, University of São Paulo, Brazil, or those collected in partnership with other institutions in the country, through serological, molecular, histopathological and immunohistochemical methods.

3.2 SPECIFIC OBJETIVES

- Survey for *Brucella* spp. exposure by detecting the presence of anti-*Brucella* spp. antibodies in serum samples from cetaceans, pinnipeds and manatees using the following serological methods: Rose Bengal Test (RBT), a commercial competitive enzyme linked immunosorbent assay (c-Elisa) and the Serum Agglutination Test (SAT, in positive cases).
- Evaluate aquatic mammal samples for *Brucella* spp. DNA by using the molecular methods: conventional polymerase chain reaction-PCR and real-time PCR (in selected cases).
- Investigate and describe using light microscopy, the histopathological lesions related with *Brucella* spp. infection on suspect/positive animals (by serological and/or molecular methods).
- Detect *Brucella* spp. positive immunolabeling by employing immunohistochemistry in tissues of suspect/positive animals (by serological and/or molecular methods).

4 BRUCELLOSIS IN A CLYMENE DOLPHIN (*STENELLA CLYMENE*) STRANDED IN BRAZIL

Used with permission from: Sánchez-Sarmiento, A.M., Carvalho, V. L., Sacristán, C., Groch, K. R., Ressio, R. A., Fernandes, N. C. C. A., Guerra, J. M., Costa-Silva, S., Díaz-Delgado, J., Favero, C. M., Silva, N. S., Ferreira Neto, J. S., Meirelles, A. C. O. and Catão-Dias, J. L. Copyright (2017) Wiley. Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil. *Transbound Emerg Dis.* 2017;1-3, © 2017 Blackwell Verlag GmbH. License number: 4275290409043.

Cetacean brucellosis has demonstrated an alarming widespread distribution and increasing spectrum of vulnerable hosts, since it was firstly diagnosed in cetaceans in 1994 (Ewalt et al., 1994). Currently, there are two recognized species of *Brucella* in marine mammals: *B. ceti* and *B. pinnipedialis* (Foster et al., 2007). To date, *B. ceti* has been identified (isolation and/or by molecular analysis) in at least 12 cetacean species from six different families (Hernández-Mora et al., 2013; West et al., 2014; McAloose et al., 2016) and seropositivity has been found in at least 35 cetacean species (Hernández-Mora et al., 2013). Among *B. ceti* strains, the ST27 has been associated with human neurobrucellosis and spinal osteomyelitis after consumption of raw seafood in the Pacific Ocean (Sohn et al., 2003; McDonald et al., 2006; Whatmore et al., 2008). This raised concern about the presence and distribution of marine mammal *Brucella* genotypes, particularly those with a zoonotic potential, in regions not extensively studied to date (Whatmore et al., 2008). In the South Atlantic Ocean, the only evidence of *Brucella* in cetaceans was the detection of *Brucella* sp. by polymerase chain reaction (PCR) in lung tissue of a newborn female southern right whale (*Eubalaena australis*) stranded in Península Valdés (Argentina) in 2003, presenting interstitial pneumonia and aspirated squames (McAloose et al., 2016). We present the first evidence of *Brucella* sp. infection in an odontocete cetacean from Brazil.

On March 10, 2012, an adult, 175 cm in body length, male Clymene dolphin (*Stenella clymene*) was found stranded alive in Parajuru, Ceará state (Brazil; 04°23'S, 37°49'W). The animal was in moderate nutritional condition and had signs of shock, including dorsal bending, muscle tremors, apnea, vomiting and mydriasis. The dolphin died shortly after stranding. A necropsy followed and tissues were collected

and fixed in formalin, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin for histopathologic studies.

Gross findings included: moderate pulmonary edema and pulmonary nematodiasis by *Halocercus* sp.; moderate gastroesophageal nematodiasis by *Anisakis typica* associated with ulcers and hemorrhage; numerous cestodes, *Phyllobotrium delphini* and *Monorygma grimaldi*, encysted in blubber of the caudal peduncle and urogenital ligaments, respectively; necrohemorrhagic colitis; mesenteric lymphadenomegaly; splenomegaly with multifocal subcapsular hemorrhage and moderate brain congestion. Histological examination of the lung revealed moderate chronic pyogranulomatous bronchopneumonia with bronchitis, bronchiolitis and adult and nematode larvae embedded in exudative inflammatory foci, along with bronchial squamous metaplasia, fibrosis and rare bronchial sclerosis, moderate edema, congestion and hemorrhage, scattered mineralizations in the mucosa and submucosa of bronchioles and bronchi, and multifocal atelectasia and hemosiderosis. Other histological findings were: moderate mesenteric and splenic lymphoid depletion; moderate mesenteric pyogranulomatous lymphadenitis; mild eosinophilic splenitis; mild lymphoplasmacytic gastritis (glandular stomach) and chronic ulcerative and hyperplastic gastritis with adult nematodes (pyloric stomach); mild to moderate, multifocal eosinophilic enterocolitis; moderate to marked, acute centrilobular congestion and minimal, multifocal hepatic portal lymphoplasmacytic infiltrates; mild to moderate, multifocal lymphoplasmacytic interstitial nephritis, focal necroulcerative glossitis and mild myocardial degeneration.

Rose Bengal Test (RBT) using inactivated suspension of *B. abortus* 1119-3 (Instituto Biológico, São Paulo, Brazil) yielded positive agglutination of serum from the dolphin. Immunohistochemical analysis was performed in 4-5µm slides cut from paraffin-embedded lung, liver, brain, heart, kidney, small intestine and mesenteric lymph node samples. Tissue sections were incubated overnight at 4°C with a polyclonal antibody against *B. abortus* (1:500, Byorbyt Ltd®, Cambridge, UK). An alkaline phosphatase polymer (Histofine®, Nichirei Biosciences Inc., Tokyo, Japan) was used for amplification and detection, and Warp Red (Biocare Medical®, California, USA) as chromogen. Positive immunolabeling was observed in the cytoplasm of macrophages in the lung (Figure 1). For PCR analysis, DNA was extracted from frozen lung, liver, brain, heart, kidney, small intestine and mesenteric lymph nodes using the DNeasy Blood & Tissue Kit (QIAGEN®, Valencia, USA).

Conventional PCR targeting a 223 bp fragment of the gene *bcsP31* encoding the 31 kDa immunogenic (cell surface) protein of the genus *Brucella* (Baily et al., 1992) was performed. Positive controls consisted of DNA from *B. abortus* (vaccine RB-51) and *B. ceti* (M644/93/1; B14/94). Nuclease-free water instead of DNA extract was used as negative control. A 182bp consensus sequence was generated from the lung, and used for homology comparison in GenBank using Basic Local Alignment Search Tool (BLAST) showing 100% identity to several *Brucella* species i.e., *B. melitensis*, *B. canis*, *B. abortus*, *B. suis* and *B. pinnipedialis*. The other tissues were PCR negative.

Figure 1. (a) Section of the lung of a Clymene dolphin (*Stenella clymene*), showing positive cytoplasmic immunolabelling for *Brucella* sp. antigen within alveolar macrophages (arrows), 209x; (b) Right upper corner (inset), 409x. (c) Positive control shows immunolabelling of *Brucella ceti* in placenta of a striped dolphin (*Stenella coeruleoalba*; provided by C. Guzman-Verri, R. Gonzalez-Barrientos and G. Hernandez-Mora; arrows), 40x

The present case provides evidence of localized *Brucella* sp. pulmonary infection, supporting a potential aerogenous route of transmission, as suggested by previous detection of *Brucella* sp. in blowhole swabs (Wu et al., 2016) and lung samples from cetaceans (Colegrove et al., 2016; Dagleish et al., 2008; Foster et al., 2002; Jauniaux et al., 2010; Muñoz et al., 2006; Prenger-Berninghoff et al., 2008).

There are no pathognomonic lesions for *Brucella*-associated lung pathology in cetaceans and its pathogenesis is yet unresolved. In this regard, different situations have been observed in cetaceans from which *Brucella* was isolated and/or detected from lung: (i) absence of lesions, (ii) different patterns of pneumonia, (iii) association with nematode infestation and (iv) fetal distress (Colegrove et al., 2016; Dagleish et al., 2008; Foster et al., 2002; Jauniaux et al., 2010; Muñoz et al., 2006; Prenger-Berninghoff et al., 2008). In the present case, we observed several commonalities with previous reports e.g., bronchopneumonia, nematode infestation, bronchiolar mineralizations, edema, small aggregates of leukocytes in the peribronchiolar connective tissue. Additional gross and histological lesions in several organs other than lung have been previously reported in *B. ceti*-infected cetaceans, not always clearly associated with *Brucella* infection (González-Barrientos et al., 2010). The lymphoid depletion suggested immunosuppression in this case, and prompted cetacean morbillivirus (CeMV) molecular investigation by reverse transcription-PCR using primers targeting the phosphoprotein (P) gene (Barrett et al., 1993). Coinfection by *Brucella* sp. and CeMV, a known immunosuppressive pathogen in these species, has been described (West et al., 2014). Moreover, a novel lineage of CeMV was recently identified in a Guiana dolphin (*Sotalia guianensis*) in Brazil (Groch et al., 2014). However, in this case, CeMV PCR was negative.

In summary, and to the best of our knowledge, we present the first immunohistochemical and molecular evidence of *Brucella* sp. infection in the lung of an odontocete cetacean from the South Atlantic Ocean. This study widens the spectrum of susceptible species and geographic range of this agent with zoonotic potential and public health concern. Ongoing research for this bacterium in Brazil is focused on host range, molecular characterization of circulating strains and associated pathogenesis.

ACKNOWLEDGMENTS

We would like to acknowledge Caterina Guzmán-Verri (Universidad Nacional, Heredia, Costa Rica), Rocio González-Barrientos and Gabriela Hernández-Mora (Servicio Nacional de Salud Animal, Ministerio de Agricultura y Ganadería, Heredia, Costa Rica) for gently providing the positive controls and useful scientific comments. We greatly appreciate the help provided by Gilbert Alvarado, Pedro Navas, Juliana

Marigo, Sândara Sguario and Jorge Oyakawa (Universidade de São Paulo). We also thank the two anonymous reviewers for their constructive comments. This research was supported in part by a Small Grant in Aid of Research from the Society for Marine Mammalogy (2015 and 2016). This study was conducted by A.M.S-S as part of her PhD at the Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil, with support from a scholarship from Coordenação de Apoio à Pesquisa de nível superior-CAPES. J.L.C.-D. is a recipient of a professorship by Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq. This research was approved by Comissão de Ética no Uso de Animais (CEUA No. 3926161213), Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo. The dolphin rescue and necropsy was performed by Aquasis' Projeto Manatí, sponsored by Petrobras.

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5 SURVEY OF *BRUCELLA* SPP. AND *LEPTOSPIRA* SPP. ANTIBODIES IN RIVERINE DOLPHINS FROM AMAZON BASIN AND CETACEANS AND MANATEES OF THE ATLANTIC OCEAN, BRAZIL

5.1 ABSTRACT

Aquatic mammals can act as sentinels of emerging and resurging pathogens in the environment. *Brucella* spp. and *Leptospira* spp. are two zoonotic pathogens of relevance in aquatic mammals and serological methods can be used as a tool to gain knowledge about exposure to both pathogens. In this study, serum from 84 individuals (63 cetaceans from families Iniidae (n=37); Delphinidae (n=22) and Kogiidae (n=4) and 21 west Indian manatees (*Trichechus manatus*) was tested by the Rose Bengal Test (RBT) and a commercial competitive enzyme-linked immunosorbent assay (c-ELISA) for detecting *Brucella* spp. antibodies and the microscopic agglutination test for screening *Leptospira* spp. exposure. Overall, 4.8% (3/63) of cetaceans were positive by RBT and 15.9% (10/63) by c-ELISA for *Brucella* spp. Serum from eight c-ELISA positive cetaceans were further tested via serum agglutination test and one individual was positive. Exposure to *Brucella* spp. was found in 5 cetacean species: Clymene dolphin (*Stenella clymene*), short-finned pilot whale (*Globicephala macrorhynchus*), pigmy killer whale (*Feresa attenuata*), melon-headed whale (*Peponocephala electra*) and Atlantic bottlenose dolphin (*Tursiops truncatus*) in the Southwestern Atlantic Ocean, Brazil, expanding the range of known *Brucella* seropositive aquatic hosts. No evidence of *Brucella* spp. exposure was found in Iniidae and Kogiidae odontocetes and manatees. Antibodies against *Leptospira* spp. were not detected in cetaceans and sirenians. These results contribute to the evaluation of different *Brucella* spp. serological methods in cetaceans and manatees and shed light to the epidemiology of pathogens with zoonotic potential in aquatic mammals of the southwestern Atlantic Ocean and Amazon basin.

Keywords: aquatic mammals, competitive ELISA, Microscopic Agglutination Test, Rose Bengal Test, Serum Agglutination Test.

5.2 INTRODUCTION

Gram-negative coccobacillus bacteria of the genus *Brucella* were isolated for the first time in 1994 from three families of marine mammals: Delphinidae, Phocoenidae and Phocidae (Ewalt et al. 1994, Ross et al. 1994). *Brucella* spp. in aquatic mammals diverge according to host preferences, with two recognized species: *Brucella ceti* and *Brucella pinnipedialis* (Foster et al. 2007), with further subdivision (Bourg et al. 2007, Groussaud et al. 2007, Dawson, Stubberfield, et al. 2008, Maquart et al. 2009). Aquatic *Brucella* seems to be endemic for some groups (e.g., pinnipeds) while, in others (riverine dolphins, manatees and dugongs) it had never been reported (Hernández-Mora et al. 2013). The range of *Brucella* spp. susceptible species in the aquatic environment is wide, with at least 54 seropositive species or subspecies of aquatic mammals (36 cetacean species, 14 pinniped species, two subspecies of sea otters, one species of freshwater otter and the polar bear) (Hernández-Mora et al. 2013, Sánchez-Sarmiento et al. 2017).

In aquatic mammals *Brucella* infection can be asymptomatic, but may lead to severe pathological processes in dolphins e.g., meningoencephalitis and placentitis in striped dolphins (*Stenella coeruleoalba*) (Hernández-Mora et al. 2008). The zoonotic potential of marine *Brucella* strains is recognized and had been related to the ST27 genotype, considered more pathogenic, associated with natural hosts or circulation through intermediaries that are more likely to have contact with humans (Whatmore et al. 2008). In South America, *Brucella* spp. exposure was found in odontocetes and pinnipeds in the Southeastern Pacific Ocean (Van Bresse et al. 2001, Jankowski et al. 2015). In addition, *Brucella* spp. was detected by PCR in a newborn female southern right whale (*Eubalaena australis*) stranded in Argentina in 2003 (McAloose et al. 2016) and more recently in Clymene dolphins (*Stenella clymene*) stranded in Brazil (Sánchez-Sarmiento et al. 2017, Attademo et al. 2018).

Another relevant zoonotic pathogen in marine mammals is *Leptospira* spp., typically associated with acute renal failure, hemorrhagic disease and death in pinnipeds. Affected species include: northern elephant seals (*Mirounga angustirostris*) (Colegrove et al. 2005), northern fur seals (*Callorhinus ursinus*) (Smith et al. 1977), harbor seals (*Phoca vitulina richardsii*) (Stamper et al. 1998) and California sea lions (*Zalophus californianus*) in which leptospirosis is endemic and

has caused high mortality epizootics (Vedros et al. 1971, Gulland et al. 1996). Recently, *Leptospira* spp. was detected by PCR in a South American pinniped, the Galapagos sea lion (*Zalophus worlebaeki*) (Denkinger et al. 2017), and a mysticete cetacean, *E. australis* from Argentina (Loffler et al. 2015). Antibodies against *Leptospira* spp. have also been detected in captive South American sirenians in South America: west Indian manatees (*Trichechus manatus*) from Brazil (Attademo 2014) and Amazonian manatees (*T. inunguis*) from Brazilian and Peruvian Amazon (Mathews et al. 2012, Delgado et al. 2015).

Aquatic mammals can act as sentinels of the presence of pathogens in the environment (Dierauf et al. 2001), some of them considered emerging and resurging, as a result of environmental distress syndrome (Bossart 2007). Little is known about the occurrence and exposure to *Brucella* spp. and *Leptospira* spp. in aquatic mammals in the Southwestern Atlantic Ocean and the Amazon basin, Brazil, a fact that summed to their zoonotic potential and supports the necessity of continuous monitoring. Thus, the aim of this study was to evaluate the exposure to both pathogens in odontocetes and manatees from Brazil.

5.3 MATERIALS AND METHODS

5.3.1 Samples

Serum samples from 84 individuals (63 cetaceans and 21 manatees) was tested. Riverine dolphins (Iniidae): Amazon river dolphin (*Inia geoffrensis*, n=16) and Bolivian river dolphin (*Inia boliviensis*, n=21) were captured, sampled and released during field expeditions performed in 2015 in Negro River (State of Amazonas) and Guaporé River (State of Rondonia). Marine cetaceans (n=26) stranded alive or dead in the State of Ceará from 2011 to 2017: melon-headed whale (*Peponocephala electra*, n=9), Clymene dolphin (*Stenella clymene*, n=4), short-finned pilot whale (*Globicephala macrorhynchus*, n=3), Guiana dolphin (*Sotalia guianensis*, n=2), pigmy killer whale (*Feresa attenuata*, n=2), Risso's dolphin (*Grampus griseus*, n=1), Atlantic bottlenose dolphin (*Tursiops truncatus*, n=1) from family Delphinidae; and dwarf sperm whale (*Kogia sima*, n=3) and pigmy sperm whale (*Kogia breviceps*, n=1) from family Kogiidae. Samples from *Trichechus manatus* (n=21) were collected from dead

individuals stranded in the coast of Ceará and from animals kept in rehabilitation after being rescued in the states of Ceará and Rio Grande do Norte by “Aquasis” and “Projeto Cetáceos da Costa Branca” (PCCB/UERN), respectively. Location of individuals tested in regard to positive and negative species is shown in Figure 1.

Stranding data and location, sex and total body length (TBL, from tip of rostrum to tail notch) in meters were recorded (Table 1 and 2). TBL was used to classify each specimen into age class, according to references for riverine dolphins (Best & da Silva, 1993) and remaining species (Rosas & Monteiro-Filho 2002, Reidenberg & Laitman 2008). Considering that some manatees rescued alive have spent considerable time in captivity at the Marine Mammal Rescue Center (CRMM/Aquasis), serological tests in this group were performed twice: one at arrival and another around a year in rehabilitation or during necropsy.

5.3.2 Serological tests

Brucella spp. antibodies were screened via the Rose Bengal Test (RBT), using 8% *B. abortus* 1119-3 whole cells suspension buffered in acid pH (3.65) as antigen (Instituto Biológico, São Paulo, Brazil) (Alton et al. 1998, Brasil 2006). Sera and antigen were allowed to equilibrate at room temperature, and 30µl of the serum was mixed with an equal volume of antigen for 4 min. The sample was considered positive when visible agglutination was observed.

Secondly, samples were tested with commercial competitive enzyme-linked immunosorbent assay (c-ELISA) kit (INGENASA®), using monoclonal antibody-Mab specific of epitope C of the lipopolysaccharide (LPS) antigen from *Brucella abortus* as antigen, following manufacturer's instructions. The c-ELISA kit has diagnostic sensitivity of 98% for bovine and 99% for ovine, caprine and swine with a diagnostic specificity of 99.9%. The analytic specificity is 100% for negative reference serum and 97% for animals infected with *Yersinia enterocolitica*.

The c-ELISA kit was validated at 1/10 dilution for cetaceans and manatees, using bovine positive and negative controls (included in the kit) and serum from PCR positive cetacean stranded in the Brazilian coast (Sánchez-Sarmiento et al. 2017). According to manufacturer, the threshold for determining seropositivity was $\geq 40\%$, with antibody titers calculated with the following formula: percentage inhibition (PI) $= 100 \times \{1 - (\text{DO sample} / \text{DO negative control})\}$. The positive/negative threshold for

these assays was in accordance with those used for testing *Brucella* spp. in several terrestrial species and similar to studies in cetacean and pinnipeds, which considered ELISA positive individuals at $\geq 30\%$ PI (Nielsen et al. 1996, Neimanis et al. 2008). Cetaceans with positive c-ELISA (upon availability {n=8; #40, #41, #55, #58, #60-63, Table 1}) were further tested by Serum Agglutination Test (SAT), used for diagnostics in bovines according the Brazilian national program for control and eradication of brucellosis (Brasil 2006). The threshold for determining positivity was 1/100. Serological tests for *Brucella* spp. were interpreted in parallel considering positive results in any of the tests as indicative of exposed or seropositive individuals.

For *Leptospira* spp. antibodies detection, the Microscopic Agglutination Test (MAT) microtechnique was used (Cole et al. 1973). Buffered Sorensen's saline solution (pH 7.6) diluted serum (1:50) was tested with a battery of live serovars (1:2): Australis, Autumnalis, Bataviae, Bratislava, Butembo, Canicola, Castellonis, Copenhageni, Cynopteri, Grippotyphosa, Guaicura, Hardjo (hardjoprajitno), Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Pomona, Pyrogenes, Sentot, Shermani, Tarassovi and Whitcombi. The sera used as positive controls were produced at the Department of Preventive Veterinary Medicine and Animal Health, FMVZ, USP. Leptospiral cultures were maintained in Ellinghausen, McCullough, Johnson and Harris - EMJH media (Becton-Dickinson Biosciences/DIFCO/Detroit/USA) modified (Alves et al. 1996) with 4 to 14 growth days, density of 100-200 microorganisms per microscopic field and visualized at 400x. Sera presenting agglutination equal to or above 50% in comparison to the positive control were considered positive (Myers 1985).

5.4 RESULTS

Brucella spp. seropositivity frequency of 15.9% (10/63) was found in cetaceans using c-ELISA, all of them presenting high %PI (51.4% to 97%). *Kogia sima*, *Kogia breviceps*, *Inia geoffrensis*, *Inia boliviensis*, *Sotalia guianensis* and *Grampus griseus* species were negative, whereas 100% (3/3) *Globicephala macrorhynchus*, 100% (1/1) *Tursiops truncatus*, 75% (3/4) *Stenella clymene*, 50% (1/2) *Feresa attenuata* and 22.2% (2/9) *Peponocephala electra* were c-ELISA positive. All c-ELISA positive cases were from the state of Ceará State (CE), Brazil.

From c-ELISA positives, 70% (7/10) were adults, 10% (1/10) juveniles and 20% (2/10) calves. Three c-ELISA positives, representing 4.8% (3/63) of cetaceans tested were positive for RBT {two *S. clymene* (#39 and #40) and one *G. macrorhynchus* (#61)}. Only one *S. clymene* (#40) was positive for SAT with titer of 100.

On the other hand, all *Trichechus manatus* individuals were negative for *Brucella* spp. via RBT and c-ELISA. All cetaceans and manatees showed no positivity for the MAT, ratifying no evidence of *Leptospira* spp. antibodies in sampled individuals. Serological results are shown on table 1 for cetaceans and table 2 for manatees, with location of positive individuals presented in Figure 1.

Figure 1. A. Location of individuals sampled in this study **B.** Serological results in regard to the different species tested in the states of Ceará and Rio Grande do Norte; positives in red; negatives in green. AM= State of Amazonas; RO= State of Rondonia; CE= State of Ceará; RN= State of Rio Grande do Norte.

Table 1. Individual data and results obtained in the serological tests for *Brucella* spp., and *Leptospira* spp. for the different cetacean species tested

Id	Species	Sex	Age class	TBL (m)	Sampling date	Location	Geographical coordinates	RBT	c-ELISA (%PI)	SAT (titer)	MAT
1	<i>Inia geoffrensis</i>	M	A	2.05	02/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
2	<i>Inia geoffrensis</i>	M	J	1.94	02/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
3	<i>Inia geoffrensis</i>	M	A	2.12	02/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
4	<i>Inia geoffrensis</i>	M	J	1.67	02/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
5	<i>Inia geoffrensis</i>	M	A	2.01	03/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
6	<i>Inia geoffrensis</i>	M	J	1.79	03/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
7	<i>Inia geoffrensis</i>	M	A	2.24	03/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
8	<i>Inia geoffrensis</i>	M	A	2.14	04/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
9	<i>Inia geoffrensis</i>	M	J	1.49	05/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
10	<i>Inia geoffrensis</i>	M	J	1.62	05/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
11	<i>Inia geoffrensis</i>	M	A	2.07	05/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
12	<i>Inia geoffrensis</i>	M	J	1.86	05/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
13	<i>Inia geoffrensis</i>	M	C	1.49	05/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
14	<i>Inia geoffrensis</i>	M	A	2.01	05/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
15	<i>Inia geoffrensis</i>	M	A	2.04	06/02/15	Praia do Davi, Rio Negro/AM	3°05'35.4"S, 60°28'31.0"W	-	-	NA	-
16	<i>Inia geoffrensis</i>	M	C	1.18	03/12/15	Cachoeira do Castanho, Rio Negro/AM	03°04' 10"S, 060°18' 41"W	-	-	NA	-
17	<i>Inia boliviensis</i>	M	A	2.10	06/02/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
18	<i>Inia boliviensis</i>	M	A	1.97	22/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
19	<i>Inia boliviensis</i>	M	C	1.00	22/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
20	<i>Inia boliviensis</i>	F	A	1.99	22/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
21	<i>Inia boliviensis</i>	M	A	2.09	22/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
22	<i>Inia boliviensis</i>	F	J	1.57	22/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
23	<i>Inia boliviensis</i>	M	J	1.69	22/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
24	<i>Inia boliviensis</i>	F	A	2.15	23/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
25	<i>Inia boliviensis</i>	M	C	1.33	23/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
26	<i>Inia boliviensis</i>	F	A	2.05	23/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
27	<i>Inia boliviensis</i>	M	A	1.93	23/09/15	Baía Grande, Rio Guaporé/RO	12°29' 32,8"S, 064°03' 26,1"W	-	-	NA	-
28	<i>Inia boliviensis</i>	M	A	2.05	23/09/15	Contra Fiado, Rio Guaporé/RO	12°28' 37,8"S, 064°07' 40,4"W	-	-	NA	-
29	<i>Inia boliviensis</i>	M	A	2.00	24/09/15	Contra Fiado, Rio Guaporé/RO	12°28' 37,8"S, 064°07' 40,4"W	-	-	NA	-
30	<i>Inia boliviensis</i>	M	J	1.77	24/09/15	Contra Fiado, Rio Guaporé/RO	12°28' 37,8"S, 064°07' 40,4"W	-	-	NA	-
31	<i>Inia boliviensis</i>	F	J	1.47	24/09/15	Contra Fiado, Rio Guaporé/RO	12°28' 37,8"S, 064°07' 40,4"W	-	-	NA	-
32	<i>Inia boliviensis</i>	M	J	1.74	24/09/15	Baía Queimada, Rio Guaporé/RO	12°27' 37.87"S, 064°17' 20,95"W	-	-	NA	-
33	<i>Inia boliviensis</i>	M	J	1.86	25/09/15	Porto França, Rio Guaporé/RO	12°27' 40,6"S, 064°17' 20,0"W	-	-	NA	-
34	<i>Inia boliviensis</i>	M	A	2.22	26/09/15	Contra Fiado, Rio Guaporé/RO	12°28' 37,8"S, 064°07' 40,4"W	-	-	NA	-
35	<i>Inia boliviensis</i>	F	A	1.94	27/09/15	Contra Fiado, Rio Guaporé/RO	12°28' 37,8"S, 064°07' 40,4"W	-	-	NA	-
36	<i>Inia boliviensis</i>	M	A	1.98	27/09/15	Contra Fiado, Rio Guaporé/RO	12°28' 37,8"S, 064°07' 40,4"W	-	-	NA	-

37	<i>Inia boliviensis</i>	M	A	2.11	27/09/15	Contra Fiado, Rio Guaporé/RO	12°28' 37,8"S, 064°07' 40,4"W	-	-	NA	-
38	<i>Stenella clymene</i>	M	A	2.01	25/03/12	Jericoacoara, Jijoca de Jericoacoara/CE	02°47'57"S, 40°31'10"W	-	-	NA	-
39	<i>Stenella clymene</i>	M	A	1.75	10/03/12	Parajuru, Beberibe/CE	04°23'28.7"S, 37°49'44.5"W	+	+ (95.1%)	NA	-
40	<i>Stenella clymene</i>	F	A [§]	1.76	25/05/16	Praia de Águas Belas, Cascavel/CE	04°03'31.3"S, 38°10'50.5"W	+	+ (96.4%)	+ (100)	-
41	<i>Stenella clymene</i>	M	A [§]	1.70	10/01/16	Praia do Japão, Aquiraz/CE	03°53'34"S, 38°21'10.5"W	-	+ (95.4%)	-	-
42	<i>Sotalia guianensis</i>	F	C	1.02	20/06/11	Praia do Diogo, Beberibe/CE	04°11'45.1"S, 38°03'59.7"W	-	-	NA	-
43	<i>Sotalia guianensis</i>	F	C	0.78	09/11/16	Porto das dunas, Aquiraz/CE	03°50'25.6"S, 38°23'27.8"W	-	-	NA	-
44	<i>Kogia breviceps</i>	M	C	1.45	29/05/12	Praia da Baleia, Itapipoca/CE	03°08'39.3"S, 039°27'39.8"W	-	-	NA	-
45	<i>Kogia sima</i>	M	A	2.22	02/08/12	Moitas, Icaraí de Amontada/CE	03°01'07.1"S, 39°39'45.9"W	-	-	NA	-
46	<i>Kogia sima</i>	F	A	2.20	04/06/13	Barra do Ceará, Fortaleza/CE	03°41'35.5"S, 38°35'07,1"W	-	-	NA	-
47	<i>Kogia sima</i>	M	C	1.18	04/06/13	Barra do Ceará, Fortaleza/CE	03°41'35.5"S, 38°35'07,1"W	-	-	NA	-
48	<i>Grampus griseus</i>	F	A	2.54	20/06/12	Retirinho, Aracati/CE	04°38'39.8"S, 37°32'15.4"W	-	-	NA	-
49	<i>Peponocephala electra</i>	F	J	2.28	09/07/11	Emboaca, Trairi/CE	03°12'26.6"S, 39°18'18.4"W	-	-	NA	-
50	<i>Peponocephala electra</i>	M	C	1.10	05/09/12	Canoa Quebrada, Aracati/CE	04°31'51.5"S, 37°41'27.6"W	-	-	NA	-
51	<i>Peponocephala electra</i>	M	A	2.39	01/10/12	Riacho Doce, Cruz/CE	02°48'11.1"S, 40°27'22.2"W	-	-	NA	-
52	<i>Peponocephala electra</i>	M	J	2.03	28/03/13	Águas Belas, Cascavel/CE	04°03'28.3"S, 38°11'48"W	-	-	NA	-
53	<i>Peponocephala electra</i>	F	A	2.30	05/11/14	Praia de Caetanós, Amontada/CE	03°01'30"S, 39°37'21.2"W	-	-	NA	-
54	<i>Peponocephala electra</i>	M	A	2.40	07/11/14	Praia das Fontes, Beberibe/CE	04°12'59.5"S, 38°02'47.1"W	-	+ (93.9%)	NA	-
55	<i>Peponocephala electra</i>	F	A	2.46	07/11/14	Praia das Fontes, Beberibe/CE	04°12'59.5"S, 38°02'47.1"W	-	+ (77.6%)	-	-
56	<i>Peponocephala electra</i>	M	C	1.21	30/12/14	Praia da Cofeco, Fortaleza/CE	03°48'28.6"S, 38°24'41.2"W	-	-	NA	-
57	<i>Peponocephala electra</i>	F	A	2.25	11/02/17	Lagoinha, Paraipaba/CE	03°18'47.8"S, 39°10'29.9"W	-	-	NA	-
58	<i>Tursiops truncatus</i>	F	A	2.71	06/07/15	Abreulândia, Fortaleza/CE	03°48'20.1"S, 38°24'45.6"W	-	+ (95.0%)	-	-
59	<i>Feresa attenuata</i>	F	C	1.13	04/05/16	Praia do Preá, Cruz/CE	02°48'46.4"S, 40°24'50.5"W	-	-	NA	-
60	<i>Feresa attenuata</i>	M	A	2.13	14/04/15	Praia da Taíba, São Gonçalo do Amarante/CE	03°30'22.0" S, 38°54'19.0"W	-	+ (51.4%)	-	-
61	<i>Globicephala macrorhynchus</i>	M	J	2.90	11/01/12	Praia do Preá, Cruz/CE	02°48'44.0"S, 40°24'54.9"W	+	+ (97%)	-	-
62	<i>Globicephala macrorhynchus</i>	F	C	1.57	09/07/16	Praia de Vila nova, Icapuí/CE	04°39'56.7"S, 37°25'40.3"W	-	+ (85.7%)	-	-
63	<i>Globicephala macrorhynchus</i>	F	C	1.30	25/01/17	Praia da Tabuba, Caucaia/CE	03°38'24.8"S, 38°42'00.1"W	-	+ (96.3%)	-	-

M=Male; F=Female; A=Adult; J=Juvenile; C=Calf; [§] sexual maturity of the individual verified by histology; TBL= Total body length; NI=Not informed; AM= State of Amazonas; RO= State of Rondonia; CE= State of Ceará; RBT= Rose Bengal test, SAT= Serum Agglutination Test; MAT= Microscopic Agglutination Test; in TBL m=meters.

Table 2. Individual data and results obtained in the serological tests for *Brucella* spp., and *Leptospira* spp. for West Indian manatees (*Trichechus manatus*).

Id	Sex	Age class	TBL (m)	Stranding date	Location	Geographical coordinates	Sampling date	RBT	c-ELISA	MAT
1	F	1y, 7m	2.02	13/08/13	Praia do Gado Bravo, Tibau/RN	04°52'37.7"S, 37°13'30"W	26/03/15	-	-	-
	F	2y, 3m	2.34		CRMM/Aquasis	-	19/11/15	-	-	-
2	F	1m	1.27	13/01/15	Morro Pintado, Areia Branca/RN	04°58'18"S, 37°11'18"W	19/02/15	-	-	-
3	F	15d	1.2	24/03/15	Praia de Alagamar, Grossos/RN	04°54'34.4"S, 37°11'42.3"W	07/04/15	-	-	-
4	M	<2y	2.01	23/09/11	Ponta Grossa, Icapuí/CE	04°37'59.9"S, 37°30'45.2"W	23/09/11	-	-	-
5	M	1m	1.08	23/03/13	Praia de Manibu, Icapuí/CE	04°48'23.4"S, 37°15'49.1"W	22/04/13	-	-	-
6	F	<3y	2.41	21/09/13	Praia de Retirinho, Aracati/CE	04°38'39.9"S, 37°32'26.7"W	21/09/13	-	-	-
7	M	1y, 3m	2.05	15/12/13	Pontal do Maceió, Fortim/CE	04°24'04.9"S, 37°46'52.9"W	26/03/15	-	-	-
	M	1y, 11m	2.19		CRMM/Aquasis	-	19/11/15	-	-	-
8	M	1y	1.79	15/10/14	Praia das Agulhas, Fortim/CE	04°23'13.4"S, 37°49'50"W	19/10/15	-	-	-
	M	1y, 1m	1.84		CRMM/Aquasis	-	19/11/15	-	-	-
9	M	1y, 1m	1.93	15/10/14	Praia das Agulhas, Fortim/CE	04°23'13.4"S, 37°49'50"W	19/11/15	-	-	-
10	M	2m	1.2	28/12/14	Rio Jaguaribe, Fortim/CE	04°25'30"S, 37°46'29"W	19/02/15	-	-	-
	M	11m	1.71		CRMM/Aquasis	-	19/11/15	-	-	-
11	F	1d	1.15	20/02/15	Praia de Manibu, Icapuí/CE	04°49'30.8"S, 37°15'22.3"W	20/02/15	-	-	-
12	M	5d	1.35	05/03/15	Praia de Ariós, Beberibe/CE	04°17'18.3"S, 37°57'52.6"W	10/03/15	-	-	-
	M	3m	1.54		CRMM/Aquasis	-	30/06/15	-	-	-
13	M	<10y	3	03/10/15	Ponta Grossa, Icapuí/CE	04°37'42.6"S, 37°30'25"W	03/10/15	-	-	-
14	NI	8m	1.6	23/04/15	Praia do Rosado, Porto do Mangue/CE	05°01'34.7"S, 36°48'41.6"W	08/12/15	-	-	-
	NI	1y	1.91		CRMM/Aquasis	-	12/04/16	-	-	-
15	M	1d	1.36	27/06/16	Praia dos Estevão, Aracati/CE	04°27'07.6"S, 37°44'56.5"W	28/06/16	-	-	-
16	F	1m	1.33	09/10/16	Praia das Agulhas, Fortim/CE	04°22'51"S, 37°50'37"W	10/10/16	-	-	-
17	M	1m	1.17	09/10/16	Praia dos Anjos, Beberibe/CE	04°16'03"S, 37°59'00"W	10/10/16	-	-	-
18	F	1m	1.42	10/11/16	Praia de Ariós, Beberibe/CE	04°12'52.5"S, 38°02'54.7"W	08/12/16	-	-	-
19	M	1d	1.31	28/12/16	Prainha do Canto Verde, Beberibe/CE	04°19'14.2"S, 37°55'40.7"W	28/12/16	-	-	-
20	F	1d	1.22	30/12/16	Praia de Fontainhas, Aracati, CE	04°37'01.7"S, 37°36'10.5"W	31/12/16	-	-	-
21	F	45d	1.31	25/03/17	Mulheres de Areia, Itarema/CE	02°52'49"S, 39°54'35"W	26/03/17	-	-	-

RBT= Rose Bengal test; MAT= Microscopic Agglutination Test; TBL= Total body length; y=year, m=months, d=days; M=Male, F=Female, NI=Not informed; RN= State of Rio Grande do Norte, CE= State of Ceará; in TBL m=meters.

5.5 DISCUSSION

Our results obtained with the described indirect tests suggest exposure to *Brucella* spp. of individuals of five odontocetes species: *Stenella clymene*, *Globicephala macrorhynchus*, *Feresa attenuata*, *Peponocephala electra* and *Tursiops truncatus* from the Southwestern Atlantic Ocean, Brazil, expanding the number of known *Brucella* seropositive aquatic mammal species. While the frequency of positive individuals was high for *G. macrorhynchus*, *T. truncatus* and *S. clymene* (75-100%), it was lower for *F. attenuata* and *P. electra* (22.2-50%). Considering the above species, the occurrence of anti-*Brucella* antibodies has previously been reported at frequencies of 100% (1/1) in *P. electra* by c-ELISA; 33% (1/3) and 27.2% (15/55) in *F. attenuata* by c-ELISA, and 17.2% (60/349) in *T. truncatus* taking in account several serological methods (Hernández-Mora et al. 2009, 2013), however it has not been previously detected in *G. macrorhynchus*.

The seropositive cetaceans species in this study are known to have oceanic habits, while riverine dolphins and manatees were negative, corroborating literature (Hernández-Mora et al. 2013). A previous serological study with 67 *Inia geoffrensis* from Tefé, state of Amazonas (Brazil) did not detect anti-*Brucella* antibodies via RBT, 2-Mercaptoethanol (2-M) and fluorescent polarization-FP (Rocca 2014). In captive *Trichechus manatus* from northeastern Brazil, 10.4% (6/58) were found positive via RBT, however the confirmatory test (complement fixation test-CFT) was negative for all individuals (Attademo 2014). In the state of Ceará, the region of seropositive individuals, *Brucella* spp. was confirmed by PCR in one *S. clymene* (#39, in this study) (Sánchez-Sarmiento et al. 2017). It was additionally detected in the same species in the state of Alagoas, also in northeastern Brazil, suggesting the circulation of bacteria in the environment. However, more studies are necessary to better understand the dynamics of brucellosis in this geographic region.

As our sample size for each species was limited, epidemiological differences intra or inter species could not be explored. The number of specimens of each species was limited by the occurrence of stranded animals, which were opportunistically sampled. Although significant differences regarding age class could not be pointed out, 30% of c-ELISA positives were young or calves. It has been suggested that seropositivity titers in calves and young animals may be related to

passive maternal transference, rather than seroconversion in direct response to *Brucella* infection (Jepson et al. 1997, Zarnke et al. 1999). On the other hand, in hooded seals (*Cystophora cristata*), seropositivity decreases with age and post-weaning environmental exposure (through diet) and subsequent infection clearance had been hypothesized (Nymo, Tryland, et al. 2013). In literature, differences among species have been related to different social structure and schooling behavior that may increase transmission (Van Bressem et al. 2001). In some marine mammal species, *Brucella* infection may occur occasionally, not persist, coming from other sources (such as predators), in which some species could be more susceptible than others (Nielsen et al. 1996, Van Bressem et al. 2001). Indeed, *Brucella* infection could lead to neurobrucellosis and cause *Stenella coeruleoalba* to strand (Hernández-Mora et al. 2008) while in other species, it has not always been linked to the disease. The influence of some parameters on the immunological response of individuals against *Brucella* infection has been also described, e.g., the disease dynamics was influenced by increased infection frequency with degraded environmental conditions (Colegrove et al. 2016).

It could be inferred that c-ELISA was more sensitive than RBT, as previously observed. Both methods are considered suitable for diagnosis of *Brucella* spp. exposure (Matope et al. 2011, OIE 2012). Moreover, c-ELISA is considered more sensitive than other techniques like serum (sFPA) and blood (BFPA) fluorescence polarization assay in cattle (Matope et al. 2011). In classical RBT false negative reactions can occur (rarely), mostly due to prozoning (Nielsen 2002, OIE 2012) or overall low avidity or reduced titers of agglutinating antibodies (Hernández-Mora et al. 2009) once agglutination tests detect mainly IgM (Godfroid et al. 2010). This must be specially considered when applied in species for which RBT was not designed. In ELISA binding patterns to common-C epitopes can be heterogeneous or much reduced/negative, depending on the *Brucella* strain tested (Baucheron et al. 2002) and this should be considered when interpreting results. This could be an explanation of differences found between c-ELISA and RBT positives in this study (15.9% versus 4.8%, respectively), while arises the possibility of further positives cetaceans not detected by c-ELISA in this study. A possible explanation of c-ELISA positives that were SAT negatives could be due to the following: (1) this test was initially developed for bovines and immunoglobulins were not appropriately detected in cetaceans; (2)

related to kinetics of the immune response, for example antibody concentrations in recent infection were too low to be detected (Maratea et al. 2003).

c-ELISA could have higher specificity compared to other methods like indirect ELISA (i-ELISA) and CFT (Muñoz et al. 2005). Its specificity is related to the displacement of low-avidity antibodies by the competing Mab against LPS-C epitope (Hernández-Mora et al. 2009). The selection of a Mab with higher affinity than cross-reacting antibody results in the capacity of eliminating some but not all false positive serological Reactions (FPSR) due to cross-reacting bacteria as *Yersinia enterocolitica* O:9-Y09 (Muñoz et al. 2005). Due to this possibility, studies in bovines argue that c-ELISA allow the detection of *Brucella* spp. specific antibodies eliminating cross reactions observed with *Y. enterocolitica* (Nielsen 1990). Additionally, it is considered a suitable choice for marine mammal species, being widely used in several serological studies on cetaceans and pinnipeds (Nielsen et al. 1996, 2001, 2005, Jepson et al. 1997, Van Bresseem et al. 2001, Dawson, Perrett, et al. 2008). Some authors consider that the occurrence of FPSR through nonspecific binding with antibodies against *Y. enterocolitica* O:9, could be less probable in marine mammals once infection caused by this microorganism has not yet been reported (Tryland et al. 1999); however, information about it is scarce. A previous study in marine mammals seropositive to *Brucella* spp. did not detect antibodies against *Y. enterocolitica* outer membrane antigens or isolated the bacteria (Tryland et al. 1999).

The performance of serological methods is in general, dependent on cross-reactivity between immunoglobulins, type of antigen employed and species tested. In order to improve serological diagnosis of *Brucella* spp. exposure in cetaceans in the last years, some studies have been developed mainly based on improvement of ELISAs, e.g., an indirect (iELISA) that recognizes immunoglobulins G (IgGs) from odontocetes as a single group (Hernández-Mora et al. 2009); a A/G iELISA protein (Nymo, Godfroid, et al. 2013) based on the complementary binding patterns among proteins A and G (Eliasson et al. 1988); and c-ELISA using whole cell antigen from *Brucella* spp. isolated from harbor seal (Meegan et al. 2010). In iELISA using *in-house* cetacean IgG, antibodies against IgG of family Delphinidae presented reduced reactivity with other cetacean families, correlating with evolutionary distance (Nollens et al. 2008). c-ELISA using marine mammal isolate as antigen has shown to be more sensitive than others using *Brucella abortus*, suggesting that whole cell assays may

be useful for antibody detection in marine mammals at the cost of specificity (Meegan et al. 2010).

Differing from this study, evidence of *Leptospira* has been previously found in cetaceans and manatees. In the Southern Atlantic Ocean, *Leptospira* spp. was isolated from a kidney sample of *Eubalaena australis* in Argentina (Löffler et al. 2015), and seropositivity was found in captive manatees (*Trichechus inunguis*) of the Brazilian Amazon at elevated rates; 31.1% (23/74) (Mathews et al. 2012) and in captive *T. manatus* from northeastern Brazil; 9.2% (5/54) (Attademo 2014). Titers against different *Leptospira* serovars have also been found in Western Indian manatees in Belize (Sulzner et al. 2012). Climatic and ecological factors are relevant to the occurrence of *Leptospira* epidemic outbreaks in Latin America (Petrakovsky et al. 2014) as they may influence pathogen or host dynamics. Seasonal association with seroprevalence to leptospirosis (higher during the dry season than the rainy season) has been detected in manatees (Sulzner et al. 2012). In that study, the effect of season on the incidence of infection was not evaluated but water levels and associated shifts in salt and fresh water availability may have seasonal influence. Considering these data and the potential of epizootics, a routine serologic and pathologic screening is recommended (Sulzner et al. 2012).

Considering studies that correlate indirect (serological) with direct methods for *Brucella* spp. infection (either by culture, molecular, immunologic and electron microscopy) (Maratea et al. 2003, Dawson, Perrett, et al. 2008, Hernández-Mora et al. 2009) and an increasing host range and location of *Brucella* spp. among cetaceans reported in literature in the last years, including in Northeastern Brazil (Sánchez-Sarmiento et al. 2017, Attademo et al. 2018), additional studies should be carried out to investigate if *Brucella* spp. was present in any tissue or involved in the cause of death of these animals. On the other hand, although no evidence of antibodies against *Leptospira* spp. was found, the occurrence of *Leptospira* infection on aquatic mammals on the locations tested and a plausible vulnerability of such species to this pathogen could not be totally ruled out. Thus, continuous monitoring of these pathogens to fully understand their ecology and role in mortality and morbidity in aquatic mammals should be performed.

ACKNOWLEDGMENTS

We would like to acknowledge Nicolle Queiroz-Hazarbassanov, Gisele Oliveira and Antonio de Sousa for the excellent technical assistance; Jacques Delbecque from INGENASA, Madrid, Spain for kindly providing the c-ELISA KIT and Nicolás Céspedes Cardenas for the map design. This research was partially supported by a Small Grant in Aid of Research from the Society for Marine Mammalogy (2015 and 2016). We greatly acknowledge support received from Dr. Vera M. F. da Silva, FIXAM/FAPEAM e UNIVERSAL/CNPq (457725/2014-1) and AMPA (Associação Amigos do Peixe-boi), for collecting Inias samples. The dolphin rescue and necropsy was performed by Aquasis' Projeto Manatí, sponsored by Petrobras. J.L.C.-D. is a recipient of a professorship by Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq (Grant No. 305349/2015-5). This research was approved by Comitê de Ética no Uso de Animais (CEUA No. 3926161213), Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brazil. This study was conducted by A.M.S-S as part of her PhD at Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil, with support from a scholarship from Coordenação de Apoio à Pesquisa de nível superior-CAPEs. A.M.S-S also acknowledge Banco Santander for the scholarship received to carry out the internship abroad.

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6 SEROLOGICAL SCREENING FOR *BRUCELLA* SPP. AND *LEPTOSPIRA* SPP. ANTIBODIES IN SOUTHERN ELEPHANT SEALS (*MIROUNGA LEONINA*) AT ELEPHANT ISLAND, ANTARCTICA.

6.1 ABSTRACT

Brucella spp. and *Leptospira* spp. antibodies were surveyed in 35 Southern Elephant Seals (*Mirounga leonina*) sampled at Elephant Island (South Shetlands Islands 61°05'S 55°20'W), Western Antarctic Peninsula in the austral summer of 2003/04. Rose Bengal Tests and a commercial competitive enzyme-linked immunosorbent assay (c-ELISA) were used to detect *Brucella* spp. exposure while the Microscopic Agglutination Test (MAT) was used with a battery of 22 live serovars to determine anti-*Leptospira* spp. antibodies. We found evidence of *Brucella* spp. exposure in 3/35 (8.6%) of the individuals tested via the c-ELISA displaying high percentage inhibition, similar to other studies in pinnipeds in which *Brucella* spp. antibodies has been determined. The c-ELISA positives were additionally tested via the Serum Agglutination Test and provided negative results. No evidence of *Leptospira* spp. exposure was found by MAT. These results contribute to the knowledge and monitoring of zoonotic pathogens with epizootic potential in Southern Ocean pinnipeds.

Keywords: competitive ELISA; brucellosis; leptospirosis; marine mammals; Phocidae; serology; Southern Ocean.

6.2 INTRODUCTION

Brucella spp. in marine mammals may lead to several pathological processes including orchitis, placentitis, myocarditis and meningitis, particularly in dolphins due to *Brucella ceti* (Dagleish et al. 2008, González-Barrientos et al. 2010). However, the strain recognized to prefer pinniped hosts (Foster et al. 2007), has not been clearly associated with lesions in pinnipeds (Nymo et al. 2011). For example, *Brucella* spp. was detected in 3/59 (5%) of placental tissues of California Sea Lions (*Zalophus californianus*) suffering from abortion and premature parturition on San Miguel Island,

California, but the classical *Brucella*-associated histological lesion, placentitis, was not present on those samples (Goldstein et al. 2009). In Common Seals (*Phoca vitulina*) and Grey Seals (*Halichoerus grypus*) *B. pinnipedialis* has been isolated, and most of the individuals were suffering from verminous pneumonia, but a clear link with *Brucella* infection was not established (Prenger-Berninghoff et al. 2008).

Since *Brucella* spp. was firstly described in pinnipeds in 1994 in Common Seals (Ross et al. 1994), the range of pinniped hosts has increased. *Brucella* spp. antibodies have been detected in at least 14 pinniped species to date (Nymo et al. 2011) including Hooded Seals (*Cystophora cristata*), Ringed Seals (*Phoca hispida*) and Harp Seals (*Phoca groenlandica*), *P. vitulina*, *H. grypus* and Walrus (*Odobenus rosmarus rosmarus*) in the North Atlantic Ocean and the Arctic (Nielsen et al. 1996, 2001, Tryland et al. 1999, 2005).

Brucella infection is considered enzootic in some populations, such as Hooded Seals in Svalbard and Greenland (North Atlantic Ocean; Greenland Sea) (Tryland et al. 2005) and a Weddell Seal (*Leptonychotes weddellii*) population from the Vestfold Hills, East Antarctica (McFarlane 2009) in which the impact of *Brucella* spp. is not clear (McFarlane 2009, Jensen et al. 2013). Additional *Brucella*-exposed Antarctic pinnipeds include Antarctic Fur Seals (*Arctocephalus gazella*) and Weddell Seals from Cape Shirreff and the San Telmo Islets in the South Shetland Islands (Retamal et al. 2000, Blank et al. 2002, Abalos et al. 2009).

Leptospirosis is a related zoonosis that may cause renal and hemorrhagic disease and death in pinnipeds. In California Sea Lions, *Leptospira* spp. infection is endemic with elevated mortality during epizootic events (Vedros et al. 1971, Gulland et al. 1996). *Leptospira* spp. have been detected in Northern Fur Seals (*Callorhinus ursinus*) (Smith et al. 1977), Harbor Seals (*Phoca vitulina richardsii*) (Stamper et al. 1998) and Northern Elephant Seals (*Mirounga angustirostris*) (Colegrove et al. 2005). Species and anti-*Leptospira* spp. antibodies that have been detected in pinnipeds include *L. interrogans* serovar Pomona, Bratislava, Canicola, Hardjo, Icterohemorrhagiae and *L. kirschneri* serovar Grippotyphosa (Smith et al. 1977, Gulland et al. 1996, Stamper et al. 1998, Colegrove et al. 2005, Delaney et al. 2014). However, no evidence of *Leptospira* spp. has been found in pinnipeds elsewhere in the Southern Ocean or in Antarctica to date (Yochem et al. 2009).

As long as anthropogenic disturbances continue to alter the natural environment dramatically, and rapid human and animal translocation occurs

increasing contact between wild and domestic animals, further outbreaks of novel and emergent pathogens in wildlife will occur (Dobson & Foufopoulos 2001, Mörner et al. 2002). Concern exists about the susceptibility of Antarctic wildlife to infectious diseases outbreaks in the future due to its behaviour, an ameliorating climate (regional warming), more intense tourism and other human related activities (Grimaldi et al. 2011).

Annex II of the Protocol on Environmental Protection to the Antarctic Treaty prohibits the introduction of non-native species to Antarctica (Anon 1991) as do the management authorities of sub-Antarctic islands (De Villiers et al. 2006). Despite this, alien species and their propagules continue to be introduced to the Antarctic and sub-Antarctic islands either via anthropogenic or natural means” (Houghton et al. 2016, Gutt 2017) The return to the wild of rehabilitated individuals to original colonies has increased potential for the propagation of invasive (non-native) species into polar regions as do movement by migratory individuals and potential propagation of zoonoses (Pacifici et al. 2015, Pauchard et al. 2016).

Many emerging pathogens have demonstrated a potential to reduce the abundance of threatened and endangered wild animal populations and monitoring and surveillance of these populations may contribute to the knowledge of their epidemiology and may prevent their spread (Dobson & Foufopoulos 2001, Mörner et al. 2002). In light of the importance of monitoring zoonotic pathogens in Antarctic pinnipeds, the aim of this study was to investigate exposure to *Brucella* spp. and *Leptospira* spp. in Southern Elephant Seals (*Mirounga leonina*) at Elephant Island, South Atlantic Ocean in 2003/04.

6.3 MATERIALS AND METHODS

6.3.1 Individuals

A total of 35 *M. leonina* were randomly captured during an expedition in 2003/04 to Elephant Island (South Shetlands Islands 61°13'S 55°23'W), Emilio Goeldi Refuge at Stinker Point, Figure 1. Individuals were chemically immobilized with Zoletil 100® via intramuscular injection in the gluteal region (Baker et al. 1990; Field et al. 2002), measured, weighed and blood samples taken from the extradural vein. The individuals were monitored until full recovery. Elephant seals are sexually

dimorphic, and the individuals' sex and age class was determined based on size, physical characters and direct observation. Upon physical examination, all animals were apparently healthy and in good condition.

Figure 1. Emilio Goeldi Refuge at Stinker Point, Elephant Island (South Shetlands Islands 61°13'S 55°23'W), the study site where our samples were collected. The island sits at the northern tip of the Western Antarctic Peninsula.

6.3.2 Serological tests

For detection of anti-*Brucella* spp. antibodies, we used two methods: (1) Rose Bengal stained *B. abortus* 1119-3 killed cells suspended in acid buffer (3.65 pH) (Rose Bengal test-RBT) (Instituto Biológico, São Paulo, Brazil) (Alton et al. 1998, Brasil 2006) and, (2) a competitive enzyme-linked immunosorbent assay (c-ELISA) commercial kit (INGENASA®), using a Monoclonal antibody-Mab specific of the epitope C of the LPS from *B. abortus* as antigen.

The c-ELISA kit was performed following manufacturer instructions and threshold for determining seropositivity as $\geq 40\%$. Antibody titers were calculated as percentage inhibition (PI) = $100 \times \{1 - (\text{DO sample} / \text{DO negative control})\}$. This approach is in accord with previous studies in pinnipeds and domestic animals

(Nielsen et al. 1996). Positive samples (n=3) were additionally tested by the Serum Agglutination Test (SAT) (Brasil 2006). We used the Microscopic Agglutination Test (MAT) for the survey of *Leptospira* spp. exposure using buffered Sorensen's saline solution (ph 7.6) diluted serum (1:50) (Cole et al. 1973), and a battery of 22 live serovars (1:2); Australis, Autumnalis, Bataviae, Bratislava, Butembo, Canicola, Castellonis, Copenhageni, Cynopteri, Grippotyphosa, Guaicura, Hardjo (hardjoprajitno), Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Pomona, Pyrogenes, Sentot, Shermani, Tarassovi and Whitcombi.

6.4 RESULTS

A total of 3 of the 35 *M. leonina* sampled (8.6%) were c-ELISA positive, with %PI of (86.6%, 70.4%, 48.8%), higher than the positive threshold ($\geq 40\%$). The positives included two pups, one male and one female, which displayed the highest %PIs and one adult female, displaying the lower %PI, respectively (Table 1.).

All additional tests for detecting *Brucella* spp. antibodies (RBT and SAT) and those directed for *Leptospira* spp. antibodies (MAT) against the 22 live serovars were negative.

Table 1. Data of *Mirounga leonina* sampled at Elephant Island in 2003/04, and results obtained in the different tests for *Brucella* spp., and *Leptospira* spp. antibodies detection.

#	Sex	Age group	Sampling date	RBT	c-ELISA (PI)	SAT	MAT
1	F	22/24d	06/12/2003	-	-	-	-
2	F	P	01/12/2003	-	+(70.4%)	-	-
3	F	P	01/12/2003	-	-	-	-
4	F	P	02/12/2003	-	-	-	-
5	M	P	02/12/2003	-	+(86.6%)	-	-
6	M	P	02/12/2003	-	-	-	-
7	M	P	02/12/2003	-	-	-	-
8	F	22/24d	04/12/2003	-	-	-	-
9	F	P	04/12/2003	-	-	-	-
10	M	P	04/12/2003	-	-	-	-
11	M	P	04/12/2003	-	-	-	-
12	M	P	04/12/2003	-	-	-	-
13	M	P	06/12/2003	-	-	-	-
14	F	P	06/12/2003	-	-	-	-
15	M	1-2y	06/12/2003	-	-	-	-
16	M	P	08/12/2003	-	-	-	-
17	F	P	08/12/2003	-	-	-	-
18	F	A	08/12/2003	-	-	-	-
19	F	J	13/12/2003	-	-	-	-
20	F	A	13/12/2003	-	-	-	-
21	M	>3y	17/12/2003	-	-	-	-
22	F	A	21/12/2003	-	-	-	-
23	F	A	21/12/2003	-	-	-	-
24	F	A	23/12/2003	-	-	-	-
25	F	A	23/12/2003	-	-	-	-
26	F	A	25/12/2003	-	-	-	-
27	F	A	25/12/2003	-	-	-	-
28	M	>3y	26/12/2003	-	-	-	-
29	M	A	26/12/2003	-	-	-	-
30	F	A	29/12/2003	-	+(48.8%)	-	-
31	F	A	30/12/2003	-	-	-	-
32	F	A	02/01/2004	-	-	-	-
33	F	A	02/01/2004	-	-	-	-
34	F	A	02/01/2004	-	-	-	-
35	F	A	03/01/2004	-	-	-	-

M=Male; F=Female; A=Adult; J=Juvenile; P=Pup; y=year; d=days. PI= percentage inhibition.

6.5 DISCUSSION

As previously recognised, c-ELISA was the most sensitive test by detecting the positive samples in this study (Blank et al. 2002, McFarlane 2009). In light of the three c-ELISA positives in this study, the low sensitivity of RBT and SAT in marine mammals may explain the previously reported negative results.

Studies in domestic animals have discussed the possibility of occurrence of false positive serological reactions-FPSR using c-ELISA due to cross-reacting Gram negative agents like *Yersinia enterocolitica* O:9 (Kittelberger et al. 1998, Muñoz et al. 2005), but see (Nielsen 1990) who believed that this method eliminates those type of cross reactions. There is presently little information regarding *Y. enterocolitica* in marine mammals. A previous attempt to investigate exposure to/and or infection by

this microorganism in marine mammals positive for *Brucella* spp. was negative (Tryland et al. 1999). Cross reactivity against *Y. enterocolitica* was found in polar bears more likely exposed to a terrestrial *Brucella* (*B. suis* biovar 4) (O'Hara et al. 2010), suggesting this cross reactivity may be dependent on presence of marine or terrestrial *Brucella*, respectively. C-ELISA has also shown to be useful and used in several surveys of *Brucella* spp. antibodies in marine mammals, including pinnipeds (Nielsen et al. 1996, 2001, 2005, Retamal et al. 2000, Blank et al. 2002, McFarlane 2009).

When comparing our data with data available in the literature regarding *Brucella* spp. antibodies prevalence in Antarctic pinnipeds (Table 2), the prevalence of positive individuals was relatively low, in agreement with previously reported for *M. leonina* collected during the same season as our sampling (Jensen et al. 2013). In addition, the %PI found in the positive animals in our study (48.8%, 70.4% and 86.6%, respectively) were similar to previous studies with c-ELISA positive Weddell Seals (presenting 49-80% PI) (Retamal et al. 2000, Blank et al. 2002). Our results were higher compared to positive *A. gazella* which presented PI values near the cutoff (32 – 45% PI) (Retamal et al. 2000). A more intense response could suggest advanced disease progression (Morrison et al. 2003). On the other hand, it is interesting to note *Brucella* spp. antibodies prevalence was reported as higher for Antarctic Fur Seals (Retamal et al. 2000) and Weddell Seals (Blank et al. 2002) farther south than this study (Livingston and Elephant Islands, South Shetlands Islands). In a Weddell Seal population from the Vestfold Hills, East Antarctica, *Brucella* spp. exposure was found in almost 100% of individuals tested, and was thus considered endemic (McFarlane 2009). However, in another study with the same species sampled at Hutton Cliffs, also in East Antarctica, the prevalence was much lower (Jensen et al. 2013), suggesting intra-specific and inter-site differences exist in the species.

The serology of an individual is informative about the infection status of the specimen (past or present), however the contemporary presence of the microorganism can be demonstrated only by direct methods such as PCR or by culturing, which is considered to be the gold standard. To date, *B. pinnipedialis* has not been isolated from any Antarctic pinniped species (Jensen et al. 2013). *Brucella* spp. may not be enzootic in this population, and may instead represent a sporadic infection from another infected pinniped. Alternatively, limited epizootics may have

occurred in the areas where seropositive animals were found, situation that has been already reported (Nielsen et al. 1996).

In general, pinniped antibodies' prevalence have been shown to differ between 1% and 100% depending on species and location (Nymo et al. 2011). It is not possible to infer any spatial, temporal and/or epidemiological differences in this study due to the low number of positives. Similar to *M. leonina* sampled at Macquarie island, Pacific Ocean and South Georgia it which differences in prevalence by age were not tested due to the very small number of positive samples, but the positive individuals were a subadult from South Georgia and a pup (<1 yr old) from Macquarie Island. In Weddell Seal a higher frequency of seropositivity was found in adults and subadults than pups (Jensen et al. 2013).

However, some variations regarding prevalence could occur; *Brucella* spp. prevalence in Antarctic Fur Seals was reported as lower (6.2%) in comparison with reports years before in the same region (Abalos et al. 2009). Previous serological studies have shown no differences in *Brucella* spp. antibodies according to sex or age in pinniped species (Tryland et al. 1999, Nielsen et al. 2001). In this regard, in species such as hooded seals, direct transmission from mother to calf during pregnancy or during the first days of life have been identified as a possible route (Tryland et al. 2005).

Table 2. Survey of *Brucella* spp. antibodies in different Antarctic pinnipeds in regard to the method employed.

Species	Location	Age class and sex (n)	Year	RBT	ELISA n (%); %PI	SAT	Reference
<i>Mirounga leonina</i>	Macquarie island, Pacific Ocean (n=37) and South Georgia (n=11)	NI	2000 (MI) and 2005 (SG)	2/48 (4.7%)	2/48 (4.7%) ¹	NT	Jensen et al. 2013
	Cape Shirreff, Livingston Island, Antarctica	NI	NI	NT	0/13	NT	Abalos et al. 2009
	South Shetlands Islands	>3y (2), 1-2y (1), 22-24d (2), P (14), A (15), J (1). Sex = F (23); M (12)	Summer of 2003-2004	0/35	8.6% (48.8-86.6) ³	0/3	This study
<i>Leptonychotes weddellii</i>	Hutton Cliffs, East Antarctica	LAF (24), P (9)	Spring (October-December) 2003-2004	21/32 (65.6%)	8/33; 24.2% (PI) ¹	NT	Jensen et al. 2013
	Davis Station, Vestfold Hills, East Antarctica	J, A. sex NI ^s	November-December 1999	22/35 (62.9%)	55/57 (96.5%) ³	24/35 (68.6%)	McFarlane 2009
	Cape Shirreff and San Telmo Islets, Norwest coast Livingston Island (South Shetland Islands)	SA (1), 1y (3), 2y (3), 3y (4), 4y (1). Sex = F (7), M (4)	January 2001	3/12 (25%)	5/12 (41.7%; 49-80% PI) ² and 4/12 (33.3; 59-76% PI) ³	NT	Blank et al. 2002
	Cape Shirreff and San Telmo islets, Livingston Island (South Shetland Islands)	NI (1)	December 1998-January 1999	0/1	1/1 (100%; 73% PI) ³	NT	Retamal et al. 2000
<i>Arctocephalus gazella</i>	Hutton Cliffs, East Antarctica	A, M	Spring (October-December) 2003-2004	0/21	0/21	NT	Jensen et al. 2013
	Cape Shirreff, Livingston Island, Antarctica	NI	NI	NT	12/52 (6.2%) ³	NT	Abalos et al. 2009
	Cape Shirreff and San Telmo islets, on Livingston Island (South Shetland Islands)	P (3), AM (1), 1yM, LAF (11)	December 1998-January 1999	1/16 ^{&}	5/16 (31.2%; 32 – 45% PI) ³	NT	Retamal et al. 2000

¹i-ELISA; ²Compelisa®; ³c-ELISA; NT=not tested; NI=not informed; P=Pup; J=Juvenile; A=Adult; F=Female; M=Male; LAF=Lactating adult female; [&]weakly positive; y=years; d=days.

Brucella spp. infection have been described in migratory species e.g., Hooded Seals that normally inhabit Arctic waters found stranded in Scotland (Foster et al. 2002). This raises the possibility and associated risks of pathogens' circulation in the environment, in species such as the migratory Southern Elephant Seal, which also migrates beyond its breeding range. The origin of the *Brucella* spp. infection to which Antarctic Fur Seals and Weddell Seals have presented antibodies is unknown, and so why there are some naïve species while others are not (McFarlane et al. 2009). However, it has been suggested that prey species may be a source of *Brucella* species (O'Hara et al. 2010).

We did not find evidence of *Leptospira* spp. exposure in this study, which agrees with a previous study in pinnipeds from Antarctica (Yochem et al. 2009). *Leptospira* spp. is a ubiquitous pathogen known to occur in several pinnipeds including *M. angustirostris* (Colegrove et al. 2005); *C. ursinus* (Smith et al. 1977), *P. vitulina richardsii* (Stamper et al. 1998) and *Z. californianus* (Vedros et al. 1971, Gulland et al. 1996). In *M. angustirostris*, Leptospirosis has been associated with kidney disease, and in *Z. californianus* it caused large mortality epizootics (Vedros et al. 1971, Gulland et al. 1996), highlighting the susceptibility of pinnipeds to this pathogen.

New diagnostic tools may contribute to the increased rate of discovery of emerging pathogens, and can provide novel and important ways to study the dynamics of pathogens in wild populations (Dobson & Foufopoulos 2001). Contemporary tests are much more sensitive to those of 10 years ago when the samples examined here were collected.

Our knowledge of the hosts associated with *Brucella* species and genotypes in marine mammals is constantly increasing. Recently Harp Seals were found to carry *B. pinnipedialis* genotypes (previously confined to Hooded Seals); *Brucella* isolates from Beluga (*Delphinapterus leucas*) were found to represent a number of distinct *B. pinnipedialis* genotypes; and the sequence type ST27 (associated with zoonotic disease) was identified in California sea lions (Whatmore et al. 2017).

The prevalence of diseases in the wild is subject to change over time, and the development of new analytical tools is key to understanding the behaviour of diseases and their dynamics. Ongoing research efforts and permanent surveillance protocols are critical for the conservation and management of wildlife facing various diseases throughout their range. In Antarctica biosecurity preventive measures (for

tourists and scientific staff) should continue being a priority to minimise the potential for introducing or spreading pathogens to the Antarctic landscape and wildlife (Grimaldi et al. 2011).

The results obtained here contribute to the knowledge of zoonotic pathogens in Southern Ocean pinnipeds. In light of the detection of *Brucella* spp. exposure in several pinnipeds species inhabiting islands in the Southern Ocean, the existence of some populations of Antarctic pinnipeds that are naïve with respect to pathogen exposure, a monitoring regime of zoonotic pathogens is warranted at various pinniped breeding and haul out sites on islands in the Southern Ocean and in Antarctica.

ACKNOWLEDGEMENTS

We would like to acknowledge Nicolle Queiroz-Hazarbassanov, Gisele Oliveira and Antonio de Sousa for the excellent technical assistance and Jacques Delbecque from INGENASA, Madrid, Spain for kindly providing the c-ELISA KIT. We are grateful to Eric Woehler for useful comments on an earlier draft of this manuscript. This study was conducted by A.M.S-S as part of her PhD program at Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil, with support from a scholarship from Coordenação de Apoio à Pesquisa de nível superior-CAPES and approved by the Ethics Committee for the Use of Animals in Research at USP (CEUA No. 3926161213), Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brazil. J.L.C.-D. is a recipient of a professorship by Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq (Grant No. 305349/2015-5). This research was conducted as part of the Brazilian Antarctic Program (PROANTAR) – Projeto Elefante Marinho do Sul (PEMS). We thank the Brazilian Navy (research vessels, the personnel from the Brazilian Antarctic Station “Comandante Ferraz”— EACF), the Brazilian Antarctic Program (PROANTAR) and SECIRM for logistical support. We would also like to thank the “Southern Elephant Seal Project” (PEMS) crew for field support, particularly Julio Jose Reynoso, Mateus Holtz Barbosa and Roberto Vilela.

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7 *BRUCELLA* SPP. IN MARINE MAMMALS OF BRAZIL: MOLECULAR, SEROLOGICAL, PATHOLOGICAL, IMMUNOHISTOCHEMICAL AND MICROBIOLOGICAL INVESTIGATION.

7.1 ABSTRACT

Brucella-exposure and infection is increasingly recognized in marine mammals worldwide. To better understand the epidemiology and health impacts of *Brucella* spp. in marine mammals of Brazil, molecular (PCR and/or qPCR), serological (RBT, c-ELISA, SAT), pathological, immunohistochemical and/or microbiological investigations were conducted in 129 animals (124 Cetartiodactyla, 4 Carnivora and 1 Sirenia). PCR/qPCR revealed 4/129 (3.1%) *Brucella*-positive Cetartiodactyla from which 10 were seropositive. Pathological, immunohistochemical and microbiological analyses conducted in PCR/qPCR-positive and seropositive cases (except for two live-stranded animals), revealed *Brucella*-type lesions in those animals including meningitis/meningoencephalitis, pneumonia, hepatic necrosis, pericarditis and osteoarthritis. In addition, all PCR/qPCR-positive and seropositive animals marked positive by immunohistochemistry. Despite unsuccessful *Brucella* sp. culture attempts, our results demonstrated *Brucella* sp. exposure, asymptomatic, acute and chronic *Brucella*-infection. Three coinfection cases involving *Brucella* sp. and cetacean morbillivirus, *Edwarsiella tarda* and *Proteus mirabilis* were detected. To our knowledge, this is the first long-term and large-scale survey of *Brucella* spp. in aquatic mammals of South America, Brazil widening the spectrum of susceptible hosts and geographic distribution range of this agent with zoonotic potential.

Key words: *Brucella* spp., cetacean morbillivirus, cetacean pathology, *Edwarsiella tarda*, immunohistochemistry, PCR, *Proteus mirabilis*, marine mammals.

7.2 INTRODUCTION

Brucellosis, a bacterial disease caused by *Brucella* spp., is a widespread zoonosis which remains an important economic and public health problem in some many areas of the world (1). This genus is comprised by 12 species, further classified into 'atypical' strains, i.e., *B. microti* (common voles [*Microtus arvalis*], red foxes

[*Vulpes vulpes*] and soil) and *B. inopinata* [a single isolate from a breast implant]), and the 'classical' strains, i.e., *B. abortus* (bovines), *B. melitensis* (sheep and goats), *B. suis* (swine, cattle, rodents, wild ungulates), *B. ovis* (sheep), *B. canis* (canines), *B. neotomae* (rodents), *B. papionis* (baboons [*Papio* spp.]), *B. vulpis* (red foxes [*V. vulpes*]), *B. ceti* (cetaceans [dolphins, whales and porpoises]), and *B. pinnipedialis* (pinnipeds [seals, sea lions and walruses] (2–10). The two latter are regarded as 'marine *Brucella* strains'. Marine *Brucella* strains can cause natural and occupational infection in humans (11–13) and domestic animals (14) indicating the zoonotic potential of these strains.

The current knowledge on susceptible species and geographic distribution of *Brucella* spp. –including *B. ceti* and *B. pinnipedialis* - in aquatic environments has increased considerably since their first detection in dolphins and seals in the early 90's (15,16). The potential aquatic mammal's hosts for *Brucella* spp. include up to 130 species and in almost half of them seropositivity has been demonstrated (17). *Brucella*-type lesions are not often apparent (17–19). However, family-specific susceptibility has been suggested for delphinids infected by *B. ceti*, where the chronic forms of the disease -characterized by meningitis and/or meningoencephalitis (resembling 'human neurobrucellosis')- are commonly reported (20–24). By contrast, the pathogenic role of *Brucella* spp. in pinnipeds is not clear and host adaptation has been proposed (25–27).

We recently reported the first case of cetacean brucellosis in the Southwest Atlantic Ocean, involving a Clymene dolphin (*Stenella clymene*) (28). A second report of *B. ceti* involving an individual of the same species (29), alerted research teams and public health services over the circulation of this bacterium in northeastern Brazilian coast. Given the zoonotic potential and the implications for marine mammal's health and conservation, we aimed to gain insight into the epidemiology and disease aspects of *Brucella* spp. in marine mammals throughout the Brazilian coast.

7.3 MATERIALS AND METHODS

7.3.1 Animals and samples included in the study

We analyzed samples from 129 live- or dead-stranded or by-caught marine mammals: 124 Cetartiodactyla, 4 Carnivora and 1 Sirenia (number of individuals by order, family and species is listed in Table 1). The samples were collected in collaboration with partner institutions along the Brazilian Coast from 1996 to 2017 and kept at the Marine Mammal Tissue Bank of the Laboratory of Wildlife Comparative Pathology, Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil. Most part of animals were from the State of Ceará (46/129; 35.6%), followed by the States of Sao Paulo (35/129; 27.1%), Santa Catarina (19/129; 14.7%), Espirito Santo (11/129; 8.5%), Bahia (10/129; 7.7%), Rio de Janeiro (6/129; 4.65%), Rio Grande do Norte and Rio grande do Sul (1/129; 0.8%), respectively. All samples used in this study were collected in full compliance with federal permits issued by the Brazilian Ministry of Environment (Biodiversity Information and Authorization System (SISBIO No 48279-1) and approved by the Ethics Committee on the Use of Animals of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (CEUA No 3926161213).

Dead animals had complete and standardized necropsies performed following international guidelines (30). Fresh tissue samples from representative organs were collected and fixed in 10% neutral buffered formalin and selected tissues, fluids and/or swabs were stored frozen (-20° C and/or -80° C) for histopathology and molecular analysis, respectively. Individual data including date and stranding location, sex, total body length (TBL, from tip of rostrum to tail notch) and origin (stranding, bycatch) is displayed in Supplementary Table 1. Age class categories were based on TBL according to published references for Guiana dolphin (*Sotalia guianensis*) (31), Franciscana (*Pontoporia blainvillei*) (32–34) and the remaining species (35,36). Additional data: condition code (for discovery and recovery carcass time) (30), body condition (good, fair, poor) (37) and gross findings were analyzed in positive cases, as recorded.

Table 1. Number and species of marine mammals analyzed in this study

Order	Family	Species	N
Cetartiodactyla	Delphinidae	<i>Sotalia guianensis</i>	23
		<i>Peponocephala electra</i>	11
		<i>Stenella clymene</i>	6
		<i>Stenella frontalis</i>	4
		<i>Globicephala macrorhynchus</i>	4
		<i>Stenella longirostris</i>	3
		<i>Tursiops truncatus</i>	3
		<i>Stenella attenuata</i>	3
		<i>Stenella sp.</i>	1
		<i>Feresa attenuata</i>	2
		<i>Grampus griseus</i>	1
		<i>Delphinus delphis</i>	1
	Pontoporidae	<i>Pontoporia blainvillei</i>	40
	Kogiidae	<i>Kogia sima</i>	7
		<i>Kogia breviceps</i>	1
	Physeteriidae	<i>Physeter macrocephalus</i>	3
	Ziphiidae	<i>Mesoplodon europaeus</i>	1
	Balaenopteridae	<i>Megaptera novaeangliae</i>	7
		<i>Balaenoptera edeni</i>	1
	Balaenidae	<i>Eubalaena australis</i>	2
Carnivora	Otariidae	<i>Arctocephalus tropicalis</i>	2
		<i>Arctocephalus australis</i>	2
Sirenia	Trichechiidae	<i>Trichechus manatus</i>	1
Total			129

7.3.2 Molecular analysis

Frozen tissues, fluids and/or swabs (-20° C and/or -80° C) from 123 aquatic mammals and sections of FFPE tissues from 6 cases (where no frozen tissues were available) were manually homogenized and 25 to 60 mg were extracted using the DNeasy Blood & Tissue Kit (QIAGEN®, Valencia, CA, USA) and/or the CTAB phenol-chloroform method (Supplementary Table 1) (38,39). DNA concentration and purity was determined via spectrophotometry with Epoch (Biotek®, Winooski, VE, USA). DNA concentration was adjusted when required by diluting template DNA up to 100-150 µM.

All extractions (n=454, Supplementary Table 1) were tested by a conventional polymerase chain reaction (PCR) was performed with primers B4 and B5 targeting a 223 bp fragment of the gene *bcsp31* which encodes the 31kDa immunogenic (cell surface) protein of the genus *Brucella* (40). Amplification was performed in 25 µl of a reaction mixture containing 4 µl of template DNA, 2x One Taq® Quick load® (New England BioLabs®, Otsu, Shiga, Japan), 0.2 µM of each primer and *qsp* nuclease-free H₂O. The PCR cycle conditions were modified from Baily et al (1992): initial denaturation at 94° C for 5 min, template denaturation at 94° C for 45 s, primer annealing at 60° C for 45 s, primer extension at 68° C for 30 s, for 40 cycles, with a

final extension at 68° C for 3 min, in a C1000 Touch Thermal Cycler (BioRad Laboratories, Hercules, CA, USA).

Additionally, selected extractions (n=134, Supplementary Table 1) were tested via quantitative (q)-PCR, targeting *Brucella* genus following previously published methodology (41). Thus, sequences of *Brucella* spp. and *B. ceti* IS711 gene were aligned for designing the primers 5'-TCAAAGTCCGGCGTATCAG-3' and 5'-GAACCGGATCGAAGCATATC-3' to amplify a fragment of approximately 190 bp, specific to the genus *Brucella*, using the program PRIMER 3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>). Amplification was performed in 20 µl of a reaction mixture containing 4 µl of template DNA, 1X SsoAdvanced™ Universal SYBR® Green Supermix (BioRad Laboratories, Hercules, CA, USA) and 20 nM of each primer and *qsp* nuclease-free H₂O. The PCR cycle conditions were: initial denaturation at 98° C for 3 min, template denaturation at 98° C for 15 s, primer annealing at 60° C for 30 s, primer extension at 65° C for 5 s, for 39 cycles, with a final extension at 72°C for 5 s, in a CFX96 Touch™ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA).

Positive controls consisted of DNA from *B. abortus* (vaccine RB-51) and *B. ceti* (M644/93/1; B14/94). Nuclease-free water instead of DNA template was used as no template control. Amplification products were read by standard 1.5% agarose electrophoresis, using SYBR® safe DNA gel stain (Thermo Fisher Scientific®). Samples presenting a PCR product of the expected size were purified by using ExoSAP-IT™ PCR product clean-up (Affymetrix®, Santa Clara, CA, USA) according to manufacturer. The products were sequenced using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, USA), and after ethanol precipitation DNA products were analyzed through Sanger method on an automated sequencer Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). Sequences were assembled and then aligned with Clustal W, available in the suite BioEdit (42). Obtained consensus sequences were used for identity comparison with other *Brucella* species available in GenBank using Basic Local Alignment Search Tool (BLAST®).

Standardization and preliminary of qPCR analyzes were done at the Institute for Animal Health and Food Hygiene, School of Veterinary Medicine, University of

Las Palmas of Gran Canaria, Spain. Additional samples were tested at the Laboratory of Molecular Pathology, Adolfo Lutz Institute, São Paulo, Brazil.

7.3.3 Serological analysis

Serological analysis (Rose Bengal Test-RBT, competitive enzyme-linked immunosorbent assay-c-ELISA and Serum Agglutination Test-SAT) performed previously on sera demonstrated 10 seropositive cetaceans (Table 2). The methods employed, and results obtained in this section constitute a parallel publication and will be published elsewhere.

7.3.4. Histopathological and immunohistochemical analysis

We conducted microscopical examinations and immunohistochemical analysis (IHC) in all PCR/qPCR- and/or serology-positive animals (n=13, Table 2). For histological analysis, formalin-fixed tissues were processed following routine procedures and 5 µm slides were cut and stained with hematoxylin and eosin. For IHC analysis 4 µm cut tissue sections on silane-coated slides were dried (37° C x 6 h - 90° C x 2 h), dewaxed and rehydrated by passing the slides through xylol, xylene, various ethanol solutions (decreasing grading), running tap water and distilled water. Next, blocking of formalin pigments in ammonium hydroxide solution 25% diluted in alcohol 95% (30 min) at room temperature, followed by tap water and PBS wash. Antibody recovery was performed in citric acid solution (PM:210.04 mmol/l) 2.1004 g/l (pH 6) in pressure cooker for 3 min. The endogenous peroxidase was blocked by immersion in hydrogen peroxide (37° C x 30 min), followed by tap water and PBS wash and blocking of non-specific binding was performed with blocking solution supplied in the kit (Novolink®) (37° C x 30 min), followed by tap water and PBS wash.

For amplification and detection of the signal we used two IHC protocols after previous standardizing: (1) tissue sections were incubated overnight at 4° C with a primary polyclonal antibody against *B. abortus* (1:2.500; Byorbyt®), incubated at 37° C (30 min) with the secondary antibody and then a Horseradish Peroxidase Polymer System-HRP (Hidex detection, Cell marque® or Novolink®) at 37° C (30 min). Signal was visualized with 3,3'-diaminobenzidine-DAB (10-40 seg) and Harris hematoxylin

(1-5 min) for counterstaining. (2) Tissue sections were incubated overnight at 4° C with a primary polyclonal antibody against *B. abortus* (1:500, Byorbyt®), incubated at 37° C (30 min) with the secondary antibody and then an Alkaline phosphatase polymer (Histofine®) at 37° C (30 min). Warp Red (Biocare®) was used as chromogen (5 min) followed by counterstaining with Mayer's hematoxylin (3 min). Slides were then mounted with Entellan®. Placenta of a *B. ceti* infected *Stenella coeruleolba* (kindly supplied by R. Gonzáles-Barrientos, G. Hernandez-Mora and C. Guzman-Verri, Costa Rica National University) was used as positive control. As negative control, we used same placenta tissue with omitted primary antibody and incubation with nonimmune homologous serum.

7.3.5 Culture and molecular analysis of isolates

According availability, we performed culture in frozen tissues from PCR/qPCR- and serology-positive animals (n=13, Table 2), following standard procedures. Briefly, 1g of each tissue was manually homogenized with 0.9% NaCl sterile solution (1:5) and 100 µL of the suspension was inoculated in duplicate, directly onto plates containing *Brucella* agar (OXOID®) supplemented with 5% of bovine fetal serum and the following antibiotic mixtures: (a) colistin methane-sulphonate (7.5 mg/l), vancomycin (3 mg/l), nitrofurantoin (10 mg/l), nystatin (100,000 International Units [IU]/l) and amphotericin B (2.5 mg/l); and (b) polymyxin B sulphate (5000 IU/l); bacitracin (25,000 IU/l); cyclohexamide (30 mg/l); nalidixic acid (5 mg/l); nystatin (100,000 IU/l); vancomycin (20 mg/l) (43).

Plates were incubated at 37°C and 10% CO₂ atmosphere, for 5 weeks. In addition, 100 µL of the suspensions were inoculated in tubes *Brucella* broth containing polymyxin B sulphate (5000 IU/l); bacitracin (25,000 IU/l); cyclohexamide (30 mg/l); nalidixic acid (5 mg/l); nystatin (100,000 IU/l); vancomycin (20 mg/l) for enrichment. The tubes were incubated at 37° C and 10% CO₂ atmosphere, for 3 weeks. Blind subcultures were performed each 4 days into plates containing *Brucella* agar supplemented with 5% of bovine fetal serum. The plates were incubated at 37° C and 10% CO₂ atmosphere, for 15 days. Plates were verified for growth each 4 days until the end period. Suspect colonies (circular, convex, shiny, 1-2 mm in diameter), when present were examined microscopically with Gram stain for purity (44), and those displaying morphology compatible with genus *Brucella* (Gram-

negative coccobacilli), were cultured in *Brucella* agar supplemented with 5% of bovine fetal serum and incubated at 37° C and 10% CO₂ atmosphere, for 5 days for further identification.

All suspect isolates were collected and submitted to DNA extraction and PCR analysis. For DNA extraction, each bacterial colony was resuspended in 300 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and boiled at 100° C for 30 min, for heat lysis of cells (45). In each extraction group, TE buffer was submitted to the same extraction procedures, as negative control. Conventional PCR of all suspect colonies were performed targeting highly conserved regions among *Brucella* genus, including the gene *bcs*p31 as described above (40) and primers IS-517F22 targeting the insertion sequence *IS*711 (46). Positive controls consisted of DNA extracted from *B. canis* reference strain RM6/66 (ATCC 23365) and nuclease-free water instead of DNA template for negative controls.

For *bcs*p31 PCR, amplification was performed in 25 µl of a reaction mixture containing 2.5 µl of template DNA, 200 µM of each dNTP, 1.5 mM of MgCl₂, buffer 1X (50 mM de KCl, 20mM Tris-HCL, Ph 8.4), 1.5 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), and 0.5 µM of each primer. The PCR cycle conditions were: initial denaturation at 94° C for 2 min, template denaturation at 94° C for 30 s, primer annealing at 60° C for 30 s, and primer extension at 72° C for 30 s, for 35 cycles, with a final extension at 72° C for 10 min. For *IS*711 PCR, amplification was performed in 25 µl of a reaction mixture containing 2.5 µl of template DNA, 200 µM of each dNTP, 1.5 mM of MgCl₂, buffer 1X (50 mM de KCl, 20mM Tris-HCL, pH 8.4), 1.5 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), and 0.6 µM of each primer. The PCR cycle conditions were: initial denaturation at 94° C for 2 min, template denaturation at 94° C for 40 s, primer annealing at 59° C for 40 s, and primer extension at 72° C for 40 s, for 40 cycles, with a final extension at 72° C for 10 min. Both *bcs*p31 and *IS*711 PCRs were performed using a Veriti™ Thermal Cycler (ThermoFisher Scientific, Applied Biosystems, Waltham, MA, US).

PCR amplification products were analyzed by standard 1.5% agarose electrophoresis, using Ethidium Bromide (Invitrogen®, Thermo Fisher Scientific, Carlsbad, CA, USA) (47). Samples presenting a PCR product of the expected size were excised from the gel and purified by using Illustra®, GFX PCR DNA and GEL Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK)

according to the manufacturer instructions. PCR sequencing and posterior analyzes were performed as described above.

Culture and molecular analysis of isolates were performed at the Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Brazil.

7.4 RESULTS

Molecular, serological, immunohistochemical and microbiological analyses along with main pathological findings in *Brucella*-positive animals are summarized in Table 2. The geographic distribution of stranding events for those positive animals is recorded in Figure 1.

7.4.1 Molecular and serological analysis

Thirteen out of 129 (10.1%) animals were positive by PCR/qPCR and/or serology. Ten of them (76.9%) were serologically positive (detailed results under publication elsewhere), including one pygmy killer whale (*Feresa attenuata*), three short-finned pilot whale (*Globicephala macrorhynchus*), two melon-headed whale (*Peponocephala electra*), three *S. clymene* and one bottlenose dolphin (*Tursiops truncatus*). Four out of 13 (30.1%) animals were *Brucella*-PCR/qPCR –positive, including one *S. clymene* (previously published in (28)), one Spinner dolphin (*Stenella longirostris*), one Franciscana and one Guiana dolphin (the three later had no sera available). Additionally, in cetaceans, *Achromobacter* sp. and *Bradyrhizobium* sp. were confirmed through sequencing in 5 of 129 (3.9%) positive results obtained by PCR using B4 and B5 primers and in 1 of 129 (0.8%) positive results obtained by qPCR using *IS711* primers, respectively (Supplementary Table 1). All individuals from families Balaenopteridae, Kogiidae, Physeteridae, Ziphiidae, Balaenidae, Otariidae, Trichechidae were negative for PCR/qPCR and/or serology.

Table 2. Individual data (species, age class, history, body condition, stranding location and date) and test results of *Brucella*-positive cetaceans. Positive results in each category are marked in bold. Individual histopathological remarks and final diagnosis of each case are included.

Case No	Individual data	Serology	Conventional and real-time PCR	Immunohistochemistry	Culture	Histopathological remarks/Final diagnosis (FD) with regards to <i>Brucella</i> sp.
No 1	<i>Feresa attenuata</i> Sex: male Age class (TBL): adult (2.13 m) History: live-stranded (COD 1), died on the same day despite being treated by veterinarians; first register of this species in State of Ceará Body condition: fair Stranding location: Praia da Taíba, São Gonçalo de Amarante, State of Ceará (03°30'22.0"S, 38°54'19.0"W) Date: April 14, 2015	C-ELISA	Blood, cerebrum, cerebellum, heart, kidney, liver, lung, spinal cord, pre-scapular lymph node, spleen	Cerebrum, cerebellum, glandular stomach, liver, lung , lymph node, mandibular lymph node, spinal cord, pre-scapular lymph node , pulmonary lymph node, spleen	Blood, cerebrum, cerebellum, lung, medula, pre-scapular lymph node	Chronic meningoencephalitis, acute pneumonia, pulmonary lymph node lymphoid depletion, multicentric reactive hyperplasia, pericholangitis, enterocolitis. FD: Despite bacterial DNA was not detected, findings are suggestive of chronic brucellosis
No 2	<i>Globicephala macrorhynchus</i> Sex: male Age class (TBL): juvenile (2.90 m) History: live-stranded (COD 1), died on the same day despite veterinary treatment Body condition: poor Stranding location: Praia do Preá, Cruz, State of Ceará (02°48'44.0"S, 40°24'54.9"W) Date: January 11, 2012	RBT, C-ELISA	Kidney, liver, lung, spleen	Glandular stomach, kidney, lymph node, small intestine , spleen, tongue	Lung	Lymphoplasmacytic gastritis and enterocolitis, necroulcerative glossitis, splenic lymphoid hyperplasia, multicentric lymphoid depletion. FD: <i>Brucella</i> -exposure and gastrointestinal infection
No 3	<i>Globicephala macrorhynchus</i> Sex: female Age class (TBL): newborn calf (1.57 m) History: live-stranded (COD 1), medicated and released but remained in the area. The animal was re-captured in Praia de Quixaba, Aracati, State of Ceará (8 days after first stranding), and was treated but died 3 days after Body condition: poor Stranding location: Praia de Vila Nova, Icapui, State of Ceará (04°39'56.7"S, 37°25'40.3"W) Date: July 9, 2016	C-ELISA	Heart, kidney, liver, lung, mesenteric lymph node, pool CNS (cerebrum, cerebellum, spinal cord), pulmonary lymph node, spleen	Cerebrum , esophagus, glandular stomach, intestine, keratinized stomach, laryngeal tonsil, laryngeal lymph node, lung, spinal cord, mesenteric lymph node, pulmonary lymph node, pyloric stomach, trachea, spleen	Blood [§] , cerebrum, cerebellum, lung, spinal cord, mesenteric lymph node, pulmonary lymph node,	Suppurative leptomeningitis, multicentric lymphoid reactive hyperplasia, subacute pneumonia, lymph node and tonsil lymphoid hyperplasia, cryptitis (tonsil), tracheitis, splenic lymphoid depletion. FD: <i>Brucella</i> sp. exposure and infection; however, other concomitant infectious pathogen remains a possibility
No 4	<i>Globicephala macrorhynchus</i> Sex: female Age class (TBL): newborn calf (1.30 m) History: dead-stranded (COD 2) Body condition: poor Stranding location: Praia da Tabuba, Caucaia, State of Ceará (03°38'24.8"S, 38°42'00.1"W) Date: January 25, 2017	C-ELISA	Heart, kidney, liver, lung, mesenteric lymph node, pulmonary lymph node, pool SNC (cerebrum, cerebellum, spinal cord) spleen	Adrenal, anterior stomach, cerebrum, cerebellum, esophagus, glandular stomach, heart, intestines, kidney, liver, lung , mandibular fat, spinal cord, melon fat, mesenteric lymph node, muscle, ovary, pancreas, pulmonary lymph node, pyloric stomach, skin, spleen, trachea, urinary bladder, uterus	Cerebrum, cerebellum, lung, spinal cord, mesenteric lymph node, pulmonary lymph node	Acute to subacute pneumonia FD: <i>Brucella</i> sp. exposure and infection; in-utero infection suspect
No 5	<i>Peponocephala electra</i> Sex: male Age class: adult (2.40 m)	C-ELISA	Blood	NA	Blood	FD: <i>Brucella</i> sp. exposure

	<p>History: Mass live-stranded (COD 1), together with case # 6. It was treated and released. Possible was the same group that stranded two days before on Praia de Caetanós, Amontada, State of Ceará (215 km away)</p> <p>Body condition: good</p> <p>Stranding location: Praia das Fontes, beberibe, State of Ceará (04°12'59.5"S, 38°02'47.1"W)</p> <p>Date: November 7, 2014</p>					
No 6	<p><i>Peponocephala electra</i></p> <p>Sex: female</p> <p>Age class: adult (2.46 m)</p> <p>History: Mass live-stranded (COD 1), together with case # 5 (see above history)</p> <p>Body condition: good</p> <p>Stranding location: Praia das Fontes, Beberibe, State of Ceará (04°12'59.5"S, 38°02'47.1"W)</p> <p>Date: November 7, 2014</p>	C-ELISA	Blood	NA	Blood	FD: <i>Brucella</i> sp. exposure
No 7	<p><i>Pontoporia blainvillei</i></p> <p>Sex: female</p> <p>Age class (TBL): newborn calf (0.62 m)</p> <p>History: by-caught (COD 2)</p> <p>Body condition: good</p> <p>Bycatch location: Praia grande, Sao Paulo state (24°01'S; 46°28'W)</p> <p>Date: November 9, 2011</p>	NA	Blood, pool SNC (cerebrum, cerebellum), hepatic lymph node, kidney, liver, lung, spinal cord, mesenteric lymph node (qPCR), pre-scapular lymph node	Cerebrum, cerebellum, colon, eye, glandular stomach, heart, keratinized stomach, kidney, laringe, liver, lung, lymph nodes (hepatic, pre-scapular, mediastinal, mesenteric and renal), ovary, skin, tongue, trachea, thymus, thyroid, uterus	Blood, pool SNC (cerebrum, cerebellum), hepatic lymph node, kidney, liver, lung, spinal cord, mesenteric lymph node, pericardial fluid, pre-scapular lymph node	Granulomatous thymitis, focal neutrophilic alveolitis FD: <i>Brucella</i> sp. lymphoid asymptomatic infection
No 8	<p><i>Sotalia guianensis</i></p> <p>Sex: female</p> <p>Age class: calf (1.08 m)</p> <p>History: dead-stranded (COD 2)</p> <p>Body condition: poor</p> <p>Stranding location: Guriri, São Mateus, Espírito Santo state (18°44'S; 39°44'W)</p> <p>Date: November 30, 2010</p>	NA	Blood, cerebrum, genitalia, kidney, liver, pulmonary lymph node (PCR/ qPCR), spleen	Adrenal, cerebrum, cerebellum, duodenum, esofagous, eye and optic nerve, kidney, large intestine, lymph nodes*, liver, lung, spinal cord, muscle, pancreas, pituitary, tongue, spleen	Blood, kidney, liver, pulmonary lymph node, spleen	Neutrophilic meningoencephalitis; pneumonia; necrotizing hepatitis and lymph node microabscesses FD: <i>Brucella</i> sp. and cetacean morbillivirus coinfection. Other concomitant infectious pathogen remains a possibility
No 9	<p><i>Stenella clymene</i></p> <p>Sex: male,</p> <p>Age class: adult (1.75 m)</p> <p>History: live-stranded (COD 1), (observed the night before). Died on the same day despite veterinary treatment</p> <p>Body condition: fair</p> <p>Stranding location: Parajuru, Beberibe, State of Ceará (04°23'28.7"S, 37°49'44.5"W)</p> <p>Date: March 10, 2012</p>	RBT, C-ELISA	Blood, cerebrum, heart, kidney, liver, lung (PCR/ qPCR), mesenteric lymph node, spleen, small intestine	Cerebrum, heart, kidney, liver, lung, mesenteric lymph node, spleen, tongue	Cerebrum, heart, kidney, liver, lung, mesenteric lymph node, spleen	Pneumonia, splenic and mesenteric lymphoid depletion, splenitis, nephritis, lymphoplasmacytic hepatitis, gastritis, glossitis, enterocolitis FD: <i>Brucella</i> sp. localized pulmonary infection
No 10	<p><i>Stenella clymene</i></p> <p>Sex: male</p> <p>Age class: adult^s (1.70 m)</p> <p>History: live-stranded (COD 1), (observed the day before), treated by veterinarians but died after 4 days</p>	C-ELISA	Blood, heart, kidney, liver, lung, mesenteric lymph node, pool SNC (cerebrum, cerebellum, spinal cord), spleen	Adrenal, cerebrum, cerebellum, glandular stomach, intestines, keratinized stomach, kidney, liver, lung**, spinal cord, mesenteric lymph node, pancreas, pre-scapular lymph node,	Cerebrum, cerebellum, lung, mesenteric lymph node, kidney, spinal cord	Acute meningitis, polyneuritis, vascular fibrinoid necrosis, neutrophilic vasculitis, multicentric lymphoid depletion, multicentric lymph node reactive hyperplasia, gastritis, pneumonia

	Body condition: fair Stranding location: Praia do Japao, Aquiraz, State of Ceará Date: January 10, 2016			skin/mucocutaneous junction, testicle, tongue, tonsila, trachea, spleen, peri-ureteral lymph node, urinary bladder		FD: <i>Brucella</i> sp. exposure; concomitant bacterial infection suspect
No 11	<i>Stenella clymene</i> Sex: female Age class: adult [§] (1.76 m) History: live-stranded (COD 1), treated and released one day after. The animal was found dead-stranded (COD 3) 4 days after first stranding Body condition: fair Stranding location: live-stranded in Praia de Águas Belas, Cascavel, State of Ceará (04°03'31.3"S, 38°10'50.5"W). Dead-stranded in Praia do Iguape, Aracati (03°58'24.7"S, 38°15'02.9"W; 12 km far way from release local) Date: May 25, 2016	RBT, C-ELISA, SAT	Heart, kidney, liver, lung, spinal cord, pulmonary lymph node, spleen	Anterior stomach, cerebrum, esophagus, glandular stomach, intestines, kidney**, lung, spinal cord, melon fat, mesenteric lymph node, pulmonary lymph node, urinary bladder	Kidney, lung, spinal cord, pulmonary lymph node	Subacute lymphocytic meningomyelitis, poliradiculoneuritis, multicentric lymphoid depletion, reactive hyperplasia, pneumonia, interstitial nephritis FD: Despite bacterial DNA was not detected, findings are suggestive of chronic brucellosis
No 12	<i>Stenella longirostris</i> Sex: male Age class: juvenile (1.21 m) History: dead-stranded (COD 2) Body condition: poor Stranding location: Praia de Quitêras, Icapuí, State of Ceará (04°43' 39.9" S / 37°17' 48.6" W) Date: April 2, 2015	NA	Blood, heart (qPCR), kidney, liver, lung (qPCR), meninges (qPCR), spleen	Atlanto-occipital abscess, adrenal, cerebellum, colon, glandular and keratinized stomach, heart, kidney, liver**, lung, lymph node*, mandibular fat, mandibular lymph node, mesenteric lymph node	Abscess and blood ^{&.1} , blood, heart, kidney, liver, lung, meninges, spleen	Fibrinosuppurative atlanto-occipital osteoarthritis with right lateral abscess formation; mild leptomeningitis, lymph node reactive hyperplasia, adenitis, pericarditis, pneumonia, nephritis, gastritis of glandular and keratinized stomach, colitis. FD: Chronic brucellosis, coinfection with <i>Proteus mirabilis</i>
No 13	<i>Tursiops truncatus</i> Sex: female Age class: adult (2.71 m) History: dead-stranded (COD 3) Body condition: fair Stranding location: Abreulândia, Fortaleza, State of Ceará (03°48'20.1"S, 38°24'45.6"W) Date: July 6, 2015	C-ELISA	Cerebrum, heart, kidney, liver, lung, mesenteric lymph node, pre-scapular lymph node, spleen, urinary bladder	Kidney**, liver, lung, mesenteric lymph node, muscle, pre-scapular lymph node, spleen, urinary bladder	Urinary bladder, blood, cerebrum, uterus ^{&.2} , kidney, lung, mesenteric lymph node, pre-scapular lymph node, urinary bladder	Pyelonephritis, cystitis, colitis, pericholangitis. gastritis, spleen lymphoid depletion. FD: <i>Brucella</i> sp. exposure and infection; coinfection with <i>Edwarsiella tarda</i> .

TBL= total body length (from tip of rostrum to tail notch). Condition codes (for discovery and recovery carcass times); COD 1= live-stranded; COD 2= freshly dead; COD 3= decomposed, but organs basically intact (1) IHC: immunolabeling in positive tissues consistently diffuse otherwise specified. [§] sexual maturity of the individual verified by histology. *several unspecified lymph nodes tested. ** inconclusive. CNS=Central nervous system; RBT=Rose Bengal Test; C-ELISA= Competitive Elisa; SAT= Serum Agglutination Test; NA= Not analyzed. [&]Independent culture done by external laboratory. ¹*Proteus mirabilis* was isolated. ²*Edwardsiella tarda* was isolated.

Figure 1. Stranding location of individuals tested in this study (white dots). Positive results (red) are displayed according species and diagnostic test used. CE= State of Ceará; ES= State of Espírito Santo; SP= State of São Paulo.

7.4.2 Pathological analysis

A wide variety of gross and microscopical findings were observed in 11 (necropsied animals) from the 13 PCR/qPCR- and/or serology *Brucella*-positives (detailed descriptions are recorded in Supplementary Table 2).

The main gross findings were: pulmonary congestion and edema in 10/11 (90.9%); gastroenteric congestion, edema and/or hemorrhage in 9/11 (81.8%); liver congestion in 9/11 (81.8%); lymphadenomegaly in 8/11 (72.7%); brain congestion, edema and/or hemorrhage, splenomegaly, splenic congestion and/or hemorrhage in 7/11 (63.4%). Grossly, case 12 (*S. longirostris*, figure 2A) presented severe fibrinosuppurative atlanto-occipital osteoarthritis with right lateral abscess formation, considered the only gross *Brucella*-type lesion (Figure 2B-D).

The main microscopical findings included: lymphoid depletion in 9/11 (81.8%), lymphoplasmacytic / neutrophilic / pyogranulomatous, interstitial pneumonia/bronchopneumonia and tonsillar, splenic and/or lymph nodes reactive hyperplasia in 7/11 (63.3%) and lymphoplasmacytic/eosinophilic entero /colitis with/without cryptitis in 6/11 (54.5%). Non-suppurative meningitis/meningoencephalitis involving the spinal cord, medulla oblongata and cerebellum (Figure 2E), necrotizing hepatitis (Figure 2F), pneumonia and pericarditis were considered as *Brucella*-type lesions.

Figure 2. Gross and histopathological findings in PCR/qPCR- and/or serology *Brucella*-positive dolphins. **A.** *Stenella longirostris* (case No 12) dead-stranded in fair body condition, **B.** Fibrinosuppurative atlanto-occipital osteoarthritis with right lateral abscess formation (*S. longirostris*, case No 12), **C.** The atlanto-occipital joint capsules are severely distended and fibrosed, and there is remodeling of the articular cartilage (*S. longirostris*, case No 12), **D.** Microscopical appearance of the lateral abscess formation. There is mineralization and chronic pyogranulomatous response (not seen at this magnification, 10x, *S. longirostris*, case No 12), **E.** Chronic lymphoplasmacytic meningoencephalitis with edema and perivascular cuffing (not seen at this magnification) in a *Feresa attenuata* (case No 1). **F.** Necrotizing hepatitis in a *Sotalia guianensis* (case No 8), 20x.

7.4.3 Immunohistochemical analysis

Positive immunolabeling of phagocytic cells and/or free bacteria in inflammatory foci (Figure 3A-F) were observed in at least one organ in 11 of the 13 PCR/qPCR and/or serology positive animals, as described above. In two cases, which tested positive for *Brucella* only by c-ELISA, necropsy was not performed, thus tissues were not available for IHC. IHC-positivity was found associated or not with *Brucella*-type lesions.

Figure 3. Immunohistochemical findings of PCR/qPCR- and/or serology *Brucella*-positive dolphins with *Brucella*-type lesions in Brazil. **A.** Marked multifocal positive immunolabeling in Kupfer and lymphocytic cells of an individual presenting necrotizing hepatitis, 40x (*Sotalia guianensis*, case No 8); **B.** Marked positive IHC in the inflammatory infiltrate around an abscess lateral to atlanto-occipital osteoarthritis, 4x, **B.1** inset, 40x (*Stenella longirostris*, case No 12); **C.** Mild to moderate, multifocal positive immunolabeling in the macrophages in lymph node of an individual with chronic brucellosis, 20x, **C.1** inset, 40x (*Stenella longirostris*, case No 12); **D.** Positive IHC in the brain of an individual presenting lymphocytic meningomyelitis and poliradiculoganglioneuritis. Positivity in inflammatory infiltrate and free bacteria attached to the nervous tissues, 20x (*S. clymene*, case No 11); **E.** Positive immunolabeling noted in the inflammatory cell population and lumen of an individual with acute to subacute interstitial pneumonia and alveolar histiocytosis associated with bacterial aggregates, 20x (*Globicephala macrorrhynchus*, case No 4); **F.** Positive IHC in an individual presenting glossitis with myriads intralesional and intravascular bacilli, 20x, (*G. macrorrhynchus*, case No 2), **F.1** Positive immunolabeling was observed in scattered cells in the intestinal submucosa with mild lymphoplasmacytic enterocolitis in the same individual.

7.4.4 Culture and molecular analysis of isolates

Brucella spp. culture attempts of frozen tissue samples from PCR/qPCR- and/or serology *Brucella*-positive dolphins were unsuccessful. Thus, further characterization including genotyping of the *Brucella* strain/s involved in the cases was not performed.

7.5 DISCUSSION

To our knowledge, this study represents the first long-term and large-scale survey of *Brucella* spp. in aquatic mammals of Brazil. We found molecular, serological, immunohistochemical and pathological evidences of *Brucella*-exposure, asymptomatic infections, and acute and chronic brucellosis in cetaceans stranded along the coastline of Brazil, widening the spectrum of susceptible hosts and geographic distribution range of this agent with zoonotic potential. All the pinnipeds and the manatees tested by PCR were negative in agreement with the literature (19).

Brucella-exposure, was confirmed serologically (c-ELISA) in two mass live-stranded *Peponocephala electra*; an adult male and an adult female (case No 5 and No 6, respectively). These animals were restrained, treated and released in the State of Ceará. PCR and culture of blood for *Brucella* were negative for both specimens. Complete blood count in these cases (data not shown) indicated leukopenia and lymphocytosis, suggestive of viral infection. One of the animals stranded dead few days after release; however, the advanced autolysis of the carcass precluded pathological examination. Additionally, a juvenile male *Globicephala macrorhynchus* stranded in State of Ceará (case No 2, suggested as exposed by RBT and c-ELISA) presented positive immunolabeling in a tongue lesion and in inflamed foci in the small intestine. These findings may suggest a gastrointestinal transmission route. The transmission of marine brucellosis is unresolved (48). Nevertheless, some potential transmission routes have been suggested e.g., direct transmission through social/sexual interaction, trans-placental, physical trauma (violent intra- and interspecific interactions) and ingestion of contaminated prey (18). In humans, the most common form of transmission is by contaminated food or direct contact with infected animals; however, sexual, aerosol, transplacental and breastfeeding routes have been reported (49). In these cases, we could not totally discard *Brucella*-

participation on stranding. *B. ceti* infection has been linked to stranding and subsequently death of striped dolphins (*Stenella coeruleoalba*) (50).

Brucella sp.-asymptomatic infection was confirmed in a newborn calf female *Pontoporia blainvillei*, by-caught in State of São Paulo (case No 7). This animal presented unspecific or no-*Brucella*-type pathological findings (e.g., pulmonary edema with alveolar histiocytosis and focal neutrophilic alveolitis, edema and/or hemorrhage in urinary bladder, thyroid, trachea, heart, brain and kidney). These hemodynamic changes as well as hepatic findings were related to bycatch. This animal also presented granulomatous thymitis which was not associated with *Brucella*-infection. In the other hand, lymphoid reactive hyperplasia with sinus histiocytosis was reported in lymph node, positive by PCR and IHC. Asymptomatic infections lacking lesions attributable to *Brucella* are common in marine mammals (18,51).

Localized *Brucella* infection, characterized by pyogranulomatous bronchopneumonia was found in an adult male *Stenella clymene* stranded in State of Ceará (case No 9), previously reported in (28). In this case, lung was PCR/qPCR and IHC positive. Immunohistochemically, intraalveolar macrophages presented cytoplasmic positive immunolabeling. Positive *Brucella* sp. antigen have been demonstrated before within inflammatory cells and nematode larvae in lung of cetaceans and pinnipeds (22,52,53), with the nematodes signalized as potential vectors for disease transmission. Additional PCR evidence of *Brucella*-lung infections have been previously reported in the literature, with or without associated pulmonary lesions (18,27,52,54).

Chronic brucellosis was diagnosed in case No 12 (*Stenella longirostris*), a juvenile male, dead-stranded in State of Ceará. This animal had severe, chronic fibrinosuppurative atlanto-occipital osteoarthritis with abscess formation. *Brucella* DNA was detected in meninges, heart and lung and microscopical examination revealed minimal leptomeningitis and chronic pericarditis. Positive immunolabeling of bacteria antigen in the inflammatory infiltrate in the abscess, and additional organs: heart, colon, glandular and keratinized stomach and mesenteric lymph node was observed. Skeletal lesions associated with localized or systemic *Brucella* infection have been previously described in marine mammals. For instance, in a white-sided dolphin (*Lagenorhynchus acutus*) with atlanto-occipital osteoarthritis (55); a *T. truncatus* with discospondylitis (56) and in a *S. coeruleoalba* with fibrinopurulent

osteoarthritis at the scapulohumeral joint that also presented meningoencephalomyelitis (50), all infected by *B. ceti*. In humans, osteoarticular disease is the most common complication of brucellosis (57) and the pattern of distribution of the existing *Brucella* arthritis forms (monoarthritis, sacroiliitis and spondylitis) relates to differences in the *Brucella* species involved (58) and a genetic predisposition have been suggested (59).

In case No 12, brain and heart inflammatory lesions differed in some extent in severity and pattern from those reported for *B. ceti* infected *S. coeruleoalba*, presenting chronic meningoencephalitis and severe endocarditis with pericardial fibrosis (50). In humans, central nervous system (CNS) involvement in *Brucella* infection occurs in about 5-7% of the cases (57), with several presentations (e.g., meningitis, encephalitis, meningovascular disease, brain abscesses and demyelinating syndrome) (60). Cardiovascular involvement is uncommon although both endocarditis and epicarditis have been described (61,62). Another interesting point of this case was the coinfection between *Brucella* sp. and *Proteus mirabilis*, which was isolated from blood and abscess, characterizing sepsis. *P. mirabilis* has been isolated from integumentary, respiratory and/or digestive system in *T. truncatus* and the beluga whale (*Delphinapterus leucas*) in association with other bacteria (63), and sometimes associated with pneumonia (64).

Case No 1 (*Feresa attenuata*, adult male) and case No 11 (*Stenella clymene*, juvenile male), both stranded on State of Ceará presented *Brucella*-type lesions in the central nervous system, recapitulating features of those reported in human neurobrucellosis (60) and in *S. coeruleoalba* neurobrucellosis by *B. ceti* (23). *B. ceti* may act as a neurotrophic agent in some species of cetaceans and CNS *Brucella*-type lesions may include chronic non-suppurative meningitis (more severe in the brainstem versus the cerebral and cerebellar cortices), periventricular encephalitis extending into the lateral, third and fourth ventricles, perivascular cuffing, scattered foci of microgliosis and mononuclear choroiditis with lymphoid follicle formation (20,23). Differential diagnosis for CNS inflammation in cetaceans include: cetacean morbillivirus (CeMV), herpesvirus, West Nile virus, *Toxoplasma gondii*, *Sarcocystis neurona* and *Nasitrema* spp. (65–71). Although the histopathological signature for most of these etiologies is not pathognomonic, the inflammatory patterns aid in the etiologic diagnosis. For instance, compared to brucellosis, cerebral toxoplasmosis produces a less severe meningitis and more severe encephalitis in striped dolphins

(65). Despite on cases No 1 and No 11, *Brucella* DNA was not detected, serology and IHC were positive. *Brucella* antigen positivity was observed in lung and lymph node (case No 1) and brain and glandular stomach (case No 11), reinforcing the diagnosis of brucellosis.

Additional pathological findings reported in this study for *Brucella* exposed or PCR/qPCR (all cases) included pneumonia, interstitial nephritis, enterocolitis and lymphoid depletion, all described before in dolphins with brucellosis (28,50). Lymphoid depletion and systemic spread or multiorgan involvement in these cases suggests immunocompromised host responses.

Coinfection by marine *Brucella* sp. and other pathogens have been reported in several cetacean species, including CeMV (72), *Toxoplasma gondii* (73) and a triple coinfection by *Brucella* sp., *T. gondii* and *Listeria monocytogenes* (74). In the present study, case No 8 (*Sotalia guianensis*, female calf dead-stranded in State of Espírito Santo), positive by PCR/qPCR in pulmonary lymph node was previously diagnosed with a novel strain of CeMV (75), thus confirming a coinfection case. This type of coinfection was previously reported in a sperm whale (*Physeter macrocephalus*) in Hawaii (72). In this *P. macrocephalus*, gross and histopathological findings suggested an in-utero infection and vertical transmission of both pathogens. We surmise an in-utero infection in case No 8 due to pathological findings (neutrophilic meningoencephalitis, pneumonia, necrotizing hepatitis and lymph node microabscesses) and positive IHC in liver, lymph nodes and lung. Similarly, in case No 4, a newborn female *Globicephala macrorhynchus*, seropositivity, pneumonia and positive immunolabeling in lung for *Brucella* sp., suggests a transplacental infection. Vertical transmission of *Brucella* sp. was also reported in an aborted bottlenose dolphin (15), neonatal *T. truncatus* presenting fetal distress and pneumonia (52) and a striped dolphin fetus without lesions (23,50). Meningitis, pericarditis, pleuritis, pneumonia, splenitis and hepatitis have been also documented in bovines with in-utero *Brucella* spp. infection (76,77).

In cases No 3 (newborn calf female *G. macrorrhynchus*, stranded in State of Ceará), No 10 (juvenile male *S. clymene*, stranded in State of Ceará), and No 8 (*S. guianensis*), the suppurative component of the inflammatory pattern in CNS diverges from previous descriptions (20,23,78). In these cases, we cannot rule out participation of other pathogens. In a striped dolphin coinfecting by *Brucella* sp., *T. gondii* and *Listeria monocytogenes*, multifocal brain microabscessation and malacic

areas were related to neurolisteriosis (74). However, these findings were not seen in case No 8. Serology confirmed *Brucella* sp. exposure in case No 3 and in case No 10. While none of these cases presented *Brucella*-type lesions in the CNS (and instead of that, presented suppurative leptomenigeal infiltrate, positive by IHC and acute meningitis, respectively), hematological analysis in case No 10 (data not shown) supported an acute bacterial infection. Case No 10 also presented multisystemic microangiopathy characterized by fibrinoid vascular necrosis and vasculitis. Similar vascular findings have been reported in dolphins (20), and humans (79) with brucellosis.

Case No 13 (*Tursiops truncatus*, adult female stranded in State of Ceará) represented another coinfection by *Brucella* sp. and *Edwardsiella tarda*. In this case, chronic ulcerative and suppurative cystitis and subacute pyelonephritis were attributed to *E. tarda*. This animal was positive by serology and IHC in liver, lung and urinary bladder. *E. tarda* is responsible for Edwardsiellosis and may lead to suppurative to necrotizing nephritis, hepatitis and splenitis in fishes (80,81). *E. tarda* has been isolated from cetaceans (82,83), and was regarded as the cause of stranding and septicemic death of a sperm whale (84). A coinfection by *E. tarda* and *Brucella* sp. was reported before in a *T. truncatus*, however, no pathological lesions were related to *E. tarda* (85).

In this study, *Achromobacter* sp. and *Bradyrhizobium* sp. were detected via PCR/qPCR in 6 cetaceans, as consequence of unspecific reaction of PCRs using the primers directed to the genes *bcs31* and *IS711*, respectively. Both are Gram-negative bacteria found in aquatic environments and soil, genetically similar to *Brucella* spp. and *Achromobacter* sp. may act as opportunistic human pathogen (86). These microorganisms have been also detected in dolphins (87,88). As in previous reports, we believe they likely represent contaminants and are unrelated to any pathological process in the present cases.

The polyclonal antibody we used was raised against whole cell *B. abortus* and reacts with antigenic domains of smooth lipopolysaccharide (S-LPS) of *Brucella* genus, but may also cross-react with antigenic domains of other Gram-negative species (89), so the interpretation of IHC positivity was carefully performed, and considered association with lesions and additional test results. As we could not isolate *Brucella* spp. from tested samples, further characterization of the *Brucella* strains involved in positive cases and the microbiological confirmation of the infection

in cases presenting *Brucella*-type lesions was precluded. Decomposition and autolysis phenomena together with postmortem bacterial overgrowth, also probably hampered our culture attempts, as described in *T. truncatus* (52) and Hector's dolphins (*Cephalorhynchus hectori*) (51). Prolonged pre-analytical frozen time intervals might also have affected negatively bacterial viability.

While most of the samples were from the States of Ceará and São Paulo, 11/13 (84.6%) of *Brucella* exposed or PCR/qPCR positive individuals were from the State of Ceará, suggesting a dissemination pattern of the disease and a plausible susceptibility of animals in this region, in special the species *S. clymene* and *S. longirostris*, which in turn represented 4/13 (30.8%) of positives. *Brucella ceti* was confirmed in a *S. clymene* in Brazil, involving an individual that stranded approximately 760 km away from the former case, also in Northeastern Brazil (29). Susceptibility for *B. ceti* infection have been suggested in *S. coeruleoalba*, another member of this genus (23).

To the knowledge of the authors, this study constitutes the first report of *Brucella* sp. in *P. blainvillei* and *S. guianensis*, both coastal species highly endangered by bycatch and pollution in Brazil (90,91). We must emphasize that the Franciscana dolphin is listed in the national Red List, the Appendix II to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and in Appendices I and II of the Convention for the Conservation of Migratory Species of Wild Animals (CMS) (90). This is of high concern considering also previous studies that demonstrated increased susceptibility to *Brucella* infection as result of negatively impacted ecosystems, for example as consequence of an oil spill (52).

In agreement with the literature, *Brucella*-exposure was found in *F. attenuata*, *P. electra* and *S. longirostris* (92). Despite *Brucella* spp. DNA was not found in this species, exposure was also found in three *G. macrorhynchus*, one of them suspect of *Brucella*-in utero infection. *B. ceti*-associated meningoencephalitis have been reported before in long-finned pilot whale (*Globicephala melas*), another member of the genus *Globicephala* (93,94). We also found *Brucella* spp. exposure in *T. truncatus*. This species shown before to be *Brucella*-seropositive and *Brucella* spp. was isolated from a purulent blubber abscess (85).

Epidemiological studies about the risks factors for *Brucella*-infection in marine mammals have not yet been performed, and the role of environmental factors in this

emerging disease is unknown (95). In general, inshore and estuarine cetaceans are at higher risks of infectious diseases than pelagic cetaceans due to habitats compromised by anthropogenic environmental changes (95). Greater density of animals increases the possibility of exposing susceptible animal populations to the source of infection, highlighting the role of animal agglomerations in the dissemination of diseases (96). According to this, it can be inferred that intra or inter specific contact between any infected cetacean may contribute to the maintenance of the infection in natural environment. Regarding age, sexually mature small cetaceans in Peru were more frequently seropositive than immatures, suggesting an age influence (95). This could be expected once, *Brucella*-type lesions, as placentitis, metritis and orchitis, are more oftenly reported in mature sexually animals (21,50,51). Nevertheless, more clues are necessary and the source, role of natural reservoirs, environmental factors as well as additional ones influencing the epidemiology of the disease in the Northeastern Brazil should be further investigated.

Our results demonstrate the presence of marine *Brucella* in stranded cetaceans of Brazil. Given the demonstrated zoonotic potential of this bacterium (11,12), and a case of laboratory acquired infection (13), proper handling (including use of personal protective equipment, avoid contact between general public and the stranded animals) during rescue, rehabilitation, necropsies and laboratorial procedures, and education is necessary. In special in the State of Ceará, which have a high occurrence of live-stranded animals, that are treated and released when possible (Meirelles, C. per. com. 2017). In those cases, culture, serology and/or additional tests should be performed whenever as possible. Considering the high number of potential marine *Brucella* hosts (17) and the fact *Brucella* infection is endemic for some species, as pinnipeds (97,98), further research efforts should be focused on bacterial culture and characterization of the circulating *Brucella* strains, not only cetacean but also pinniped and other aquatic organisms in Brazil. Our results raise public health concerns.

ACKNOWLEDGMENTS

We acknowledge Caterina Guzman-Verri (Universidad Nacional, Heredia, Costa Rica), Rocio Gonzalez-Barrientos and Gabriela Hernandez-Mora (Servicio Nacional de Salud Animal, Ministerio de Agricultura y Ganadería, Heredia, Costa

Rica) for gently provide the positive controls. We also acknowledge Sandara Squario, Jorge Oyakawa and Mayara Caiaffa, Giorgia Corazzola, Nubia Soares and Julian Céspedes, students from LAPCOM, for their help during the analyses. This research was partially supported by a Small Grant in Aid of Research from the Society for Marine Mammalogy (2015 and 2016). We acknowledge all the partnership institutions and collaborators from Associação de Pesquisa e Preservação de Ecossistemas Aquáticos - Aquasis, Projeto Biopesca, Instituto Baleia Jubarte-IBJ, Projeto Toninhas Univille, Projeto Maqua, Projeto Baleia Franca-PBF, R3 Animal, Aquario de Santos, Gemars, Atlantis, Instituto Oceanografico USP, Instituto de Pesquisas Ecologicas-IPEC, Universidade Estadual de Santa Catarina-UDESC, for kindly provide samples to the MMTB/LAPCOM/VPT/FMVZ. The dolphin rescue and necropsy was performed by Aquasis' Projeto Manatí, sponsored by Petrobras. Conselho Nacional de Desenvolvimento Científico e Tecnológico–CNPq (Brazil) provided scholarship to M.J.C. (PQ 313419/2014-0) and J.L.C.-D (Grant No. 305349/2015-5). This study was conducted by A.M.S-S as part of her PhD at Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil, with support from a scholarship from Coordenação de Apoio à Pesquisa de nível superior-CAPEs. A.M.S-S also acknowledge Banco Santander for the scholarship received to carry out the internship abroad.

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Supplementary Table 1. Cetaceans tested by PCR and real-time PCR. Tissues tested are listed along with form of conservation of the samples, extraction method and results.

No	Species	Sex	Length (cm)	Age class	Origin	Location	Brazilian State	Date	Tissues	Extraction method	Conventional PCR	Real-time PCR
1	<i>Stenella clymene</i>	F	189.2	A	S	N.I	RS	09-Apr-01	liver, spleen ¹ SNC ¹	PH PH	- -	NA -
2	<i>Pontoporia blainvillei</i>	M	113.7	A	B	Cananéia	SP	12-Sep-98	lung ¹	PH	-	NA
3	<i>Pontoporia blainvillei</i>	M	124	A	B	Tupi em terra, Praia Grande	SP	09-Nov-01	pool liver+intestine+lung+ cerebrum ²	K	-	NA
4	<i>Pontoporia blainvillei</i>	M	69	NC	B	Tupi em terra, Praia Grande	SP	23-Nov-01	pool kidney+lung+intestine ²	K	-	NA
5	<i>Pontoporia blainvillei</i>	M	117	A	B	Aviação, Praia Grande	SP	25-Apr-02	pool kidney+liver ²	K	-	NA
6	<i>Pontoporia blainvillei</i>	M	57.5	NC	S	Itamambuca (Ubatuba)	SP	20-Dec-02	pool kidney+liver+lung+inte stine+spleen+spinal cord ²	K	-	NA
7	<i>Pontoporia blainvillei</i>	M	93.3	J	B	Guarujá	SP	14-May-01	lung ¹	PH	-	NA
8	<i>Pontoporia blainvillei</i>	F	130	A	S	N.I	SP	16-Jun-99	lung ¹	PH	-	NA
9	<i>Sotalia guianensis</i>	F	168	A	B	Praia Grande	SP	06-Jul-04	spinal cord ¹ liver, spleen ¹	PH PH	- -	- NA
10	<i>Eubalaena australis</i>	F	14.25	A	S	Itapirubá Sul, Laguna	SC	14-Sep-10	lymph node ²	K	-	-
									lung, lymph node ²	PH	-	NA
									kidney, liver ²	K	-	NA
11	<i>Eubalaena australis</i>	M	430	C	S	Campo Bom, Jaguaruna	SC	17-Sep-10	blood, lung ²	PH	-	NA
12	<i>Peponocephala electra</i>	F	218	J	S	Barro preto, Aquiraz	CE	28-May-10	spleen ²	PH	-	-
									mediastinal lymph node ²	K	-	-
									lung, mandibular lymph node, mesenteric lymph node, pulmonary lymph node, pre-scapular lymph node ²	PH	-	NA

										liver, spinal cord, spleen ²	K	-	NA
										spleen, mesenteric lymph node ²	K	-	-
13	<i>Stenella longirostris</i>	F	172	A	S	Abreulândia, Fortaleza	CE	05-Aug-10		lung, lymph node, mesenteric lymph node, spleen ²	PH	-	NA
										lung, spleen ²	PH	-	NA
14	<i>Sotalia guianensis</i>	F	122	C	S	Tabuba, Caucaia	CE	18-Aug-10		cerebrum, liver ²	K	-	NA
										lung, spleen ²	K	-	-
15	<i>Physeter macrocephalus</i>	F	407	NC	S	Praia da Galheta, Laguna	SC	21-Feb-11		blood, lung, spleen ²	PH	-	NA
										cerebrum, liver, spinal cord ²	K	-	NA
										spleen ²	K	-	-
16	<i>Pontoporia blainvillei</i>	M	91	C	B	Praia Grande, Santos	SP	23-Jun-11		cerebrum, lung, mediastinal lymph node ²	K	-	NA
										blood, lung, mesenteric lymph node, spleen ²	PH	-	NA
										spleen ²	K	-	-
17	<i>Pontoporia blainvillei</i>	F	141	A	B	Praia grande, Canto do Forte	SP	23-Jun-11		blood, lung, mesenteric lymph node, spleen ²	PH	-	NA
										cerebrum+cerebellum, lung, mediastinal lymph node, pre-scapular lymph node ²	K	-	NA
18	<i>Pontoporia blainvillei</i>	F	26.5	Fetus	B	Praia grande, Canto do Forte	SP	23-Jun-11		amniotic fluid, lung ²	PH	-	NA
										placenta ²	K	-	NA
										spleen ²	K	<i>Achromobacter</i> sp.	-
										liver ²	K	-	<i>Bradyrhizobium</i> sp.
19	<i>Delphinus delphis</i>	M	208	A	B	Praia Grande	SP	12-Jul-11		blood, cervical lymph node, lung, mediastinal lymph node, mesenteric lymph node, pancreatic lymph node, pulmonary lymph node, spleen ²	PH	-	NA

									mediastinal lymph node, mesenteric lymph node ²	K	-	-
									cerebellum, skin, skin lesions ²	K	-	NA
20	<i>Pontoporia blainvillei</i>	M	82	C	B	Praia Grande	SP	25-Jul-11	blood, lung, mediastinal lymph node, mesenteric lymph node, pre-scapular lymph node, spleen ²	PH	-	NA
21	<i>Pontoporia blainvillei</i>	M	111	A	B	Praia Grande	SP	31-Aug-02	lung, spinal cord ²	PH	-	NA
									spleen ²	K	-	NA
22	<i>Mesopodion europeus</i>	F	422	A	S	Praia do Preá, Cruz	CE	27-Jan-11	amniotic fluid, placenta, spleen, uterus ²	PH	-	NA
									lung, placenta, uterus, spleen ²	K	-	NA
23	<i>Stenella clymene</i>	M	199	A	S	Canoa Quebrada, Aracati	CE	12-Mar-11	blood, spinal cord, mesenteric lymph node, spleen ²	PH	-	NA
									lung, spleen ²	K	-	NA
									liver ²	K	-	-
24	<i>Pontoporia blainvillei</i>	M	106	A	S	Ubatuba	SP	22-Oct-01	liver, lung ²	PH	-	NA
25	<i>Pontoporia blainvillei</i>	M	87	C	B	Praia Grande	SP	06-Oct-11	blood, lung, pulmonary lymph node, spleen ²	PH	-	NA
									cerebrum+cerebellum, spinal cord ²	K	-	NA
									spleen ²	K	-	-
26	<i>Pontoporia blainvillei</i>	F	97	J	B	Praia Grande	SP	08-Oct-11	blood, lung, lymph node, mesenteric lymph node, spleen ²	PH	-	NA
									cerebrum+cerebellum, mediastinal lymph node ²	K	-	NA
									lymph node ²	K	<i>Achromobacter</i> sp.	-
27	<i>Pontoporia blainvillei</i>	M	114	A	B	Boutique, Praia Grande	SP	18-Oct-11	blood, lymph node ²	PH	-	NA
									cerebrum, mediastinal lymph node, mesenteric lymph node, penile ulcer ²	K	-	NA
28	<i>Pontoporia</i>	M	85	C	N.I	Boutique,	SP	18-Oct-11	lymph node ²	K	-	NA

	<i>blainvillei</i>					Praia Grande			lymph node ²	PH	-	-
									blood, lung ²	PH	-	NA
									mesenteric lymph node ²	PH	-	NA
									cerebrum, mediastinal lymph node ²	K	-	NA
29	<i>Pontoporia blainvillei</i>	M	120	A	B	Itanhaém	SP	16-Dec-00	lung ²	PH	-	NA
									hepatic lymph node, liver, lung, mesenteric lymph node, pre-scapular lymph node ²	PH	-	NA
30	<i>Pontoporia blainvillei</i>	F	62	NC	B	Praia Grande	SP	09-Nov-11	mesenteric lymph node ²	K	-	<i>Brucella</i> sp.
									blood ²	PH	-	-
									cerebrum+cerebellum, hepatic lymph node, kidney, liver, lung, spinal cord, pre-scapular lymph node ²	K	-	-
31	<i>Pontoporia blainvillei</i>	M	66	NC	B	Praia Grande	SP	14-Nov-11	cerebrum, cervical lymph node ²	K	-	NA
									blood ²	PH	-	NA
32	<i>Pontoporia blainvillei</i>	F	139	A	B	Ocian, Praia Grande	SP	09-Jan-12	cerebrum+cerebellum, cervical lymph node, mediastinal lymph node ²	K	-	NA
33	<i>Sotalia guianensis</i>	M	143	J	S	Tabuba, Caucaia	CE	12-Nov-10	lung, mesenteric lymph node ²	K	-	NA
34	<i>Sotalia guianensis</i>	F	181	A	S	Pacheco, Caucaia	CE	07-Sep-11	liver, lung ²	K	-	NA
35	<i>Stenella clymene</i>	M	201	A	S	Jericoacoara, Jijoca de Jericoacoara	CE	25-Mar-12	cerebrum, spleen ²	K	-	NA
									lung, mesenteric lymph node, tongue lesion ²	K	-	-
36	<i>Kogia breviceps</i>	M	145	C	S	Praia da Baleia, Itaipoca	CE	29-May-12	spleen ²	K	-	-
									blood, lung, spleen ²	PH	-	NA
									cerebrum ²	K	-	NA
37	<i>Sotalia guianensis</i>	F	100	C	S	Praia do Uraú, Beberibe	CE	24-Oct-11	liver ²	K	-	NA
38	<i>Stenella clymene</i>	M	175	A	S	Parajuru, Beberibe	CE	10-Mar-12	spleen ²	K	<i>Achromobacter</i> sp.	-
									lung ²	K	<i>Brucella</i> sp.	<i>Brucella</i> sp.

									lung ²	PH	<i>Brucella</i> sp.	<i>Brucella</i> sp.
									cerebrum, heart, kidney, liver, mesenteric lymph node, small intestine ²	K	-	-
									blood ²	PH	-	-
39	<i>Pontoporia blainvillei</i>	F	59	NC	S	Riviera de São Lourenço, Bertioga	SP	17-Nov-12	lung ²	K	-	-
									lung ²	PH	-	NA
									cerebrum, spinal cord ²	K	-	NA
40	<i>Pontoporia blainvillei</i>	F	83	C	S	Praia do Luz, Imbituba	SC	21-Nov-12	cerebrum, lung ²	K	-	NA
41	<i>Pontoporia blainvillei</i>	M	124	A	S	Itapirubá Norte, Imbituba	SC	23-Feb-12	liver ²	K	-	NA
42	<i>Sotalia guianensis</i>	F	98	C	B	Baía de Sepetiba	RJ	19-Aug-10	liver ²	K	-	NA
43	<i>Stenella frontalis</i>	F	194	A	N.I	Baía de ilha grande (Praia das laranjeiras-Trindade)	RJ	27-Apr-12	lung ²	K	-	NA
44	<i>Globicephala macrorhynchus</i>	M	290	J	S	Praia do Preá, Cruz	CE	10-Jan-12	kidney, liver, lung, spleen ²	K	-	-
									lung ²	PH	-	NA
45	<i>Globicephala macrorhynchus</i>	M	410	A	S	Praia das agulhas, Fortim	CE	13-Jun-11	liver ²	K	-	NA
46	<i>Sotalia guianensis</i>	F	102	C	S	Praia do Diogo, Beberibe	CE	20-Jun-11	cerebrum+cerebellum, liver ²	K	-	NA
									blood ²	PH	-	NA
47	<i>Peponocephala electra</i>	F	228	A	S	Praia da Emboaca, Trairi	CE	08-Jul-11	cerebrum, heart, kidney, liver, lung, mesenteric lymph node, pre-scapular lymph node, spleen ²	K	-	-
									pulmonary lymph node ²	K	<i>Achromobacter</i> sp.	-
									blood ²	PH	-	-
									blood ²	PH	-	NA
48	<i>Pontoporia blainvillei</i>	F	134	A	B	Baía da Babitonga, São Francisco do	SC	10-Apr-13	cerebrum, lung ²	K	-	NA

Sul													
49	<i>Pontoporia blainvillei</i>	M	83	C	B	Baía da Babitonga, São Francisco do Sul	SC	10-Apr-13	cerebrum, lung, mesenteric lymph node ²	K	-	NA	
									blood ²	PH	-	NA	
50	<i>Pontoporia blainvillei</i>	M	112	A	B	Baía da Babitonga, São Francisco do Sul	SC	10-Apr-13	lung, mesenteric lymph node ²	K	-	-	
									cerebrum, mediastinal lymph node ²	K	-	NA	
51	<i>Pontoporia blainvillei</i>	M	103	A	S	Praia Grande	SP	31-Jul-13	blood ²	PH	-	NA	
									mediastinal lymph node, mesenteric lymph node, para-rectal lymph node, pool SNC, pre-scapular lymph node ²	K	-	NA	
52	<i>Pontoporia blainvillei</i>	M	113.5	A	B	Praia Grande	SP	06-Nov-13	cerebrum+cerebellum, testis ²	K	-	NA	
53	<i>Pontoporia blainvillei</i>	M	104	A	B	Praia Grande	SP	06-Nov-13	cerebrum, lung, pulmonary lymph node ²	K	-	-	
54	<i>Kogia sima</i>	M	222	A	S	Praia das Moitas, Icarai de Amontada	CE	02-Aug-12	cerebrum+cerebellum, lung ²	K	-	NA	
									spleen ²	K	-	-	
									blood ²	PH	-	NA	
55	<i>Kogia sima</i>	F	208	A	S	Praia das Agulhas, Fortim	CE	29-Mar-13	lung, placenta, pool SNC, uterus ²	K	-	NA	
									blood ²	PH	-	NA	
56	<i>Peponocephala electra</i>	M	239	A	S	Praia do Riacho Doce, Cruz	CE	01-Oct-12	pulmonary lymph node ²	K	-	NA	
									blood ²	PH	-	NA	
									lung ²	PH	-	NA	
57	<i>Kogia sima</i>	F	220	A	S	Barra do Ceará, Fortaleza	CE	04-Jun-13	lung, pool SNC ²	K	-	NA	
									blood ²	PH	-	NA	
58	<i>Peponocephala electra</i>	M	203	J	S	Praia de Águas Belas, Cascavel	CE	28-Mar-13	blood ²	PH	-	NA	
59	<i>Kogia sima</i>	M	118	C	S	Barra do Ceará,	CE	04-Jun-13	lung ²	K	<i>Acromobacter</i> sp.	NA	
									cerebrum ²	K	-	NA	

						Fortaleza			blood ²	PH	-	NA
60	<i>Peponocephala electra</i>	M	110	C	S	Praia de Canoa Quebrada, Aracati	CE	05-Sep-12	blood ²	PH	-	NA
61	<i>Grampus griseus</i>	F	254	J	S	Praia Retirinho, município de Aracati	CE	20-Jun-12	cerebrum+cerebellum, lung, pulmonary lymph node ²	K	-	NA
									blood ²	PH	-	NA
62	<i>Pontoporia blainvillei</i>	F	110	J	S	Boutique, Praia Grande	SP	11-Aug-12	pararectal lymph node ²	K	-	NA
63	<i>Pontoporia blainvillei</i>	M	110	A	B	Boutique, Praia Grande	SP	22-Jan-14	mediastinal lymph node, pre-scapular lymph node ²	K	-	NA
64	<i>Pontoporia blainvillei</i>	M	82	C	B	Boutique, Praia Grande	SP	22-Jan-14	lung ²	K	-	NA
65	<i>Pontoporia blainvillei</i>	M	81.5	C	B	Astúrias próximo ao farol Moela, Guarujá	SP	31-Jan-14	lung, spinal cord, mesenteric lymph node, pre-scapular lymph node, pulmonary lymph node ²	K	-	NA
66	<i>Pontoporia blainvillei</i>	F	71	NC	B	Praia Grande	SP	19-Mar-14	cerebrum+cerebellum, lung, mediastinal lymph node+thymus, mesenteric lymph node, pre-scapular lymph node ²	K	-	NA
									blood ²	PH	-	NA
67	<i>Kogia sima</i>	M	200	A	S	Santos	SP	04-May-14	medullary bulb, cortex, epididymis, eye, intestines, kidney, liver, lung, spinal cord, mesenteric lymph node, pre-scapular lymph node, testis ²	PH	-	NA
68	<i>Pontoporia blainvillei</i>	M	N.I	N.I	N.I	Cananéia	SP	N.I	tracheobronchial lymph node ²	K	-	NA
69	<i>Stenella longirostris</i>	M	225	A	S	Praia Grande, São Francisco do Sul	SC	20-Aug-12	mesenteric lymph node, pulmonary lymph node, testis ²	K	-	NA

70	<i>Sotalia guianensis</i>	M	183	A	S	Praia de Ubatuba, São Francisco do Sul	SC	02-Mar-13	lung, mesenteric lymph node, pulmonary lymph node, testis ²	K	-	NA
71	<i>Balaenoptera edeni</i>	M	1320	A	S	Praia do Molhe, São Francisco do Sul	SC	23-Jun-14	blood ² lung, lymph node, mesenteric lymph node, testis ²	PH K	- -	NA NA
72	<i>Pontoporia blainvillei</i>	M	88	C	S	São Francisco do Sul	SC	03-Aug-13	blood ² lung, mesenteric lymph node, pool SNC ²	PH K	- -	NA NA
73	<i>Sotalia guianensis</i>	M	109	J	S	São Francisco do Sul	SC	15-Oct-13	cerebrum, mesenteric lymph node, pulmonary lymph node ²	K	-	NA
74	<i>Stenella frontalis</i>	N.I	190	A	S	Praia de Itaguaçu, São Francisco do Sul	SC	29-Jun-12	cerebellum, lung, spinal cord, pre-scapular lymph node, pulmonary lymph node ²	K	-	NA
75	<i>Arctocephalus tropicalis</i>	M	157	A	S	São Francisco do Sul	SC	N.I	laryngeal lymph node, lung, mandibular lymph node, mesenteric lymph node, tracheal lymph node ²	K	-	NA
76	<i>Tursiops truncatus</i>	M	257	A	S	Portinho, Laguna	SC	18-Oct-14	cerebrum, lung, mesenteric lymph node, pararectal lymph node, pre-scapular lymph node, pulmonary lymph node ²	K	-	NA
77	<i>Pontoporia blainvillei</i>	F	86	C	S	Ribanceira, Imbituba	SC	08-Nov-11	blowhole swab, vaginal swab ²	K	-	NA
78	<i>Arctocephalus australis</i>	M	93	J	S	Morro das Pedras	SC	23-Aug-14	lung ²	K	-	NA
79	<i>Arctocephalus australis</i>	F	N.I	C	S	Campeche	SC	21-Sep-14	lung ²	K	-	NA
80	<i>Sotalia guianensis</i>	M	190	A	S	Pontal do Ipiranga, Linhares	ES	10-Feb-14	aqueous humor ²	PH	-	NA

81	<i>Pontoporia blainvillei</i>	F	120	J	S	Guriri, São Mateus	ES	11-Feb-14	aqueous humor ²	PH	-	NA
82	<i>Sotalia guianensis</i>	M	157	J	S	Praia de Degredo, Pontal do Ipiranga	ES	01-Aug-14	aqueous humor ²	PH	-	NA
83	<i>Sotalia guianensis</i>	F	183	A	N.I	Baía de Sepetiba, Ilha de Jaguanum	RJ	09-Feb-10	blood ²	PH	-	NA
84	<i>Stenella frontalis</i>	N.I	N.I	N.I	N.I	N.I	RJ	N.I	abscess ²	K	-	-
85	<i>Sotalia guianensis</i>	M	170	A	N.I	Baía de Sepetiba, Ilha de Jaguanum	RJ	28-May-11	lung ¹	PH	-	NA
86	<i>Stenella attenuata</i>	M	198	A	S	Praia de Curimãs, Barroquinha	CE	04-Feb-14	axilar lymph node, cerebrum, lung ²	K	-	NA
87	<i>Stenella longirostris</i>	M	121	J	S	Praia de Quitérias, Icapuí	CE	02-Apr-15	kidney, liver, spleen	K	-	-
									heart, lung, meninges	K	-	<i>Brucella</i> sp.
									liver, lung, meninges, spleen	PH	-	NA
									blood ²	PH	-	-
88	<i>Stenella</i> sp.	M	92	C	S	Praia do Estevão, Acaratí	CE	04-Jun-15	blood ²	PH	-	NA
89	<i>Sotalia guianensis</i>	F	169	A	B	Itacuruca, Mangaratiba	RJ	22-Dez-12	blood ²	PH	-	NA
90	<i>Sotalia guianensis</i>	F	119	C	S	Praia da Taiba, São Gonçalo do Amarante	CE	12-Jun-15	cerebrum, lung	K	-	NA
									blood ²	PH	-	NA
91	<i>Peponocephala electra</i>	M	121	C	S	Praia da Cofeco, Fortaleza	CE	30/12/14	cerebrum + spinal cord+medullary bulb, lung, mandibular lymph node, pre-scapular lymph node+lymph node, spleen ²	K	-	NA
92	<i>Feresa attenuata</i>	M	213	A	S	Praia da Taiba, São Gonçalo do Amarante	CE	14-Apr-15	blood ²	PH	-	-
									cerebrum+cerebellum, heart, kidney, liver, lung, spinal cord, pre-scapular lymph node, spleen ²	K	-	-

93	<i>Tursiops truncatus</i>	F	271	A	S	Praia da Abreulandia, Fortaleza	CE	06/07/15	cerebrum, heart, kidney, liver, lung, mesenteric lymph node, pre-scapular lymph node, urinary bladder, spleen ²	K	-	-
94	<i>Sotalia guianensis</i>	M	175	A	S	Praia do Estevão, Acarati	CE	23-May-14	lung ²	K	-	NA
95	<i>Stenella frontalis</i>	M	200	A	S	Praia Grande	SP	06-Oct-15	cerebrum, liver, lung, pulmonary lymph node, spleen ²	PH	-	NA
96	<i>Tursiops truncatus</i>	M	146	C	S	Tororão, Prado	BA	28-Jan-15	lung ²	K	-	NA
97	<i>Megaptera novaeangliae</i>	M	450	NC	S	Praia do Terminal, Belmonte	BA	22-Aug-12	lung ²	K	-	NA
98	<i>Megaptera novaeangliae</i>	M	1300	A	S	Barra Velha, Nova Viçosa	BA	11-Jun-13	blood ²	PH	-	NA
99	<i>Megaptera novaeangliae</i>	M	383	C	S	Alcobaça	BA	05-Jul-08	lung ²	K	-	NA
100	<i>Sotalia guianensis</i>	F	116	C	S	Pontal do Ipiranga, Linhares	ES	06-Jan-15	blood, kidney, liver ²	K	-	NA
101	<i>Physeter macrocephalus</i>	N.I	700	J	S	Guriri, São Mateus	ES	02-Dec-13	lung ²	K	-	NA
102	<i>Peponocephala electra</i>	M	228	A	S	Arraial D'Ajuda, Porto Seguro	BA	09-Apr-11	liver ²	K	-	-
103	<i>Sotalia guianensis</i>	F	172	A	S	Guriri, São Mateus	ES	26-Feb-10	liver ²	K	-	-
104	<i>Megaptera novaeangliae</i>	M	390	C	S	Itaúnas, Conceição da Barra	ES	14-Aug-08	liver ²	K	-	NA
105	<i>Physeter macrocephalus</i>	M	410	NC	S	Belmonte	BA	19-Mar-15	lung ²	K	-	NA
106	<i>Pontoporia blainvillei</i>	M	63	NC	S	Guriri, São Mateus	ES	12-May-11	lung ²	K	-	NA
107	<i>Arctocephalus tropicalis</i>	M	151	J	S	Corumbau, Prado	BA	14-Aug-10	liver, lung ²	K	-	NA
108	<i>Megaptera novaeangliae</i>	F	918	J	S	Guriri, São Mateus	ES	04-Jul-09	liver kidney, liver ²	K PH	- -	- NA
109	<i>Sotalia</i>	F	137	J	S	Cumuruxatiba	BA	14-Nov-08	liver ²	K	-	-

	<i>guianensis</i>					, Prado						
110	<i>Sotalia guianensis</i>	M	172	A	S	Praia do Mangue Alto (Km 16.5), Belmonte	BA	27-Sep-11	lung ²	K	-	NA
111	<i>Megaptera novaeangliae</i>	M	428	NC	S	Praia da Cacimba, Povoação	ES	16-Sep-10	liver ²	K	-	-
112	<i>Megaptera novaeangliae</i>	F	320	NC	S	Praia dos Coqueiros, Trancoso	BA	25-Sep-12	liver, lung ²	K	-	NA
113	<i>Sotalia guianensis</i>	F	108	C	S	Guriri, São Mateus	ES	06-Dec-10	cerebrum, genitalia, liver, spleen	K	-	-
									lymph node	K	<i>Brucella</i> sp.	-
									lymph node	PH	-	<i>Brucella</i> sp.
									blood, kidney ²	PH	-	-
114	<i>Peponocephala electra</i>	F	230	A	S	Praia de Caetanós, Amontada	CE	05-Nov-14	blood ²	PH	-	NA
115	<i>Peponocephala electra</i>	M	240	A	S	Praia das Fontes, Beberibe	CE	07-Nov-14	blood ²	PH	-	-
116	<i>Peponocephala electra</i>	F	246	A	S	Praia das Fontes, Beberibe	CE	07-Nov-14	blood ²	PH	-	-
117	<i>Kogia sima</i>	M	192	J	S	Praia da Lagoinha, Paraipaba	CE	08-Aug-15	lung, pool SNC ²	K	-	NA
118	<i>Sotalia guianensis</i>	M	178	A	B	Praia de Taiba, Sao Goncalo do Amarante	CE	09-Dec-15	lung ²	K	-	NA
119	<i>Trichechus manatus</i>	F	121	C	S	Praia de Alagamar, Grossos	RN	24-Mar-15	liver, lung ²	K	-	NA
120	<i>Stenella clymene</i>	M	170	A	S	Praia do Japao, Aquiraz	CE	10-Jan-16	cerebrum+cerebellum +spinal cord, heart, kidney, liver, lung, mesenteric lymph node, spleen ²	K	-	-
121	<i>Kogia sima</i>	M	215	A	S	Praia de Almofala, Itarema	CE	07-Feb-16	lung ²	K	-	NA
122	<i>Feresa</i>	F	113	J	S	Praia do	CE	04-May-16	lung ²	K	-	NA

<i>attenuata</i>						Prea, Cruz						
123	<i>Stenella clymene</i>	F	176	A	S	Praia de Águas Belas, Cascavel	CE	25-May-16	heart, kidney, liver, lung, spinal cord, pulmonary lymph node, spleen ²	K	-	-
124	<i>Stenella attenuata</i>	F	178	J	S	Praia de Cana Brava, Trairi	CE	25-May-16	lung ²	K	-	NA
125	<i>Globicephala macrorhynchus</i>	F	157	NC	S	Vila Nova, Icapui	CE	9-Jul-16	cerebrum+cerebellum + spinal cord, heart, kidney, liver, lung, mesenteric lymph node, pulmonary lymph node, spleen ²	K	-	-
126	<i>Stenella attenuata</i>	F	106	C	S	Praia de Quixaba, Aracati	CE	23-Jul-16	lung ²	K	-	NA
127	<i>Sotalia guianensis</i>	F	78	C	S	Porto das Dunas, Aquiraz	CE	09-Nov-16	lung ²	K	-	NA
128	<i>Globicephala macrorhynchus</i>	F	130	NC	S	Praia da Tabuba beach, Caucaia	CE	25-Jan-17	cerebrum+cerebellum + spinal cord, heart, kidney, liver, lung, mesenteric lymph node, pulmonary lymph node, spleen ²	K	-	-
129	<i>Peponocephala electra</i>	F	225	A	S	Lagoinha, Paraipaba	CE	11-Feb-17	lung, mesenteric lymph node, pulmonary lymph node ²	K	-	NA

F=Female; M=Male; A=Adult; J=Juvenile; C=Calf; NC=Newborn calf; S=Stranding; B=Bycatch; CE=State of Ceara; BA= State of Bahia; ES= State of Espirito Santo, RJ= State of Rio de Janeiro; RN= State of Rio Grande do Norte; RS= State of Rio Grande do Sul; SC= State of Santa Catarina; SP= State of Sao Paulo; N.I=Not informed; ¹preserved in formalin; ²frozen at -20 °C or -80 °C. Extraction method: PH= CTAB Phenol chloroform method; K= DNeasy Blood & Tissue Kit (QIAGEN®, Valencia, CA); -=Negative; NA=Not analyzed.

Supplementary Table 2. Gross and microscopical findings observed in the PCR/qPCR- and/or serology *Brucella*-positive cetaceans

Case No	Macroscopic findings	Histopathological findings
No 1 <i>Feresa attenuata</i>	<p>External examination: multifocal healed cookie-cutter shark (<i>Isistius brasiliensis</i>) bites; corneal ulceration (right eye); <i>Phyllobothrium delphini</i> encysted in blubber of perianal region</p> <p>Lungs: difusse edema and congestion, and apical atelectasis</p> <p>Heart: depletion of coronary fat; congestion and thickening of the mitral valve with adhered thrombus in the tendinous cords and left atrium; focal mural hemorrhage in left ventricle</p> <p>Spleen: splenomegaly</p> <p>Scapular, mesenteric and pulmonary lymph nodes: lymphadenomegaly</p> <p>Abdominal cavity: <i>Monorygma grimaldii</i> encysted in peri testicular ligaments</p> <p>Gastrointestinal system: multifocal mucosal congestion on glandular stomach; multifocal petechiae in the pyloric stomach and proximal small intestine</p> <p>Omentum: opaque, presenting light tanned spots</p> <p>Liver: hepatomegaly and congestion</p> <p>Kidney: perirrenal edema; mild bilateral hydronephrosis</p> <p>Urinary bladder: distended with cloudy, brownish urine; mucosal congestion</p> <p>Adrenal: subcortical hemorrhage (right adrenal)</p> <p>Cranium: white frothy foam and <i>Crassicauda</i> sp. on the left pterygoid sinus; petechiae in the right tympanic bulla</p> <p>CNS: meningeal congestion with scattered hemorrhage in cerebral white mater neuroparenchyma</p>	<p>Cerebellum, cerebrum: mild to moderate, multifocal, chronic lymphoplasmacytic meningoencephalitis with perivascular cuffing and edema</p> <p>Spinal cord: mild to moderate, multifocal lymphoplasmacytic meningitis</p> <p>Lung: mild, multifocal, subacute suppurative and histiocytic bronchopneumonia with mild edema, hemorrhage, and interstitial fibrosis</p> <p>Heart: congestion and scattered hemorrhage</p> <p>Adrenal: mild, diffuse cortical hyperplasia</p> <p>Pulmonary lymph node: mild, diffuse lymphoid depletion with moderate cortical/paracortical hyalinosis and sinus histiocytosis; capsular fibrosis and scattered sinus mineralizations</p> <p>Pre-scapular lymph node: mild sinus histiocytosis; rare sinus mineralizations</p> <p>Lymph nodes (not identified): moderate, multifocal cortical and paracortical hyalinosis and mild interstitial fibrosis; mild multifocal hemosiderosis; mild interstitial fibrosis</p> <p>Mesenteric lymph node: mild, multifocal sinus histiocytosis; multifocal, acute perinodal hemorrhage</p> <p>Mandibular lymph node: mild, diffuse lymphoid reactive hyperplasia and mild sinus histiocytosis</p> <p>Adipose tissue: multifocal, acute hemorrhage</p> <p>Liver: moderate, multifocal, acute bridging centrilobular and midzonal congestion with hepatocyte atrophy, vacuolar degeneration, loss and hemorrhage; mild, multifocal, chronic lymphoplasmacytic pericholangitis</p> <p>Small and large intestine: mild, multifocal, chronic lymphoplasmacytic enterocolitis</p> <p>Spleen: mild, diffuse lymphoid reactive hyperplasia</p> <p>Urinary bladder, pancreas, glandular stomach, keratinized stomach, adipose tissue, testicle, epididymis, spermatic cord: NSLO</p>
No 2 <i>Globicephala macrorhynchus</i>	<p>External examination: small numbers of <i>Cyamus</i> spp. on the spiracle and focal peribuccal ulcer; multifocal deep abrasions on the head (live-stranding); teeth marks (conspecific interaction) on peduncle and dorsal fin; multiple healed cookie-cutter shark (<i>I. brasiliensis</i>) bites; multifocal irregular nodules with slightly defined edges in abdominal region; mild <i>P. delphini</i> encysted in blubber of perianal region</p> <p>Tongue: focal necrotic ulcer (left lateral)</p> <p>Thoracic cavity: mild hydrothorax</p> <p>Respiratory system: tracheal and pulmonary congestion, edema and multifocal atelectasis</p> <p>Heart: mild hydropericardium</p> <p>Abdominal cavity: mild <i>M. grimaldii</i> encysted in ligaments of perianal region</p> <p>Esophagus: multifocal mucosal congestion</p> <p>Anterior and glandular stomachs: fetid brownish liquid; diffuse congestion and scattered necrotic ulcers on mucosa of the glandular stomach</p> <p>Liver: diffuse congestion and mildly enhanced lobular pattern</p> <p>Spleen: mild splenomegaly</p> <p>Urinary bladder: diffuse mucosal congestion</p> <p>Kidney: congestion and multifocal corticomedullary hemorrhage</p> <p>Adrenal: multifocal subcortical hemorrhage</p>	<p>Skeletal muscle: mild, multifocal, acute segmental hyaline myocyte degeneration and necrosis</p> <p>Glandular and pyloric stomach: minimal to mild, multifocal, chronic lymphoplasmacytic gastritis</p> <p>Small and large intestine: mild, multifocal lymphoplasmacytic enterocolitis with moderate apical fibrosis and villi atrophy</p> <p>Tongue: marked, focal, subacute necroulcerative glossitis with thrombosis and myriads intralesional and intravascular bacilli</p> <p>Spleen: mild, diffuse lymphoid reactive hyperplasia, follicular hyalinosis and mild hemosiderosis</p> <p>Liver: mild, diffuse congestion and edema, hepatocyte vacuolar change, eosinophilic globules, pink points and strands, and mild dissociation</p> <p>Kidney: mild, multifocal, acute tubular degeneration; scattered peri-renal hemorrhage</p> <p>Lymph node: mild, diffuse lymphoid depletion; scattered trabecular mineralizations; perinodal edema</p> <p>Heart (including isolated cardiac valve), Unidentified dense connective tissue, Keratinized stomach, Testicle, Testicle, Adrenal: NSLO.</p>

	<p>Cranium: severe infestation by <i>Stenurus</i> sp. in pterygoid sinuses and tympanic bullae; <i>Crassicauda</i> sp. in the left pterygoid sinus</p> <p>CNS: NE</p>	
<p>No 3 <i>Globicephala macrorhynchus</i></p>	<p>External examination: evident folds and umbilical scar; healing bites from cookie-cutter shark (<i>I. brasiliensis</i>) in cervical and thoracic ventral area; subcutaneous emphysema in the dorsal phase of the left lobe of the caudal fin, regurgitation in oral cavity</p> <p>Lungs: mild edema, congestion, and presence of <i>Halocercus</i> sp. and mucus obstructing the airways of the left lung; multifocal calcified nodules and emphysema</p> <p>Pulmonary and mesenteric lymph node: lymphadenomegaly</p> <p>Heart: depletion of coronary fat; apparent increase and dilatation of the aortic arc; a mixed thrombus in right atrium; pale coloration of the endocardium</p> <p>Spleen: splenomegaly and congestion</p> <p>Gastrointestinal system: Bilious content, edema and multifocal hemorrhages on duodenal ampola mucosa; mucous yellowish content in the medial portion of intestines; the final portion contained large amount of greenish liquefied feces; congestion of mesenteric vessels</p> <p>Liver: congestion; mildly enhanced lobular pattern; hepatomegaly</p> <p>Kidneys: apparently enlarged; connective tissue between reniculi opaque and thickened</p> <p>Urinary bladder: mucosal congestion</p> <p>CNS: meningeal congestion and opacity; gaseous emboli in the superficial cerebral blood vessels in the occipital region; soft texture and brain consistency; apparent right ventricle enlargement</p>	<p>Cerebrum: minimal, focal suppurative leptomeningeal infiltrate; scattered hemorrhage; mild, multifocal perivascular edema with astrocytosis; rare foci of gliosis</p> <p>Cerebellum: pachymeningeal and neuroparenchymal hemorrhage</p> <p>Lung: diffuse atelectasia; mild, multifocal, subacute interstitial lymphohistiocytic pneumonia with focal granuloma, scattered larvae, mild edema, rare neutrophils and scattered multinucleated giant cells in alveolar spaces</p> <p>Tonsil: marked lymphoid hyperplasia associated with neutrophilic cryptitis, erosion and exocytosis (tonsillitis)</p> <p>Colon: minimal lymphoplasmacytic infiltrates in the lamina propria</p> <p>Pulmonary and laryngeal lymph nodes: mild, diffuse lymphoid reactive hyperplasia with scattered hemorrhage</p> <p>Trachea: marked, diffuse, chronic lymphoplasmacytic tracheitis with erosion and ulcers</p> <p>Uterus: mucoid plug</p> <p>Liver: centrilobular congestion; scattered areas have portal fibrosis with rare bile duct hyperplasia</p> <p>Kidney: multifocal, acute interrenicular hemorrhage; scattered acute tubular degeneration</p> <p>Spleen: diffuse congestion; mild, diffuse lymphoid depletion</p> <p>Connective and adipose tissue: marked, multifocal, acute hemorrhage</p> <p>Mucosal tissue: serosal hemorrhage</p> <p>Spinal cord, keratinized, glandular and pyloric stomach, small intestine, mesenteric lymph node, ovary, esophagus, heart, skeletal muscle, tongue, adrenal, pancreas: NSLO</p>
<p>No 4 <i>Globicephala macrorhynchus</i></p>	<p>External exam: rectal mucosa with greenish fezes (meconio) and superficial abrasions in the spiracle; dorsal fin curved to the right side; fetal marks present in the left side of the body and caudal fin; vibrissae present; healing umbilical button; teeth marks (conespecific interaction), superficial and deep in various regions of the body; cookie-cutter shark (<i>I. brasiliensis</i>) bites in face and ventro lateral region</p> <p>Oral cavity: hypercoloration of tongue and oral mucosa</p> <p>Respiratory system: tracheal and bronchial multifocal moderate edema; slight pulmonary <i>Halocercus</i> sp. nematodiasis (caudal portion); diffuse pulmonary congestion, multifocal emphysema and atelectasia (dorsal surface) and hemorrhage; lymphadenomegaly of pulmonary lymph nodes</p> <p>Cardiovascular system: pronounced apex, congenital aortopulmonary septal defect (communication between the pulmonary artery and aorta)</p> <p>Spleen: splenomegaly and diffuse congestion</p> <p>Gastrointestinal system: mesenteric vessels were congested and engorged; anterior stomach containing small amount of milk; bilious content in the pyloric stomach; yellowish pasty content in the initial third of the intestine; greenish gelatinous fezes in the final portion (meconium)</p> <p>Liver: congested and when cut grayish</p> <p>Kidneys: renal capsules opacity and irregular coloration; yellowish when cut</p> <p>Urinary bladder: focal mucosal congestion, next to the opening of the urethra</p> <p>CNS: diffuse congestion of superficial vessels, multifocal hemorrhage; apparent slight dilation of the ventricles</p>	<p>Lung: mild, multifocal, acute to subacute interstitial pneumonia with alveolar histiocytosis associated with bacterial aggregates, rare multinucleate giant cells, edema and scattered hemorrhage</p> <p>Skin: mild, multifocal epidermal hyperplasia with keratinocyte hydropic change and rare intracytoplasmic eosinophilic inclusion bodies</p> <p>Liver: diffuse congestion with mild leukocytosis and mild hepatocellular dissociation; mild, diffuse steatosis and occasional cytoplasmic hyaline globules</p> <p>Cerebellum: focal, acute leptomeningeal hemorrhage and congestion</p> <p>Kidney: mild, multifocal, acute tubular degeneration and congestion; scattered hemorrhage and tubular proteinosis</p> <p>Heart: scattered acute apical epicardial hemorrhage</p> <p>Lymph node: mild, multifocal cortical lymphoid depletion and sinus histiocytosis</p> <p>Spleen: extramedullary hematopoiesis; mild lymphoid depletion</p> <p>Cerebrum: mild, multifocal perivascular edema</p> <p>Small intestine: mild, multifocal villi apical hemosiderosis</p> <p>Uterine cervix/cranial vagina: mucous plug</p> <p>Spinal cord, nerve plexus, colon, urinary bladder, adrenal, esophagus, trachea, pancreas, ovary, nerve, pharynx/esophagus, glandular stomach, uterine horn, skeletal muscle: NSLO</p>

No 7 <i>Pontoporia blainvillei</i>	External examination: marks in maxilla and pectoral fins (net marks), scar on the internal side on peduncle Stomach: milk content Heart: pale coloration Kidneys: corticomedullary congestion	Cerebrum, cerebellum: rare perivascular neuroparenchymal hemorrhage and edema Lymph nodes: mild to moderate, diffuse lymphoid reactive hyperplasia with mild to moderate sinus histiocytosis and minimal hemosiderosis; multifocal acute perinodal hemorrhage Thymus: mild, multifocal, chronic medullary granulomatous thymitis with scattered mineralization; multifocal perithymic hemorrhage Lung: mild, diffuse edema with mild alveolar histiocytosis (binucleated and multinucleated) with focal neutrophilic alveolitis and rare alveolar hemorrhage Urinary bladder: apical congestion and scattered hemorrhage Thyroid: congestion and scattered hemorrhage Trachea: multifocal, acute peritracheal hemorrhage Heart: multifocal, acute intramyocardial hemorrhage Kidney: mild, multifocal, acute tubular degeneration; congestion and multifocal acute inter-renal hemorrhage Liver: mild, diffuse congestion with minimal hepatocellular dissociation and scattered eosinophilic globules with pink points Uterus, ovary, glandular stomach, pancreas, eye: NSLO
No 8 <i>Sotalia guianensis</i>	External examination: multifocal ulcers in the oral mucosa and genital slit Lungs: diffusely dark red coloration and edema Brain: congestion and edema	Cerebrum: multifocal, acute neutrophilic meningoencephalitis with scattered neutrophilic and lymphocytic perivascular cuffing, hypophysitis, astrocytosis, microgliosis and neutrophilic leukocytosis Eye: marked, focally extensive, acute suppurative optic perineuritis with edema; minimal, focal lymphocytic conjunctivitis Lung: mild, multifocal, acute lymphocytic and neutrophilic interstitial pneumonia with edema and scattered neutrophilic and histiocytic alveolar exudate; scattered bronchial/olar mucosal calcifications Liver: mild to moderate, multifocal, subacute necrotizing hepatitis; mild, multifocal, chronic lymphocytic pericholangitis with mild bile duct hyperplasia; mild, multifocal, chronic proliferative serositis Pancreas: minimal, focal periductal lymphocytic infiltrate Spleen: mild, diffuse lymphoid depletion with rare hemosiderosis, fibrosis and hyalinosis (may include mild multifocal neutrophilic splenitis) Colon: minimal to mild, multifocal, chronic lymphocytic colitis with scattered crypt abscesses (cryptitis), ectasia and necrosis Tongue: mild, mucosal hyperplasia with scattered single cell epithelial ballooning degeneration Kidney: interrenal edema Adrenal: minimal, multifocal nodular cortical hyperplasia Skeletal muscle: rare segmental myocyte (hyaline) degeneration Thyroid, spinal cord, cerebellum, heart: NSLO
No 9 <i>Stenella clymene</i>	External examination: scars from cookie-cutter shark (<i>I. brasiliensis</i>) bites; lamprey bite on the lateral abdominal region; multifocal <i>P. delphini</i> encysted in blubber of the caudal peduncle; light tanned soft nodule in the tongue dorsal medial surface and palate Lungs: congestion, an irregular light tanned lesion (1cm) on right lung dorsal surface; moderate edema and <i>Halocercus</i> sp. infestation Abdominal cavity: <i>M. grimaldii</i> encysted in urogenital ligaments Mesenteric lymph node: lymphadenomegaly Glandular stomach: mucosal diffuse hemorrhage and ulceration associated to	Lung: moderate, chronic pyogranulomatous bronchopneumonia with bronchitis, bronchiolitis and adult and nematodes larvae (Pseudalidae), bronchial squamous metaplasia, fibrosis and rare bronchial sclerosis; moderate edema, congestion and hemorrhage, scattered mineralizations in mucosa and submucosa of bronchioles and bronchi, multifocal atelectasia and hemosiderosis Spleen: moderate, diffuse lymphoid depletion, mild eosinophilic splenitis congestion, multifocal hemorrhages and mild hemosiderosis; Mild, multifocal, chronic vascular siderocalcinosis with scattered multinucleated giant cells Pyloric stomach: moderate to focally marked, multifocal, chronic ulcerative and hyperplastic

	<p>intralesional <i>Anisakis</i> sp.</p> <p>Piloric stomach: biliar content and extensive vulcanic ulcer in the mucosa (aprox. 4cm) with intralesional <i>Anisakis</i> sp.</p> <p>Small intestine: congestion in the initial portion of the mucosa; diffuse catarrhal enteritis and edema</p> <p>Large intestine: final portion displaying necrotic aspect (necrohemorrhagic colitis)</p> <p>Liver: hepatomegaly and congestion</p> <p>Spleen: splenomegaly with multifocal subcapsular hemorrhage</p> <p>Kidneys: congestion, capsule adherence</p> <p>Urinary bladder: diffuse mucosal congestion</p> <p>Brain: moderate brain congestion, scatered hemorrhage in cerebral gray matter neuroparenchyma</p>	<p>gastritis with intralesional anisakid adult nematodes and few bacteria, fibrin, necrosis and mild fibrosis</p> <p>Urinary bladder: scattered serosal hemorrhage</p> <p>Kidney: mild to moderate, multifocal, chronic lymphoplasmacytic interstitial nephritis with minimal fibrosis and rare glomerulosclerosis; mild, multifocal, acute tubular degeneration, congestion and scattered hemorrhage</p> <p>Liver: moderate to marked, multifocal, acute centrilobular (bridging) congestion with hepatocyte degeneration and loss and edema; minimal multifocal portal lymphoplasmacytic infiltrates</p> <p>Pancreas: multifocal, acute parenchymal hemorrhage</p> <p>Glandular stomach: mild, multifocal, chronic lymphoplasmacytic gastritis</p> <p>Tongue: marked, focal, subacute necroulcerative glossitis with myriads intralesional bacilli</p> <p>Esophagus: rare submucosal edema and hemorrhage</p> <p>Heart: mild, multifocal acute cardiomyocyte degeneration</p> <p>Small and large intestine: mild to moderate, multifocal eosinophilic enterocolitis</p> <p>Mesenteric lymph node: Mild to moderate, diffuse moderate lymphoid depletion with mild sinus eosinophilia, histiocytosis, moderate pyogranulomatous lymphadenitis with multinucleated giant cells, and interstitial fibrosis</p> <p>Cerebrum: scattered satellitosis; scattered perivascular edema and hemorrhage</p> <p>Skeletal muscle: minimal, focal myocyte basophilic degeneration</p> <p>Testicle, keratinized stomach, skin: NSLO</p>
<p>No 10 <i>Stenella clymene</i></p>	<p>External examination: cookie-cutter shark (<i>I. brasiliensis</i>) bite scars on the ventral abdominal region; bilateral phlebitis on peduncle; <i>P. delphini</i> encysted in blubber of perianal region; edema on left dorso- subcutaneous tissue; epaxial musculature with dark coloration</p> <p>Tongue: circular and dark lesion in the central portion</p> <p>Lungs: congestion, firm with multifocal areas of emphysema and apical atelectasis (dorsal bilateral); infestation by <i>Halocercus</i> sp.</p> <p>Heart: opacity and thickening of superficial coronary vessels; left ventricle rigidity and slight thickening and opacity of tendinous chords and bilateral atrioventricular valves (endocardiosis)</p> <p>Anterior stomach: multifocal healing ulcers</p> <p>Glandular stomach: mucosal congestion</p> <p>Urinary bladder: hemorrhagic mucosa</p> <p>Intestines: apparent edema and congestion; catharral enteritis on the medial segment</p> <p>Adrenal: subcortical hemorrhage (right adrenal)</p> <p>Rectal tonsils: megalia</p> <p>Abdominal cavity: opacity and mild infestation by <i>M. grimaldii</i> encysted in testicular ligaments</p> <p>Omentum: opacity</p> <p>Spleen: splenomegaly and subcapsular hemorrhage</p> <p>Pre-scapular, pulmonar and mesenteric lymph nodes: lymphadenomegaly</p> <p>Liver: congestion; hepatomegaly; multifocal steatosis</p> <p>CNS: NSLO.</p>	<p>Spinal cord: moderate, multifocal, subacute lymphocytic meningitis and polyneuritis with rare chromatolysis</p> <p>Cerebellum, cerebrum: mild, multifocal, acute neutrophilic (suppurative) meningitis with perivascular edema and rare meningeal and parenchymal hemorrhage</p> <p>Skin: marked, focal, acute dermal necrotizing (neutrophilic) vasculitis with thrombosis and hemorrhage</p> <p>Keratinized stomach: moderate to marked, multifocal subacute ulcerative gastritis with fibrinoid vascular necrosis and thrombosis</p> <p>Mesenteric lymph node: marked, diffuse lymphoid depletion and mild hemosiderosis</p> <p>Kidney: mild, multifocal, acute tubular degeneration and minimal proteinosis; mild, multifocal, chronic interstitial fibrosis with rare lymphocytic infiltrates, scattered tubular ectasia and atrophy; multifocal perirrenal and interrenicular acute hemorrhage</p> <p>Lymph node (close to ureter): mild to moderate, multifocal lymphoid depletion with interstitial fibrosis and scattered hemosiderosis</p> <p>Pre-scapular and scapular lymph nodes: mild multifocal lymphoid reactive hyperplasia with scattered areas of depletion, sinus histiocytosis and erythrocytosis and occasional erythrophagia</p> <p>Adrenal: focal periadrenal hemorrhage</p> <p>Urinary bladder: mild interstitial edema with rare hemorrhage</p> <p>Spleen: moderate to marked, diffuse lymphoid depletion</p> <p>Liver: moderate, acute congestion and edema with scattered single cell hepatocyte degeneration; focal intraductal metacestode</p> <p>Glandular stomach: mild diffuse congestion with scattered hemorrhage</p> <p>Melon and mandibular (acoustic) fat: congestion (scattered intravascular round clear spaces)</p> <p>Lung: moderate, multifocal, subacute suppurative bronchopneumonia with intralesional adult nematodes and larvae</p>

		<p>Skeletal muscle: focal, acute intrafascicular vascular fibrinoid necrosis with myonecrosis and hemorrhage</p> <p>Trachea: focal peritracheal hemorrhage</p> <p>Heart: mild multifocal acute degenerative changes with epicardial acute hemorrhage</p> <p>Testicle, pancreas: NSLO</p>
<p>No 11 <i>Stenella clymene</i></p>	<p>External examination: bite scars from cookie-cutter shark (<i>I. brasiliensis</i>) in left lateral abdominal region, the left basis of dorsal fin and basis of right penduncle, including a healing lesion in left peduncle; musculature edema and dark coloration</p> <p>Mouth: fracture of two lower teeth and one upper one</p> <p>Thoracic cavity: bloodish fluid accumulation in pleural cavity; adherence of the cranial portion of the left lung to the pleura</p> <p>Lungs: moderate <i>Halocercus</i> sp. infestation, firm and irregular consistency; multifocal emphysema and atelectasis; congestion, edema and multifocal yellowish nodules (aprox. 0.2 x 0.4)</p> <p>Pulmonary and mesenteric lymph node: lymphadenomegaly</p> <p>Heart: flacid, depletion of coronary fat</p> <p>Spleen: congestion</p> <p>Esophagus: multifocal congestion</p> <p>Anterior stomach: mild infestation by <i>Anisakis</i> sp.</p> <p>Glandular stomach: flacid, mucosal edema and congestion</p> <p>Intestines: multifocal hemorrhages and bilious content in the initial portion, greenish feces in the final portion; mucosa detaching, with orangish coloration</p> <p>Liver: slightly rounded edges and congestion</p> <p>Reproductive system: light tanned mucus in the cervix; no scars in ovaries; <i>M. grimaldii</i> encysted in uterine ligaments</p> <p>Urinary bladder: mucosal focal congestion in the lap</p> <p>Kidneys: thickening and opacity of the renal capsules; coalescence of the reniculi (caudal portion, left kidney); when cut, pale diffuse color and discreet loss of cortical-medullar distinction; opacity of reniculi pelvis, ureteral tubules and connective tissues</p> <p>Adrenals: NSLO</p> <p>Pancreas: AA</p> <p>CNS: NE</p>	<p>Spinal cord: marked, diffuse, subacute lymphocytic meningomyelitis and poliradiculoganglioneuritis</p> <p>Lung: mild, multifocal, subacute suppurative bronchopneumonia with multifocal interstitial mononuclear infiltrates; multifocal bronchial mineralization</p> <p>Kidney: moderate, multifocal, chronic interstitial mononuclear nephritis with fibrosis, nephron loss and atrophy</p> <p>Pulmonary lymph node: mild, multifocal lymphoid depletion with areas of hyperplasia</p> <p>Mesenteric and rectal lymph node: diffuse lymphoid depletion with mild histiocytosis and interstitial fibrosis</p> <p>Skeletal muscle: mild, multifocal myocyte (sarcoplasmic) basophilic degeneration</p> <p>Uterus, ovary, melon, urinary bladder, keratinized stomach, liver, pancreas, adrenal, heart, esophagus: NSLO</p> <p>Spleen, glandular stomach, small intestine: AA</p>
<p>No 12 <i>Stenella longirostris</i></p>	<p>External examination: multiple bites from cookie-cutter shark (<i>I. brasiliensis</i>) and lamprey; larger hematoma on mandibular left region and irregular blackish lesions on right mandibular; <i>P. delphini</i> encysted in perianal blubber</p> <p>Lungs: congestion and edema; right lung firm, displaying brownish heterogenous coloration; left lung presented emphysematous and atelectatic areas</p> <p>Heart: thickening of tendinous cords and valves on the right ventricle</p> <p>Abdominal cavity: <i>M. grimaldii</i> encysted in ligaments of perigenital region</p> <p>Spleen: splenomegaly, congestion and subcapsular hemorrhage</p> <p>Mandibular, pulmonar and mesenteric lymph nodes: lymphadenomegaly</p> <p>Esophagus: free abundant <i>Anisakis</i> sp.</p> <p>Anterior stomach: congestion and mucosal ulceration with intralesional <i>Anisakis</i> sp.</p> <p>Glandular stomach: diffuse hemorrhage and edema</p>	<p>Spinal cord: marked, multifocal, acute hemorrhage (hematomyelia, within white and grey matter, meningeal, arachnoideal, epidural, perineurial, intranerve and periganglionic) associated with liquefactive necrosis, axonal spheroids and perivascular edema; gliosis of spinal ganglion</p> <p>Cerebellum: multifocal, acute leptomenigeal hemorrhage</p> <p>Cerebrum: minimal lymphocytic infiltrates in leptomeninges</p> <p>Keratinized stomach: marked, focally extensive, chronic ulcerative and granulomatous gastritis with intralesional nematodes, marked mucosal hyperplasia and granulation tissue</p> <p>Glandular stomach: moderate to marked, hyperplastic lymphoplasmacytic, eosinophilic and granulomatous gastritis with intralesional <i>Anisakis</i> sp. nematodes and bacteria</p> <p>Abcess: marked, diffuse, chronic pyogranulomatous and calcifying reaction</p> <p>Kidney: multifocal (interrenicular and intraparenchymal) acute hemorrhage; minimal, multifocal, chronic interstitial nephritis with fibrosis and rare glomerulosclerosis; diffuse congestion with scattered acute tubular degeneration</p>

Pyloric stomach: bilious content and diffuse hemorrhage
Omentum: greenish and opaque coloration, with purulent foci
Duodenum: presence of mucus and *Tetraphoridae* sp.
Large intestine: diffuse catarrhal enteritis in the medial portion and mucosal congestion
Liver: diffuse congestion and presence of *Scolex pleuronectis* in hepatobiliary ducts
Adrenals: subcortical hemorrhage
Musculoskeletal system: extended abscess in the atlanto-occipital joint with abundant seropurulent content, osteoarthritis and adjacent myonecrosis
CNS: meningeal congestion and edema. Superficial brain blood vessels with gas embolus
Pancreas: AA

Lung: mild, multifocal, chronic lymphoplasmacytic interstitial pneumonia with scattered fibrosis and multifocal angiomatosis; atelectasia and diffuse congestion with scattered acute hemorrhage, mild edema and alveolar histiocytosis
Heart: moderate, multifocal, chronic lymphoplasmacytic pericarditis with fibrosis; multifocal acute intramyocardial hemorrhage, congestion and multifocal acute hemorrhage; multifocal myocardial fibrosis
Liver: moderate to marked, centrilobular to diffuse congestion with hepatocellular atrophy and hepatocellular hyaline globules with pink points; mild, multifocal, chronic lymphoplasmacytic pericholangitis with mild fibrosis and minimal bile duct hyperplasia
Adrenal: minimal to mild, multifocal, chronic lymphocytic cortical adrenalitis; multifocal acute hemorrhage associated with cortical degeneration, necrosis and loss; contralateral adrenal: diffuse congestion with subcapsular hemorrhage
Lymph node (non-specified): mild, multifocal, chronic interstitial fibrosis; mild, multifocal sinus erythrocytosis (hemorrhage drainage)
Lymph node (non-specified): mild paracortical and medullary lymphoid reactive hyperplasia with mild sinus histiocytosis
Colon: mild to moderate, multifocal, chronic lymphoplasmacytic colitis with multifocal acute mural hemorrhage
Skeletal muscle: rare acute single cell segmental myocyte degeneration
Testicle, epididymis, cerebellum, urinary bladder: NSLO

CNS = Central Nervous System; NSLO = No significant lesions observed; NE = No examined; AA = Advanced autolysis

8 FINAL COMMENTS

- This study represents the first long-term survey of *Brucella* spp. in aquatic mammals in Brazil, widening the spectrum of susceptible hosts and the geographic distribution range of this agent with zoonotic potential, and contributing with the knowledge of pathological and epidemiological aspects of its infection.
- We found evidences of exposure to *Brucella* spp. in the Southern elephant seal (*Mirounga leonina*) and various cetacean's species: short-finned pilot whale (*Globicephala macrorhynchus*), Melon-headed whale (*Peponocephala electra*) and Atlantic bottlenose dolphin (*Tursiops truncatus*). Cases of asymptomatic *Brucella*-infection as well as cases of acute and chronic brucellosis were confirmed in a Franciscana (*Pontoporia blainvillei*), a Clymene dolphin (*Stenella clymene*) and a Spinner dolphin (*Stenella longirostris*) and a Guiana dolphin (*Sotalia guianensis*), respectively.
- The Guiana dolphin (*Sotalia guianensis*) with chronic brucellosis, was previously diagnosed with cetacean morbillivirus, constituting probably an intra-uterine coinfection. *Edwardsiella tarda* was isolated from one *Brucella* spp. exposed *T. truncatus*, and *Proteus mirabilis* from a *S. longirostris* with chronic brucellosis representing concomitant infections.
- We gained information about morbidity of pathogens in endangered cetaceans in Brazil. To the knowledge of the authors, this study constitutes the first report of *Brucella* spp. in *P. blainvillei* and *S. guianensis*, coastal species highly endangered in Brazil, specially by bycatch and pollution in Brazil (DI BENEDITTO et al., 2010; BARRETO et al., 2011). It is important to highlight that *P. blainvillei* is listed in the national Red List, the Appendix II to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and in Appendices I and II of the Convention for the Conservation of Migratory Species of Wild Animals (CMS) (DI BENEDITTO et al., 2010).
- Our data suggests that members of South Atlantic Ocean Delphinidae family seems to be susceptible to *Brucella* spp. infection, as previously discussed in some populations of Striped dolphins (*Stenella coeruleolaba*) (HERNÁNDEZ-

MORA et al., 2008) and Hector's dolphins (*Cephalorhynchus hectori*) (BUCKLE et al., 2017).

- Despite the fact that no antibodies against *Brucella* spp. were found in riverine dolphins and manatees, continuous sanitary monitoring of those species is recommended.
- *Brucella*-type lesions in the central nervous system were detected in pigmy killer whale (*Feresa attenuata*) and various individuals of *S. clymene*. In those cases, further investigation of additional pathogenic agents, including cetacean morbillivirus, herpesvirus, West Nile virus and *Toxoplasma gondii* should be considered for the differential diagnosis (KENNEDY et al., 1992; RESENDES et al., 2002; ESPERÓN; FERNÁNDEZ; SÁNCHEZ-VIZCAÍNO, 2008; DI GUARDO et al., 2010; ST. LEGER et al., 2011; VAN BRESSEM et al., 2014) as previously indicated (SIERRA et al., 2014).
- Considering proved occurrence of *Brucella* spp. in cetaceans inhabiting the Brazilian coast, further studies should focus on characterization of the circulating *Brucella* strains and investigation of its relevance to public health in the country. In order to achieve this, it should be prioritized routine culture of samples from stranded cetaceans, with special attention to lung, lymph nodes, central nervous system and abscess/granulomatous lesions adopting strategies to minimize time between samples storing and processing, especially when samples are frozen.
- Considering that the potential number of host for *Brucella* spp. is very high in the aquatic environment (HERNÁNDEZ-MORA; PALACIOS-ALFARO; GONZÁLEZ-BARRIENTOS, 2013) and, moreover, that *Brucella* infection is endemic in some species, such as pinnipeds (TRYLAND; SØRENSEN; GODFROID, 2005; MCFARLANE, 2009), is of great interest to gain additional information about *Brucella* spp. in other species of aquatic mammals in Brazil.
- Finally, and considering natural cases of human brucellosis that have been associated with marine *Brucella* species without previous contact with marine mammals (SOHN et al., 2003; MCDONALD et al., 2006), and cases of laboratory acquired infection (BREW et al., 1999), is always recommended proper handling of stranded animals during rescue, rehabilitation, necropsies and laboratorial procedures.

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ANNEX A

Authorization of the co-authors of the article “Brucellosis in a clymene dolphin (*Stenella clymene*) stranded in Brazil” for the inclusion in this thesis.

Fortaleza, 20 de dezembro de 2017

Eu, Vitor Luz Carvalho, CPF 00388387378, co-autor do artigo “Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil”, publicado em 2017 pela revista: “Transboundary and Emerging Diseases”, declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada “*Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation” da autoria de Angélica Maria Sanchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Vitor Luz Carvalho

São Paulo, 20 de dezembro de 2017

Eu, Carlos Sacristán Yagüe, CPF: 236.451.518-18, co-autor do artigo “Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil”, publicado em 2017 pela revista: “Transboundary and Emerging Diseases”, declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada “*Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation” da autoria de Angélica María Sánchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

__Carlos Sacristán Yagüe__

(Assinatura)

São Paulo, 02 de Janeiro de 2018

Eu Kátia Regina Groch, CPF73975141972, co-autor do artigo “Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil”, publicado em 2017 pela revista: “Transboundary and Emerging Diseases”, declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada “*Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation” da autoria de Angélica Maria Sanchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Kátia R. Groch

São Paulo, 04 de janeiro de 2018

Eu Rodrigo Albergaria Ressio, CPF:26753933808, co-autor do artigo “Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil”, publicado em 2017 pela revista: “Transboundary and Emerging Diseases”, declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada “ *Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation” da autoria de Angélica Maria Sanchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Rodrigo A Ressio

São Paulo, 05 de Janeiro de 2018.

Eu, Natália Coelho Couto de Azevedo Fernandes, CPF 351815908-96, co-autora do artigo “Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil”, publicado em 2017 pela revista “Transboundary and Emerging Diseases”, declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada “*Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation” da autoria de Angélica Maria Sanchez Sarmiento, aluna 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

São Paulo, 20 de dezembro de 2017

Eu Juliana Mariotti Guerra, CPF: 228038578-39, co-autor do artigo "Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil", publicado em 2017 pela revista: "Transboundary and Emerging Diseases", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada " *Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation" da autoria de Angélica Maria Sanchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

A handwritten signature in dark ink, reading "Juliana Mariotti Guerra", is written over a horizontal line. The signature is cursive and fluid.

(Assinatura)

São Paulo, 20 de dezembro de 2017

Eu, Samira Costa da Silva, CPF:100140327-40, co-autor do artigo "Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil", publicado em 2017 pela revista: "Transboundary and Emerging Diseases", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "Brucella spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation" da autoria de Angélica Maria Sanchez Sarmiento, aluna 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, 04 de janeiro de 2017

Eu Josué Díaz Delgado, CPF: 07395245180, co-autor do artigo "Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil", publicado em 2017 pela revista: "Transboundary and Emerging Diseases", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada " *Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation" da autoria de Angélica Maria Sanchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Josué Díaz Delgado

DVM, MSc, PhD, Dipl. ACVP (Anatomic Pathology)

Wildlife Comparative Pathology Laboratory, Department of Pathology,
College of Veterinary Medicine and Animal Science,
University of São Paulo

São Paulo, 20 de dezembro de 2017

Eu Cíntia Maria Favero, CPF 277.163.428-78, co-autor do artigo "Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil", publicado em 2017 pela revista: "Transboundary and Emerging Diseases", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada "*Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation" da autoria de Angélica Maria Sanchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

A handwritten signature in black ink, reading "Cíntia M Favero", is written over a horizontal line.

(Assinatura)

São Paulo, 20 de dezembro de 2017

Eu Nairléia dos Santos Silva, CPF: 003639245 66, co-autor do artigo "Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil", publicado em 2017 pela revista: "Transboundary and Emerging Diseases", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada " *Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation" da autoria de Angélica Maria Sanchez Sarmiento, aluna 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, 20 de dezembro de 2017

Eu José Soares Ferreira Neto, CPF: 048863088-64, co-autor do artigo "Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil", publicado em 2017 pela revista: "Transboundary and Emerging Diseases", declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada " *Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation" da autoria de Angélica Maria Sanchez Sarmiento, aluna 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, 20 de dezembro de 2017

Eu, Ana Carolina Oliveira de Meirelles, CPF 814.279.053-04, co-autor do artigo “Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil”, publicado em 2017 pela revista: “Transboundary and Emerging Diseases”, declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada “ *Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation” da autoria de Angélica Maria Sanchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

Ana Carolina Meirelles

São Paulo, 21 de dezembro de 2017

Eu Jose Luiz Catão-Dias CPF:02959788800, co-autor do artigo “Brucellosis in a Clymene dolphin (*Stenella clymene*) stranded in Brazil”, publicado em 2017 pela revista: “Transboundary and Emerging Diseases”, declaro estar de acordo com a inclusão e publicação do acima referido artigo na tese de doutorado intitulada “*Brucella* spp. in aquatic mammals of Brazil: serological, molecular, histopathological and immunohistochemical investigation” da autoria de Angélica Maria Sanchez Sarmiento, aluna de doutorado do programa de Patologia Experimental e Comparada do Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Atenciosamente,

A handwritten signature in black ink, consisting of a large loop on the left and a more complex, scribbled shape on the right, crossing itself.

Jose Luiz Catão-Dias

ANNEX B

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