SHORT COMMUNICATIONS

DOI: 10.7589/2017-03-050

Journal of Wildlife Diseases, 54(1), 2018, pp. 000–000

Retrospective Survey for Pathogens in Stranded Marine Mammals in Northeastern Brazil: *Brucella* spp. infection in a Clymene Dolphin (*Stenella clymene*)

Fernanda L. N. Attademo,[1,2] Jean C. R. Silva,[2,3] Fábia O. Luna,[1] Joana Ikeda,[1,4] Ernesto F. C. Foppel,[1,5] Gláucia P. Sousa,[1,6] Augusto C. Bôaviagem-Freire,[7] Rodrigo M. Soares,[6] Thalita Faita,[6] Maria C. A. Batinga,[6] and Lara B. Keid[8,9] [1]Centro Nacional de Pesquisa e Conservação de Mamíferos Aquáticos, Instituto Chico Mendes de Conservação da Biodiversidade, Ilha de Itamaracá, Brazil; [2]Instituto Brasileiro de Medicina da Conservação - Tríade, Recife, Brazil; [3]Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Recife, Brazil; [4]Instituto Mamíferos Aquáticos, Salvador, Brazil; [5]Programa em Desenvolvimento e Meio Ambiente (PRODEMA), Universidade Federal de Sergipe, Sergipe, Brazil; [6]Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, Brazil; [7]Projeto Cetáceos da Costa Branca, Universidade Estadual do Rio Grande do Norte, Brazil; [8]Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, Brazil; [9]Corresponding author (email: larakeid@usp.br)

ABSTRACT: We surveyed 13 carcasses of marine mammals (12 *Trichechus manatus* and one *Stenella clymene*) that had stranded in northeastern Brazil during 1990–2013 for infectious diseases by screening tissues from the collection of the Brazilian National Center of Research and Conservation of Aquatic Mammal, Chico Mendes Institute for Biodiversity Conservation. *Brucella* spp. and *Mycobacterium* spp. were investigated by culturing and PCR of tissue samples, whereas Sarcocystidae parasites, *Leptospira* spp., and *Morbillivirus* were surveyed for using specific PCR assays. *Brucella* spp. and *Mycobacterium* spp. were not isolated through microbiologic culturing, and all animals were negative for detection of Sarcocystidae parasites, *Leptospira* spp., *Mycobacterium* spp., and *Morbillivirus* by PCR assays. All manatees were negative for *Brucella* spp. infection, but *Brucella ceti* was detected in the brain tissue of an *S. clymene* calf by using a PCR assay.

Key words: *Brucella*, cetacean, *Leptospira*, *Morbillivirus*, *Mycobacterium*, Sarcocystidae, sirenian, *Stenella clymene*.

Several pathogens, including some with zoonotic or epizootic potential, have been reported in marine mammal species worldwide. Some pathogens potentially can cause large outbreaks with mass mortality; severe clinical manifestations; or impairment of reproduction, posing serious threats to endangered species (Bossart 2011). Despite the length of the Brazilian coast, there is sparse information regarding the health status of marine mammals in this area. We aimed to investigate the occurrence of infectious and parasitic pathogens in cetaceans and sirenians stranded on the northeastern coast of Brazil by screening the tissue collection of Brazilian National Center of Research and Conservation of Aquatic Mammal (CMA), Chico Mendes Institute for Biodiversity Conservation (ICMBio).

This study was conducted on animals found on Itamaracá Island, Pernambuco, Brazil (7°48′33″S, 34°50′16″W). Thirteen carcasses of marine mammals were necropsied during 2011–2013: 12 manatees (*Trichechus manatus*) and one clymene dolphin (*Stenella clymene*) (Table 1). Twenty-four tissue samples were stored at –20 C at CMA/ICMBio and were available to us. Samples were macerated using sterile mortar and pestle to obtain a 1:5 suspension in 0.9% sterile sodium chloride solution. The suspension was used in microbiological culturing and RNA extraction. A fragment of 25 ng of each tissue was used in DNA extraction.

Suspensions of liver, lungs, uterus, brain, heart, kidney, and intestine were divided equally into two aliquots of 100 µL. Samples were streaked directly onto plates containing two selective media used for *Brucella* spp. isolation: tryptose agar supplemented with 5% fetal bovine serum and an antibiotic mixture consisting of vancomycin (3 mg/L), colistin methane sulfonate (7.5 mg/L), and nystatin (100,000 IU/L); and Farrell's medium. Plates were incubated at 37 C under microaerophilic atmosphere with 10% CO_2 (v/v) for 15 d and

TABLE 1. Tissues of a clymene dolphin (*Stenella clymene*) and manatee (*Trichechus manatus manatus*) tested for *Brucella* spp., *Mycobacterium* spp., *Leptospira* spp., *Morbillivirus*, and Sarcocystidae protozoa infections. All animals had stranded on the northeastern coast of Brazil from 1990 to 2013, were necropsied, and had tissues stored until testing.

Case no.	Species	Sex	Age	Location	Year	Analyzed sample(s)
1	*T. manatus*	Male	Newborn	Pernambuco (stranded)	2003	Muscle
2	*T. manatus*	Male	Adult	Paraíba (stranded)	2011	Muscle, liver, intestine
3	*T. manatus*	Male	Calf	Pernambuco (captive)	2010	Muscle, liver, brain and kidney
4	*T. manatus*	Male	Adult	Alagoas (stranded)	1990	Muscle and urinary tract
5	*T. manatus*	Female	Young	Pernambuco (captive)	2011	Muscle, kidney, and reproductive tract
6	*T. manatus*	Male	Calf	Alagoas (stranded)	2012	Liver and heart
7	*T. manatus*	Female	Adult	Rio Grande do Norte (stranded)	1990	Liver and brain
8	*S. clymene*	Male	Calf	Alagoas (stranded/rehabilitation)	2012	Brain
9	*T. manatus*	Male	Young	Pernambuco (captive)	2013	Liver and lungs
10	*T. manatus*	Male	Stillborn	Pernambuco (captive)	2012	Placenta
11	*T. manatus*	Male	Young	Alagoas (stranded)	2011	Kidney
12	*T. manatus*	Female	Calf	Ceará (stranded)	2012	Muscle
13	*T. manatus*	Male	Adult	Pernambuco (captive)	2012	Liver

inspected for growth every 4 d (World Organization for Animal Health 2012).

A 1-mL aliquot of each suspension was decontaminated using 1-hexadecylpyridinium at 1.5% (w/v) after Corner and Trajstman (1988) and then inoculated in duplicate onto two egg-based media for *Mycobacterium* isolation: Stonebrink and Lowenstein-Jensen with sodium pyruvate (Centro Panamericano de Zoonosis 1985). Each duplicate was incubated at 25 and 37 C under an aerobic atmosphere for 90 d.

We extracted DNA from 25 ng of tissues by using a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, with a previous lysis step using lysozyme (20 mg/mL). Total RNA was extracted from 250 µL of each tissue suspension BY using the Trizol-LS reagent (Invitrogen, Carlsbad, California, USA). An MMLV Reverse Transcription Kit (Invitrogen) was used to synthesize cDNA from extracted RNA BY using random primers (Invitrogen). The reaction was performed according to the manufacturer's instructions, and the cDNA obtained was stored at –80 C.

We used a pair of primers directed to the *bcsp31* gene to detect *Brucella* spp. DNA through PCR, yielding a fragment of 223 base pairs (bp; Baily et al. 1992). To detect *Leptospira* spp., PCR was conducted using primers targeting DNA coding the 16S rRNA gene, yielding 331 bp (Mérien et al. 2002). To detect Sarcocystidae parasites, a nested-PCR assay was used to amplify a 500-bp fragment of the DNA coding the 18S-5.8S rRNA interspace region (Soares et al. 2011). To detect *Mycobacterium* spp. DNA, a pair of primers targeting DNA coding the 16S rRNA gene was used to amplify a 439-bp fragment (Telenti et al. 1993). *Morbillivirus* detection was conducted through a PCR assay using primers directed to the L-protein–coding sequences targeting the *Respirovirus-Morbillivirus-Henipavirus* group (Tong et al. 2008). Platinum Taq DNA Polymerase (Invitrogen) was used to amplify DNA extracts by using the previously described protocols for reagent concentrations and cycling conditions. A negative control (ultrapure water) was included with every second sample, during all the molecular procedures, to avoid aerosol cross-contamination. Positive controls included 1) Onderstepoort vaccine strain for canine distemper virus; and 2) extracted DNA of *Brucella abortus* biovar 1 (strain 544), *T.*

gondii strain RH, *Neospora caninum* strain NC1, *Sarcocystis neurona* strain 138, *Leptospira interrogans* serovar Pomona, and *Mycobacterium bovis* strain AN5. We separated PCR products by electrophoresis on 2% agarose gels stained with ethidium bromide and viewed the gels under UV light (Ausubel et al. 1999).

We excised amplicons of the expected size from the gel; purified them by using Illustra, GFX GEL Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK); and directly sequenced them in both forward and reverse directions by using the same primers as for PCR, for confirmation of results. We purified DNA by alcohol precipitation, and sequencing products were analyzed using the ABI PRISM BigDye® Terminator v3.1 Kit (Thermo Fisher, Carlsbad, California, USA). Sequences were assembled and the contig formed with the phred-base calling and the phrap-assembly tool available in Codoncode aligner v.4.2.1 software (Codon Code, Dedham, Massachusetts, USA). The sequences were aligned with Clustal W, available in the suite BioEdit Sequence Alignment Editor (Hall 1999), based on homologous sequences available in GenBank.

Brucella spp. and *Mycobacterium* spp. were not isolated through microbiologic culturing, and all the animals were negative by PCR for Sarcocystidae parasites, *Leptospira* spp., *Mycobacterium* spp., and *Morbillivirus*. All manatees were negative by PCR for *Brucella* spp. infection.

Brucella spp. DNA was amplified using B4/B5 genus primers in the brain of a clymene dolphin, and the sample was characterized using multilocus sequencing typing (MLST) scheme (Whatmore et al. 2007). The sample was successfully amplified and sequenced using three of the nine primers used in the MLST panel (directed to *omp25*, *trpE*, and *aroA* genes). Obtained sequences were queried against the National Center for Biotechnology Information nucleotide sequence database using the BLAST program, allowing retrieval of homologous fragments from *Brucella* with equal length. Obtained sequences were deposited in GenBank under the following accessions: KY657244, KY657245, and KY657246. The sample showed 100% of similarity with marine *Brucella* sequence type 26 (ST26) in *aroA*, *omp25*, and *trpE* genes, suggesting the bacterium was *B. ceti*.

The positive animal was a male clymene dolphin calf, about 102 cm long and weighing 9.5 kg, values considered normal for this age (Jefferson et al. 1993). It was found stranded alive on Maragogi Beach (9°0′39″S, 35°13′9″W), Alagoas, Brazil, on 23 February 2012. The animal remained in the CMA/ICMBio Center for 9 d before dying, after showing vomiting, regurgitation, and the inability to maintain buoyancy.

The following hematologic parameters were altered in the dolphin calf: hemoglobin (20 g/dL; reference value [RV]: 15.25–18.7 g/dL), hematocrit (56%; RV: 42.1–51.9%), white blood cell count ($3.3 \times 10^3/mm^3$; RV: 5.95–$18.6 \times 10^3/mm^3$), eosinophils ($66 \times 10^3/mm^3$; RV: 424–$5580 \times 10^3/mm^3$), neutrophils ($2400 \times 10^3/mm^3$; RV: 2448–$8265 \times 10^3/mm^3$), and reticulocytes (0.30%; RV: 0.5–2.5%). We used reference values established for *S. attenuata* (St. Aubin et al. 2013). During necropsy, the macroscopic examination revealed petechiae on the external wall of the large and small intestine, and peritonitis. Stomach mucosa was dark red, suggesting gastritis, and the final third was partially obstructed with food debris and tablets used for treatment. Lungs were emphysematous and left lung was hemorrhagic. Fluid with foamy material was present in trachea. Neurologic signs were not verified.

Three sequence types (STs) have been described among *B. ceti* isolates: ST26, predominant in dolphins; ST23, associated with porpoise infections; and ST27, isolated from a common bottlenose dolphin (*Tursiops truncatus*) and from human cases (Groussaud et al. 2007; Whatmore et al. 2007). Brucellosis in cetaceans can be asymptomatic, or it can cause chronic disease characterized by abortion, infertility, skin and bone lesions, endocarditis, and neurobrucellosis (Guzmán-Verri et al. 2012); little is known about its epidemiology. Regarding sirenians, there are few studies investigating brucellosis. *Brucella* in-

fection was reported in 7% of manatees in Florida by Geraci et al. (1999), but the infection was not evidenced by serologic tests in another study conducted in Belize (Sulzner et al. 2012) or by PCR in the present study.

Brucella ceti has been isolated from the central nervous system of several cetacean species, with relatively high numbers of reports in striped dolphins (*Stenella coeruleoalba*) in Europe and South America, where it caused opisthotonus, tremors, seizures, disorientation, and the inability to maintain buoyancy (Hernández-Mora et al. 2008; Davison et al. 2009; Isidoro-Ayza et al. 2014), suggesting that this dolphin species has an increased susceptibility to neurobrucellosis (González-Barrientos et al. 2010; Guzmán-Verri et al. 2012). Brain was the only organ available for examination of the clymene dolphin we surveyed, and it yielded a positive result in the brucellosis test. Neurologic impairment was not observed on clinical and postmortem gross examination, and histopathologic examination was not conducted. Although it was not possible to associate the clinical signs presented with *Brucella* infection, this result highlighted the relevance of routine examinations for *Brucella* infection when investigating dead marine mammals in Brazil. Our results also raised the question as to the possible degree of susceptibility of other species of the *Stenella* genus to neurobrucellosis, and they highlighted the need for postmortem examinations of *Stenella* species.

We thank the staff of the Brazilian National Center of Research and Conservation of Aquatic Mammals of Chico Mendes Institute for Biodiversity Conservation. J.C.R.S. and R.M.S. received a fellowship from the National Center for Scientific and Technological Development.

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Submitted for publication 1 February 2017.
Accepted 14 August 2017.