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Survey of *Brucella* spp. and *Leptospira* spp. antibodies in cetaceans and manatees of the Amazon basin and Atlantic Ocean, Brazil

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ABSTRACT: Aquatic mammals can act as sentinels of emerging and resurging pathogens in the environment. *Brucella* spp. and *Leptospira* spp. are 2 zoonotic pathogens relevant to aquatic mammals, and their detection can be used to assess pathogen exposure. In this study, serum from 84 individuals — 63 cetaceans (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 (MAT) 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 8 c-ELISA positive cetaceans (with available serum) was further tested via serum agglutination test (SAT) and 1 individual was positive. c-ELISA was more sensitive than RBT. Exposure to *Brucella* spp. was found in 5 cetacean species: Clymene dolphin *Stenella clymene*, short-finned pilot whale *Globicephala macrorhynchus*, pygmy killer whale *Feresa attenuata*, melon-headed whale *Peponocephala electra* and Atlantic bottlenose dolphin *Tursiops truncatus* in the 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 by MAT. These results contribute to the evaluation of different *Brucella* spp. serological methods in cetaceans and manatees and highlight the epidemiology of zoonotic pathogens in aquatic mammals of the southwestern Atlantic Ocean and the Amazon basin.

KEY WORDS: Aquatic mammals · Competitive ELISA · Microscopic agglutination test · Rose Bengal test · Serum agglutination test

1. INTRODUCTION

Gram-negative coccobacillus bacteria of the genus *Brucella* were initially isolated in 1994 from 3 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 2 recognized species: *B. ceti* and *B. pinnipedialis* (Foster et al. 2007), with further subdivision into sequence types (ST) (Bourg

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et al. 2007, Groussaud et al. 2007, Dawson et al. 2008b, Maquart et al. 2009). A broad range of species have been confirmed to be susceptible to *Brucella* spp., with at least 55 seropositive species or subspecies of aquatic mammals (36 cetacean species, 15 pinniped species, 2 subspecies of sea otters, 1 species of freshwater otter and the polar bear) (Hernández-Mora et al. 2013, Foster et al. 2018, Sánchez-Sarmiento et al. 2018). Interestingly, in some groups of aquatic mammals (riverine dolphins, manatees and dugongs) infection by or exposure to *Brucella* spp. has never been reported (Hernández-Mora et al. 2013).

In aquatic mammals, *Brucella* infection can be asymptomatic, but may led 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). Along the southeastern Pacific coast of South America, *Brucella* spp. exposure was found via competitive and indirect enzyme-linked immunosorbent assay (c-ELISA and i-ELISA) in odontocetes and via card test and c-ELISA in pinnipeds (Van Bressem 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 (Attademo et al. 2018, Sánchez-Sarmiento et al. 2018).

Another relevant zoonotic pathogen in marine mammals is *Leptospira* spp., typically associated with renal disease, stranding and death in pinnipeds (Vedros et al. 1971, Gulland et al. 1996, Colegrove et al. 2005). 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 epizooties (Vedros et al. 1971, Gulland et al. 1996). Recently, antibodies against *Leptospira* spp. have been detected in captive sirenians in South America: in West Indian manatees *Trichechus manatus* from Brazil and in Amazonian manatees *T. inunguis* from Brazil and Peru (Mathews et al. 2012, Attademo 2014, Delgado et al. 2015). *Leptospira* spp. was also detected by PCR in a southern right whale from Argentina (Grune Loffler et al. 2015) and in a South American pinniped species, the Galapagos sea lion *Zalophus wollebaeki* (Denkinger et al. 2017).

Aquatic mammals can act as sentinels of environmental health by indicating the presence of pathogens, some of them of emerging and resurging character due to environmental distress syndrome (Dierauf & Gulland 2001, Bossart 2007). The aim of this study was to evaluate the presence of antibodies against *Brucella* spp. and *Leptospira* spp. in odontocetes and manatees from Brazil.

2. MATERIALS AND METHODS

2.1. Individuals and blood collection

Serum samples were tested from 84 individuals (63 cetaceans and 21 manatees). Of these, 37 were riverine dolphins (Iniidae) — Amazon river dolphin *Inia geoffrensis* (n = 16) and Bolivian river dolphin *I. boliviensis* (n = 21) — that were captured, sampled and released during field expeditions performed in 2015 in Negro River (state of Amazonas) and Guaporé River (state of Rondonia). The remaining 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 (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 the family Delphinidae; and dwarf sperm whale *Kogia sima* (n = 3) and pigmy sperm whale *Kogia breviceps* (n = 1) from the family Kogiidae. Samples from West Indian manatees (n = 21) were collected from dead individuals stranded on the coast of Ceará (n = 4) and from animals kept in rehabilitation (n = 17) after being rescued alive in the states of Ceará and Rio Grande do Norte by 'Aquasis' and 'Projeto Cetáceos da Costa Branca' (PCCB/UERN), respectively. Considering that the manatees rescued alive have spent considerable time in captivity at the Marine Mammal Rescue Center (MMRC/Aquasis), when possible, serological tests in this group were performed twice: one at arrival and another after around 1 yr in rehabilitation or during necropsy.

Stranding data including location, sex, total body length (TBL; from tip of rostrum to tail notch) and animal condition/carcass decomposition code (COD; 1 = live-stranded; 2 = freshly dead; 3 = decomposed, but

organs basically intact) (Geraci & Lounsbury 2005) were recorded (see Tables 1 & 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). Locations of individuals tested are shown in Fig. 1.

From Iniidae (COD 1), blood samples were drawn from the tail flukes or the ventral caudal peduncle, with the dolphin restrained in the river margin, generally within 5–20 min after they were captured following health examination. After that, specimens were released. From Delphinidae and Kogiidae (COD 1), blood samples were collected from tail fluke or ventral caudal peduncle while the animal was under restraint during rehabilitation. From live manatees (COD 1), blood samples were collected from the brachial vascular bundle. From the remaining animals (COD 2 and 3), blood samples were obtained directly from the heart during necropsy. Samples were placed directly in Vacutainer® tubes with EDTA and without anticoagulant, and centrifuged for harvesting serum. Samples were placed immediately on cold packs, transferred to the shipboard laboratory (Iniidae)/laboratory and then frozen at −20°C until analysis.

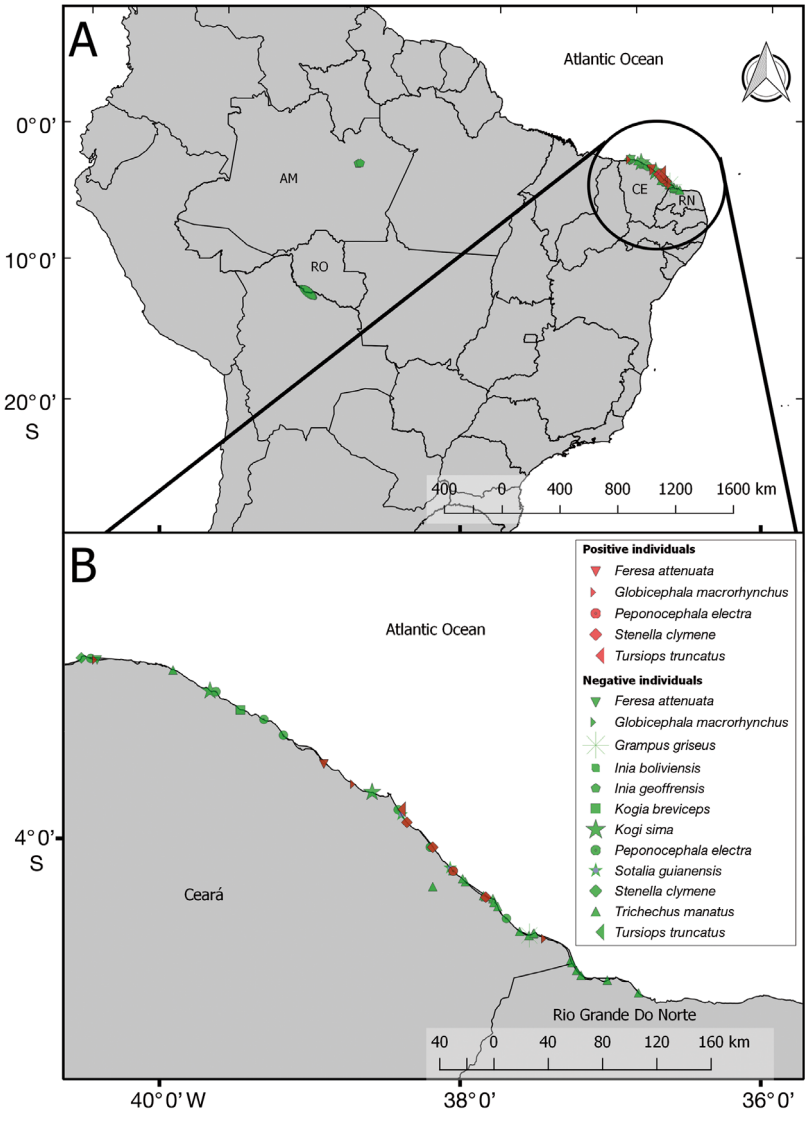

Fig. 1. (A) Location of individuals sampled in this study (AM: state of Amazonas; RO: state of Rondonia; CE: state of Ceará; RN: state of Rio Grande do Norte). (B) Serological results for the different species tested in the states of Ceará and Rio Grande do Norte. Red: positives; green: negatives

2.2. 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) (Alton et al. 1998, Ministério da Agricultura, Pecuária e Abastecimento 2006). Sera and antigen were allowed to equilibrate at room temperature, and 30 µl of the serum was mixed with an equal volume of antigen for 4 min. The sample was considered positive when visible agglutination was noted by the observer.

Secondly, samples were tested with a commercial competitive c-ELISA kit (INGENASA®), using monoclonal antibody (Mab) specific to epitope C of the lipopolysaccharide (LPS) antigen from *B. 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 *Yernisia 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 a PCR-positive cetacean stranded on the Brazilian coast (Sánchez-Sarmiento et al. 2018). According to the manufacturer, the threshold for determining seropositivity was ≥40%, with antibody titers calculated according to optical density (OD) with the

following formula: percentage inhibition (PI) = 100 × [1 – (OD sample / OD 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 ≥30% PI (Nielsen et al. 1996, Neimanis et al. 2008). Cetaceans with positive c-ELISA (considering also serum 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 (Ministério da Agricultura, Pecuária e Abastecimento 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. antibody 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, University of Sáo Paulo. Leptospiral cultures were maintained in Ellinghausen, McCullough, Johnson and Harris media (Becton-Dickinson Biosciences) modified (Alves et al. 1996) with 4 to 14 growth days, density of 100–200 microorganisms per microscopic field and visualized at 400×. Sera presenting agglutination equal to or above 50% in comparison to the positive control were considered positive (Myers 1985).

3. 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%). Dwarf sperm whale, pigmy sperm whale, Amazon river dolphin, Bolivian river dolphin, Guiana dolphin and Risso's dolphin were negative, whereas all short-finned pilot whale (3/3) and Atlantic bottlenose dolphin (1/1), 3 out of 4 Clymene dolphin, 1 out of 2 pigmy killer whale and 22.2% (2/9) of melon-headed whale were c-ELISA positive. All c-ELISA positive cases were from the state of Ceará (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 (2 Clymene dolphins [#39 and #40] and 1 short-finned pilot whale [#61]). Only one Clymene dolphin (#40) was positive for SAT with titer of 100.

On the other hand, all manatees 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 in Table 1 for cetaceans and Table 2 for manatees, with location of positive individuals presented in Fig. 1.

4. DISCUSSION

Our results suggest exposure to *Brucella* spp. in 5 odontocete species (Clymene dolphin, short-finned pilot whale, pigmy killer whale, melon-headed whale and Atlantic bottlenose dolphin) from the southwestern Atlantic Ocean, Brazil, expanding the number of known *Brucella* seropositive aquatic mammal species. Considering the above species, *Brucella* exposure has not been reported in the short-finned pilot whale. The occurrence of anti-*Brucella* antibodies has previously been reported in one melon-headed whale and in 1 out of 3 and 27.2% (15/55) of pigmy killer whales by c-ELISA, and in 17.2% (60/349) of Atlantic bottlenose dolphins by several serological methods (Hernández-Mora et al. 2009, 2013).

The seropositive cetacean species in this study are known to have oceanic and/or coastal habits, while riverine dolphins and manatees were negative, corroborating the existing literature (Hernández-Mora et al. 2013). A previous serological study with 67 Amazon river dolphins from Tefé, state of Amazonas, Brazil, did not detect anti-*Brucella* antibodies via RBT, 2-mercaptoethanol or fluorescence polarization assay (FPA) (Rocca 2014). In captive West Indian manatees 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 previously confirmed by PCR in one Clymene dolphin (#39, included in this study) (Sánchez-Sarmiento et al. 2018). It was also detected in the same species in the state of Alagoas, also in northeastern Brazil (Attademo et al. 2018). Addi-

Table 1. Individual data and results obtained in the serological tests for *Brucella* spp. and *Leptospira* spp. for the different cetacean species tested. M: male; F: female; A: adult; J: juvenile; C: calf. TBL: total body length. COD: animal condition/carcass decomposition code (1 = live animal; 2 = freshly dead; 3 = decomposed, but organs basically intact; Geraci & Lounsbury 2005). AM: state of Amazonas; RO: state of Rondonia; CE: state of Ceará. RBT: Rose Bengal test; c-ELISA: competitive enzyme-linked immunosorbent assay; SAT: serum agglutination test; MAT: microscopic agglutination test. (+) positive; (–) negative; na: not analysed

ID	Sex	Age class	TBL (m)	COD	Sampling date (dd/mm/yy)	Location	Geographical coordinates	RBT	c-ELISA (%PI)	SAT (titer)	MAT
Inia geoffrensis											
1	M	A	2.05	1	02/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
2	M	J	1.94	1	02/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
3	M	A	2.12	1	02/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
4	M	J	1.67	1	02/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
5	M	A	2.01	1	03/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
6	M	J	1.79	1	03/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
7	M	A	2.24	1	03/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
8	M	A	2.14	1	04/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
9	M	J	1.49	1	05/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
10	M	J	1.62	1	05/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
11	M	A	2.07	1	05/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
12	M	J	1.86	1	05/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
13	M	C	1.49	1	05/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
14	M	A	2.01	1	05/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
15	M	A	2.04	1	06/02/15	Praia do Davi, Rio Negro, AM	3° 5′ S, 60° 28′ W	–	–	na	–
16	M	C	1.18	1	03/12/15	Cachoeira do Castanho, Rio Negro, AM	3° 4′ 10″ S, 60° 18′ 41″ W	–	–	na	–
Inia boliviensis											
17	M	A	2.10	1	06/02/15	Baía Grande, Rio Guaporé, RO	12° 29′ S, 64° 3′ W	–	–	na	–
18	M	A	1.97	1	22/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
19	M	C	1.00	1	22/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
20	F	A	1.99	1	22/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
21	M	A	2.09	1	22/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
22	F	J	1.57	1	22/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
23	M	J	1.69	1	22/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
24	F	A	2.15	1	23/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
25	M	C	1.33	1	23/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
26	F	A	2.05	1	23/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
27	M	A	1.93	1	23/09/15	Baía Grande, Rio Guaporé, RO	12° 29′′ S, 64° 3′ W	–	–	na	–
28	M	A	2.05	1	23/09/15	Contra Fiado, Rio Guaporé, RO	12° 28′ S, 64° 7′ W	–	–	na	–
29	M	A	2.00	1	24/09/15	Contra Fiado, Rio Guaporé, RO	12° 28′ S, 64° 7′ W	–	–	na	–
30	M	J	1.77	1	24/09/15	Contra Fiado, Rio Guaporé, RO	12° 28′ S, 64° 7′ W	–	–	na	–
31	F	J	1.47	1	24/09/15	Contra Fiado, Rio Guaporé, RO	12° 28′ S, 64° 7′ W	–	–	na	–
32	M	J	1.74	1	24/09/15	Baía Queimada, Rio Guaporé, RO	12° 27′ 38″ S, 64° 17′ 21″ W	–	–	na	–
33	M	J	1.86	1	25/09/15	Porto França, Rio Guaporé, RO	12° 27′ 41″ S, 64° 17′ 20″ W	–	–	na	–
34	M	A	2.22	1	26/09/15	Contra Fiado, Rio Guaporé, RO	12° 28′ S, 64° 7′ W	–	–	na	–
35	F	A	1.94	1	27/09/15	Contra Fiado, Rio Guaporé, RO	12° 28′ S, 64° 7′ W	–	–	na	–
36	M	A	1.98	1	27/09/15	Contra Fiado, Rio Guaporé, RO	12° 28′ S, 64° 7′ W	–	–	na	–
37	M	A	2.11	1	27/09/15	Contra Fiado, Rio Guaporé, RO	12° 28′ S, 64° 7′ W	–	–	na	–

Table 1 continued on next page

Table 1. (continued)

ID	Sex	Age class	TBL (m)	COD	Sampling date (dd/mm/yy)	Location	Geographical coordinates	RBT	c-ELISA (%PI)	SAT (titer)	MAT
Stenella clymene											
38	M	A	2.01	2	25/03/12	Jericoacoara, Jijoca de Jericoacoara, CE	2° 47′ 57″ S, 40° 31′ 10″ W	–	–	na	–
39	M	A	1.75	2	10/03/12	Parajuru, Beberibe, CE	4° 23′ 29″ S, 37° 49′ 45″ W	+	+ (95.1 %)	na	–
40	F	A[a]	1.76	1	25/05/16	Praia de Águas Belas, Cascavel, CE	4° 3′ 31″ S, 38° 10′ 51″ W	+	+ (96.4 %)	+ (100)	–
41	M	A[a]	1.70	1	10/01/16	Praia do Japão, Aquiraz, CE	3° 53′ 34″ S, 38° 21′ 11″ W	–	+ (95.4 %)	–	–
Sotalia guianensis											
42	F	C	1.02	2	20/06/11	Praia do Diogo, Beberibe, CE	4° 11′ 45″ S, 38° 4′ 0″ W	–	–	na	–
43	F	C	0.78	2	09/11/16	Porto das dunas, Aquiraz, CE	3° 50′ 26″ S, 38° 23′ 28″ W	–	–	na	–
Kogia breviceps											
44	M	C	1.45	2	29/05/12	Praia da Baleia, Itapipoca, CE	3° 8′ 39″ S, 39° 27′ 40″ W	–	–	na	–
Kogia sima											
45	M	A	2.22	3	02/08/12	Moitas, Icaraí de Amontada, CE	3° 1′ 7″ S, 39° 39′ 46″ W	–	–	na	–
46	F	A	2.20	2	04/06/13	Barra do Ceará, Fortaleza, CE	3° 41′ 36″ S, 38° 35′ 7″ W	–	–	na	–
47	M	C	1.18	1	04/06/13	Barra do Ceará, Fortaleza, CE	3° 41′ 36″ S, 38° 35′ 7″ W	–	–	na	–
Grampus griseus											
48	F	A	2.54	2	20/06/12	Retirinho, Aracati, CE	4° 38′ 40″ S, 37° 32′ 15″ W	–	–	na	–
Peponocephala electra											
49	F	J	2.28	2	09/07/11	Emboaca, Trairí, CE	3° 12′ 27″ S, 39° 18′ 18″ W	–	–	na	–
50	M	C	1.10	2	05/09/12	Canoa Quebrada, Aracati, CE	4° 31′ 52″ S, 37° 41′ 28″ W	–	–	na	–
51	M	A	2.39	2	01/11/12	Riacho Doce, Cruz/CE	2° 48′ 11″ S, 40° 27′ 22″ W	–	–	na	–
52	M	J	2.03	2	28/03/13	Águas Belas, Cascavel/CE	4° 3′ 28″ S, 38° 11′ 48″ W	–	–	na	–
53	F	A	2.30	1	05/11/14	Praia de Caetanos, Amontada, CE	3° 1′ 30″ S, 39° 37′ 21″ W	–	–	na	–
54	M	A	2.40	1	07/11/14	Praia das Fontes, Beberibe, CE	4° 13′ 0″ S, 38° 2′ 47″ W	–	+ (93.9 %)	na	–
55	F	A	2.46	1	07/11/14	Praia das Fontes, Beberibe, CE	4° 13′ 0″ S, 38° 2′ 47″ W	–	+ (77.6 %)	–	–
56	M	C	1.21	2	30/12/14	Praia da Cofeco, Fortaleza, CE	3° 48′ 29″ S, 38° 24′ 41″ W	–	–	na	–
57	F	A	2.25	1	11/02/17	Lagoinha, Paraipaba, CE	3° 18′ 48″ S, 39° 10′ 30″ W	–	–	na	–
Tursiops truncatus											
58	F	A	2.71	2	06/07/15	Abreulândia, Fortaleza, CE	3° 48′ 20″ S, 38° 24′ 46″ W	–	+ (95.0 %)	–	–
Feresa attenuata											
59	F	C	1.13	2	04/05/16	Praia do Preá, Cruz, CE	2° 48′ 46″ S, 40° 24′ 51″ W	–	–	na	–
60	M	A	2.13	2	14/04/15	Praia da Taíba, São Gonçalo do Amarante, CE	3° 30′ 22″ S, 38° 54′ 19″ W	–	+ (51.4 %)	–	–
Globicephala macrorhynchus											
61	M	J	2.90	1	11/01/12	Praia do Preá, Cruz, CE	2° 48′ 44″ S, 40° 24′ 55″ W	+	+ (97 %)	–	–
62	F	C	1.57	1	09/07/16	Praia de Vila nova, Icapuí, CE	4° 39′ 57″ S, 37° 25′ 40″ W	–	+ (85.7 %)	–	–
63	F	C	1.30	2	25/01/17	Praia da Tabuba, Caucaia, CE	3° 38′ 25″ S, 38° 42′ 0″ W	–	+ (96.3 %)	–	–

[a]Sexual maturity verified by histology

Table 2. Individual data and results obtained in the serological tests for *Brucella* spp. and *Leptospira* spp. for West Indian manatees. M: male; F: female; ni: no information. yr: year; mo: month; d: days. TBL: total body length. COD: animal condition/carcass decomposition code (1 = live animal; 2 = freshly dead; Geraci & Lounsbury 2005). RN: state of Rio Grande do Norte; CE: state of Ceará; MMRC: Marine Mammal Rescue Center/Aquasis. RBT: Rose Bengal test; c-ELISA: competitive enzyme-linked immunosorbent assay; MAT: microscopic agglutination test; (–) negative

ID	Sex	Age class	TBL (m)	COD	Stranding date (dd/mm/yy)	Location	Geographical coordinates	Sampling date (dd/mm/yy)	RBT	c-ELISA	MAT
1	F	1 yr, 7 mo	2.02	1	13/08/13	Praia do Gado Bravo, Tibau, RN	4° 52' 38'' S, 37° 13' 30'' W	26/03/15	–	–	–
	F	2 yr, 3 mo	2.34			MMRC, Aquasis	–	19/11/15	–	–	–
2	F	1 mo	1.27	1	13/01/15	Morro Pintado, Areia Branca, RN	4° 58' 18'' S, 37° 11' 18'' W	19/02/15	–	–	–
3	F	15 d	1.2	1	24/03/15	Praia de Alagamar, Grossos, RN	4° 54' 34'' S, 37° 11' 42'' W	07/04/15	–	–	–
4	M	<2 yr	2.01	2	23/09/11	Ponta Grossa, Icapuí, CE	4° 38' 0'' S, 37° 30' 45'' W	23/09/11	–	–	–
5	M	1 mo	1.08	1	23/03/13	Praia de Manibu, Icapuí, CE	4° 48' 23'' S, 37° 15' 49'' W	22/04/13	–	–	–
6	F	<3 yr	2.41	2	21/09/13	Praia de Retirinho, Aracati, CE	4° 38' 40'' S, 37° 32' 27'' W	21/09/13	–	–	–
7	M	1 yr, 3 mo	2.05	1	15/12/13	Pontal do Maceió, Fortim, CE	4° 24' 5'' S, 37° 46' 53'' W	26/03/15	–	–	–
	M	1 yr, 11 mo	2.19			MMRC, Aquasis	–	19/11/15	–	–	–
8	M	1yr	1.79	1	15/10/14	Praia das Agulhas, Fortim, CE	4° 23' 13'' S, 37° 49' 50'' W	19/10/15	–	–	–
	M	1 yr, 1 mo	1.84			MMRC, Aquasis	–	19/11/15	–	–	–
9	M	1 yr, 1 mo	1.93	1	15/10/14	Praia das Agulhas, Fortim, CE	4° 23' 13'' S, 37° 49' 50'' W	19/11/15	–	–	–
10	M	2 mo	1.2	1	28/12/14	Rio Jaguaribe, Fortim, CE	4° 25' 30'' S, 37° 46' 29'' W	19/02/15	–	–	–
	M	11 mo	1.71			MMRC, Aquasis	–	19/11/15	–	–	–
11	F	1 d	1.15	1	20/02/15	Praia de Manibu, Icapuí, CE	4° 49' 31'' S, 37° 15' 22'' W	20/02/15	–	–	–
12	M	5 d	1.35	1	05/03/15	Praia de Ariós, Beberibe, CE	4° 17' 18'' S, 37° 57' 53'' W	10/03/15	–	–	–
	M	3 mo	1.54			MMRC, Aquasis	–	30/06/15	–	–	–
13	M	<10 yr	3	2	03/10/15	Ponta Grossa, Icapuí, CE	4° 37' 43'' S, 37° 30' 25'' W	03/10/15	–	–	–
14	ni	8 mo	1.6	1	23/04/15	Praia do Rosado, Porto do Mangue, CE	5° 1' 35'' S, 36° 48' 42'' W	08/12/15	–	–	–
	ni	1 yr	1.91			MMRC, Aquasis	–	12/04/16	–	–	–
15	M	1 d	1.36	1	27/06/16	Praia dos Estevão, Aracati, CE	4° 27' 8'' S, 37° 44' 57'' W	28/06/16	–	–	–
16	F	1 mo	1.33	1	09/10/16	Praia das Agulhas, Fortim, CE	4° 22' 51'' S, 37° 50' 37'' W	10/10/16	–	–	–
17	M	1 mo	1.17	1	09/10/16	Praia dos Anjos, Beberibe, CE	4° 16' 3'' S, 37° 59' 0'' W	10/10/16	–	–	–
18	F	1 mo	1.42	1	10/11/16	Praia de Ariós, Beberibe, CE	4° 12' 53'' S, 38° 2' 55'' W	08/12/16	–	–	–
19	M	1 d	1.31	1	28/12/16	Prainha do Canto Verde, Beberibe, CE	4° 19' 14'' S, 37° 55' 41'' W	28/12/16	–	–	–
20	F	1 d	1.22	2	30/12/16	Praia de Fontaínhas, Aracati, CE	4° 37' 2'' S, 37° 36' 11'' W	31/12/16	–	–	–
21	F	45 d	1.31	1	25/03/17	Mulheres de Areia, Itarema, CE	2° 52' 49'' S, 39° 54' 35'' W	26/03/17	–	–	–

tional serologically positive cetaceans identified in this study (#40, #58, #60–63) were suspected of *Brucella*-infection following histopathology and immunohistochemistry results (data not shown).

As our sample size for each species was limited, intra- or interspecies epidemiological differences 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. Vertical transmission of *Brucella* spp. has been reported in several cetacean species (Ewalt et al. 1994, Hernández-Mora et al. 2008, González-Barrientos et al. 2010, Colegrove et al. 2016). 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. 2006). In hooded seals *Cystophora cristata*, seropositivity decreases with age and post-weaning environmental exposure (through diet), and subsequent infection clearance had been hypothesized (Nymo et al. 2013). Histopathological and imunohistochemical findings (data not shown) revealed *in utero* infection as the most plausible transmission route for both short-finned pilot whale calves (#62, #63) in this study.

In the current literature, differences among species have been related to different social structures and schooling behavior that may increase transmission (Van Bressem et al. 2001). In some marine mammal species, *Brucella* infection may occur intermittently, lacking a persistent infection; a result of other sources (such as predators), to which some species

could be more susceptible than others (Nielsen et al. 1996, Van Bressem et al. 2001). The relation of some parameters with the immunological response of individuals against *Brucella* infection has been also described, e.g. disease dynamics influenced by increased infection frequency with degraded environmental conditions (Colegrove et al. 2016).

c-ELISA and RBT are considered suitable methods for diagnosis of *Brucella* spp. exposure (Matope et al. 2011, OIE 2012). However, c-ELISA has demonstrated more sensitivity than RBT and other techniques such as serum and blood FPA in cattle (Matope et al. 2011). 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). RBT detects either IgM, IgG or IgA, but 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) since agglutination tests detect mainly IgM (Godfroid et al. 2010). The performance of serological methods is, in general, dependent on cross-reactivity between immunoglobulins, the type of antigen employed and species tested. There is enough evidence of the cross-reactivity of antibodies of cetaceans to recognize *B. abortus* as an antigen (Hernández-Mora et al. 2009). However, a possible explanation of c-ELISA positives that were RBT and SAT negatives in this study could be due to the following: (1) those tests were initially developed for bovines, and immunoglobulins were not appropriately detected in cetaceans; related to standardization and origin; or (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 i-ELISA and CFT (Muñoz et al. 2005). Its specificity is related to the displacement of low-avidity antibodies by the competing Mab against the LPS-C epitope (Hernández-Mora et al. 2009). The selection of a Mab with higher affinity than cross-reacting antibody results in the capacity to eliminate some false positive serological reactions (FPSR) due to cross-reacting bacteria such as *Yersinia enterocolitica* O:9 (Muñoz et al. 2005). Due to this possibility, studies in bovines argue that c-ELISA allows the detection of *Brucella* spp. specific antibodies, eliminating cross reactions observed with *Y. enterocolitica* (Nielsen 1990). Since c-ELISA is not animal species-specific and can be used on poor-quality samples unsuitable for conventional tests, as usually observed with marine mammals, it is considered a suitable choice for those species, and has been widely used in several serological studies on cetaceans and pinnipeds (Nielsen et al. 1996, 2001, 2005, Jepson et al. 1997, Van Bressem et al. 2001, Dawson et al. 2008a, Perrett et al. 2010). 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 since 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, nor did it isolate the bacteria (Tryland et al. 1999).

Differing from this study, evidence of *Leptospira* spp. has been previously found in cetaceans and manatees. In the Southern Atlantic Ocean, *Leptospira* spp. was isolated from a kidney sample of southern right whale in Argentina (Grune Loffler et al. 2015), and seropositivity was found in captive Amazonian manatees from Brazil at elevated rates (31.1%, 23/74; Mathews et al. 2012) and in captive West Indian manatees from northeastern Brazil (9.2%, 5/54; Attademo 2014). Titers against different *Leptospira* serovars have also been found in Antillean manatees *Trichechus manatus manatus* in Belize (Sulzner et al. 2012). Climatic and ecological factors are relevant to the occurrence of *Leptospira* spp. 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 Antillean 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 freshwater availability were indicated to have seasonal influence. Considering these data and the potential of epizootics, a routine serologic and pathologic screening was recommended (Sulzner et al. 2012).

The increase of host range and geographical distribution of *Brucella* spp. among cetaceans has previously been reported in the literature. Our study provides additional evidences of circulation of *Brucella* spp. in northeastern Brazil, as noted recently (Attademo et al. 2018, Sánchez-Sarmiento et al. 2018). Additional studies are required to better understand the dynamics of *Brucella*-infection in this geographic region. The routine investigation of *Brucella* spp. in stranded or bycaught mammals in Brazil is highly encouraged via indirect (serological) and/or direct

methods to detect the infection (either by culture, molecular assays, immunological tests or electron microscopy) (Maratea et al. 2003, Dawson et al. 2008a, Hernández-Mora et al. 2009). Although no evidence of antibodies against *Leptospira* spp. was found, the occurrence and a plausible vulnerability of aquatic mammals to this pathogen at the locations tested 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.

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