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ORIGINAL ARTICLE

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Molecular, serological, pathological, immunohistochemical and microbiological investigation of *Brucella* spp. in marine mammals of Brazil reveals new cetacean hosts

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Summary

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 (conventional PCR and/or real-time PCR), serological (Rose Bengal Test [RBT], Competitive [c]ELISA, Serum Agglutination Test [SAT]), pathological, immunohistochemical (IHC) and/or microbiological investigations were conducted in samples of 129 stranded or by-caught marine mammals (orders Cetartiodactyla [*n* = 124], Carnivora [*n* = 4] and Sirenia [*n* = 1]). Previous serological tests performed on available sera of 27 of the 129 animals (26 cetaceans and one manatee), indicated 10 seropositive cetaceans. Conventional PCR and/or real-time PCR performed in cases with available organs (*n* = 119) and/or blood or swabs (*n* = 10) revealed 4/129 (3.1%) *Brucella*-infected cetaceans (one of them with positive serology; the remaining three with no available sera). Pathological, IHC and/or

microbiological analyses conducted in PCR/real-time PCR and/or seropositive cases (*n* = 13) revealed *Brucella*-type lesions, including meningitis/meningoencephalitis, pneumonia, necrotizing hepatitis, pericarditis and osteoarthritis in some of those animals, and positive IHC was found in all of them (excepting two live-stranded animals without available organs). *Brucella* spp. culture attempts were unsuccessful. Our results demonstrated exposure, asymptomatic, acute and chronic *Brucella* sp. infection in several cetacean species in the Brazilian coast, highlighting the role of this pathogen in stranding and/or death, particularly in Clymene dolphin (*Stenella clymene*) and short-finned pilot whale (*Globicephala macrorhynchus*) off Ceará State. Novel hosts susceptible to *Brucella* included the franciscana (*Pontoporia blainvillei*), the Guiana dolphin (*Sotalia guianensis*) and the spinner dolphin (*Stenella longirostris*). Additionally, three coinfection cases involving *Brucella* spp. and cetacean morbillivirus, *Edwarsiella tarda* and *Proteus mirabilis* were detected. To the best of our knowledge, this is the first long-term and large-scale survey of *Brucella* spp. in marine mammals of South America, widening the spectrum of susceptible hosts and geographical distribution range of this agent with zoonotic potential.

KEYWORDS

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

1 | INTRODUCTION

Brucellosis, a widespread zoonosis caused by bacteria belonging to genus *Brucella* spp., remains an important economic and public health problem in many areas of the world (Corbel, 2006). This genus is comprised by 12 species, further classified into 'atypical' strains, that is, *B. microti* and *B. inopinata*, and the 'classical' strains, that is, *B. abortus* (bovines), *B. melitensis* (sheep and goats), *B. suis* (swine, cattle, rodents, wild ungulates), *B. ovis* (sheep), *B. canis* (canids), *B. neotomae* (rodents), *B. papionis* (baboons [*Papio* spp.]), *B. vulpis* (red foxes [*Vulpes vulpes*]), *B. ceti* (most commonly associated with porpoises and dolphins) and *B. pinnipedialis* (predominantly associated with seals) (Foster, Osterman, Godfroid, Jacques, & Cloeckert, 2007; Hubálek et al., 2007; Kazmierczak, 2012; Osterman & Moriyón, 2006; Scholz, 2008; Scholz et al., 2009, 2010, 2016; Whatmore et al., 2014, 2017). The two latter are regarded as 'marine *Brucella* strains', and are classified into three major groupings with five to 18 genotypes or sequence types (STs) depending on BruMLSA9 and BruMLSA21 results respectively (Whatmore et al., 2017; Whatmore, Perrett, & MacMillan, 2007). Marine mammal *Brucella* have the potential to infect and cause disease in domestic animals (Rhyan et al., 2001) and humans (Brew, Perrett, Stack, MacMillan, & Staunton, 1999; McDonald et al., 2006; Sohn et al., 2003). In humans, naturally acquired infection is associated with genotype ST27 and possibly with raw fish and seafood ingestion (McDonald et al., 2006; Sohn et al., 2003), while laboratory acquired infection with ST23 (Brew et al., 1999; Whatmore et al., 2008, 2007).

Knowledge about susceptible hosts and geographical distribution of marine *Brucella* strains has increased considerably since first detection in dolphins and seals in the early 1990s (Ewalt, Payeur, Martin, Cummins, & Miller, 1994; Ross, Foster, Reid, Jahans, & MacMillan, 1994). The potential marine mammal hosts for *Brucella* spp. include up to 130 species; in almost half of them seropositivity has been demonstrated (Hernández-Mora, Palacios-Alfaro, & González-Barrientos, 2013). *Brucella*-type lesions are not often apparent (Foster et al., 2002; Guzmán-Verri et al., 2012; Hernández-Mora et al., 2013). Furthermore, 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 (Dagleish, Barley, Finlayson, Reid, & Foster, 2008; Dagleish et al., 2007; González et al., 2002; Hernández-Mora et al., 2008; Jauniaux et al., 2010). By contrast, the pathogenic role of *Brucella* spp. in pinnipeds is unclear and host adaptation with different infection patterns between eared and true seals has been proposed (Goldstein et al., 2009; Nymo et al., 2018; Nymo, Tryland, & Godfroid, 2011; Prenger-Berninghoff et al., 2008).

We recently reported the first case of cetacean brucellosis in the Southwest Atlantic Ocean, involving a Clymene dolphin (*Stenella clymene*) (Sánchez-Sarmiento et al., 2017). A contemporary study identified *B. ceti* in an individual of the same species that stranded approximately 760 km away (Attademo et al., 2018) alerting researchers and public health services over the circulation of this bacterium in the northeastern Brazilian coast. Given the zoonotic potential and the implications for marine mammal health and conservation,

TABLE 1 Number and species of marine mammals analysed in this study

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

we aimed to gain insight into the epidemiology and disease aspects of *Brucella* spp. in marine mammals throughout the Brazilian coast.

2 | MATERIALS AND METHODS

2.1 | Animals and samples included in the study

We analysed samples from 129 live- or dead-stranded or by-caught marine mammals: orders Cetartiodactyla (n = 124), Carnivora (n = 4) and Sirenia (n = 1; Table 1). In 10 of the 129 cases only blood or swabs were available for analyses. Standard necropsies were performed (Geraci & Lounsbury, 2005). Representative tissue samples from major organs were collected and fixed in 10% neutral buffered formalin for histological analysis, and selected tissues, fluids and/or swabs were stored frozen (−20°C, −80°C) until molecular analysis respectively. The samples were collected in collaboration with partner institutions along the Brazilian Coast from 1998 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.

Individual data including date and location, sex, total body length (TBL; from tip of rostrum to tail notch) and origin (stranding, bycatch) were recorded (Table S1). Age class categories were based on TBL according to published references for Guiana dolphin (*Sotalia guianensis*) (Rosas & Monteiro-Filho, 2002a), franciscana (*Pontoporia blainvillei*) (Danilewicz, 2003; Di Beneditto & Ramos, 2001; Rosas & Monteiro-Filho, 2002b) and remaining species (Marmontel, 1993; Reidenberg & Laitman, 2008). Decomposition code (Geraci & Lounsbury, 2005) and body condition (Pugliares et al., 2008) data as well as gross pathological findings were recorded in *Brucella*-exposed and/or infected cetaceans.

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).

2.2 | Molecular analysis

Samples from 129 animals were analysed, consisting of manually homogenized frozen tissues from 123 marine mammals and formalin-fixed, paraffin-embedded (FFPE) tissue sections from six cases (without available frozen tissue). DNA was extracted from the samples described previously using the DNeasy Blood & Tissue Kit (QIAGEN®, Valencia, CA, USA) and/or the CTAB phenol-chloroform method (Table S1; Kamel, Helmy, & Hafez, 2014; Vejarano et al.,

2013). DNA concentration and purity was determined via spectrophotometry with Epoch (Biotek®, Winooski, VE, USA). DNA concentration was adjusted when required by diluting template DNA down to 100–150 μM. All extractions (n = 454, Table S1) were tested by conventional polymerase chain reaction (PCR) with primers B4 and B5 targeting a 223 bp fragment of the *bcsp31* gene which encodes the 31kDa immunogenic (cell surface) protein of the genus *Brucella* (Baily, Krahn, Drasar, & Stoker, 1992). Amplification was performed in 25 μl of a reaction mixture containing 4 μl of template DNA, 1x One Taq® Quick load® (New England BioLabs®, Otsu, Shiga, Japan), 0.2 μM of each primer and *qsp* nuclease-free H_2O. 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). Amplification products were analysed by standard 1.5% agarose electrophoresis, using SYBR® safe DNA gel stain (Thermo Fisher Scientific®, Applied Biosystems, Waltham, MA).

As an alternative diagnostic approach, selected extractions (n = 134, Table S1) were submitted to a real-time PCR for the detection of *Brucella* spp. targeting the *IS711* gene. For that purpose, sequences of *Brucella* spp. and *B. ceti IS711* gene were aligned to design the primers 5′-TCAAAGTCCGGCGTATCAG-3′ and 5′- GAACCGGATCGAAGCATATC-3′, amplifying a fragment of approximately 234 bp, specific to the genus *Brucella*, using PRIMER 3 software (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), 0.375 μM of each primer and *qsp* nuclease-free H_2O. The real-time PCR cycle conditions were: initial denaturation at 98°C for 3 min, template denaturation at 98°C for 15 s, followed by 40 amplification cycles of template denaturation at 98°C for 15 s, primer annealing at 60°C for 30 s and primer extension at 65°C for 5 s, with a final extension at 72°C for 5s, in a CFX96 Touch™ Real-Time PCR Detection System. The melting curve analysis was generated immediately after the amplification protocol by heating from 55°C to 95°C in increments of 0.5°C/5 s.

Positive controls consisted of DNA from *B. abortus* (vaccine RB-51) and *B. ceti* (M644/93/1; B14/94). Nuclease-free water was used as no template control. Positive samples were identified through purification and direct sequencing of all amplicons detected either by PCR and/or real-time PCR. All products were purified with ExoSAP-IT™ PCR product clean-up (Affymetrix®, Santa Clara, CA, USA) according to the manufacturer's instructions, then sequenced using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, USA), and analysed through Sanger method on an automated sequencer Applied Biosystems 3500 Genetic Analyser (Applied Biosystems, Foster city, CA, USA). Sequences were assembled and aligned using Clustal W. After that, sequence identities between the obtained consensus sequences and other *Brucella* species available in GenBank were calculated based on the p-distance. All sequence analyses were performed with MEGA version 7.0 (Kumar, Stecher, & Tamura, 2016).

Standardization and preliminary real-time PCR analyses 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.

2.3 | Serological analysis

Serological analysis (Rose Bengal Test [RBT], competitive enzyme-linked immunosorbent assay [c-ELISA], and Serum Agglutination Test [SAT]) previously performed on available sera (27 of 129; 26 cetaceans and one manatee) demonstrated 10 seropositive cetaceans (Table 2). The methods employed and results obtained in this section constitute a parallel publication (Sánchez-Sarmiento et al., 2018).

2.4 | Histopathological and immunohistochemical analysis

We conducted microscopic examinations and immunohistochemistry (IHC) in all PCR/real-time PCR- and/or serology-positive animals (n = 13, Table 2). For histological analysis, FFPE tissues were processed following routine procedures and 5 μm-thick sections were cut and stained with haematoxylin and eosin. For IHC, 4 μm-thick cut tissue sections on silane-coated slides were dried (37°C × 6 hr–90°C × 2 hr), dewaxed and rehydrated by passing the slides through xylol, various ethanol solutions (decreasing grading), running tap water and distilled water. Next, formalin pigments were blocked in 25% ammonium hydroxide solution diluted in 95% alcohol (30 min), followed by tap water and distilled water wash. Antigen retrieval was performed in 10 mmol/L citrate buffer (pH 6) in a pressure cooker for 3 min. The endogenous peroxidase was blocked by immersion in hydrogen peroxide (37°C for 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®, Leica Biosystems, Newcastle, UK) (37°C for 30 min), followed by tap water and PBS wash. For amplification and detection of the signal we used two IHC protocols after previous standardization: (a) tissue sections were incubated overnight at 4°C with a primary polyclonal antibody against whole *B. abortus* (1:2,500; produced in rabbit, Byorbyt®, Cambridge, UK), incubated at 37°C for 30 min with a secondary antibody (rabbit anti mouse IgG) and then a Horseradish Peroxidase Polymer (HRP) System (anti-rabbit Poly-HRP-IgG, Novolink™ Polymer Detection System, Leica Biosystems®, Newcastle, UK) at 37°C for 30 min. Signal was visualized with 3,3'-diaminobenzidine (DAB) chromogen and sections were counterstained with Harris' haematoxylin; (b) tissue sections were incubated overnight at 4°C with a primary polyclonal antibody against whole *B. abortus* (1:500; produced in rabbit, Byorbyt®, Cambridge, UK), incubated at 37°C for 30 min with an alkaline phosphatase polymer (Simple stain AP [Multi] Histofine®, Nichirei Biosciences Inc., Tokyo, Japan) at 37°C for 30 min. Warp Red® (Biocare Medical, Pacheco, CA) was used as chromogen followed by counterstaining

TABLE 2 Individual data (species, age class, history, body condition, stranding location, date) and test results of *Brucella*-exposed and/or infected cetaceans. Individual pathological remarks and case definition are included

Case #	Individual data	Serology	PCR/real-time PCR	Immunohistochemistry	Culture	Pathological remarks/case definition (CD)/additional comments
1	*Feresa attenuata* **Sex:** male **Age class (TBL):** adult (2.13 m) **History:** live-stranded (code 1), died on the same day despite veterinary treatment. First register for 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, prescapular lymph node, spleen	Cerebrum, cerebellum, glandular stomach, liver, **lung**, lymph node, mandibular lymph node, spinal cord, **prescapular lymph node**, pulmonary lymph node, spleen	Blood, cerebrum, cerebellum, lung, spinal cord, prescapular lymph node	Chronic meningoencephalitis and meningitis, subacute pneumonia, lymphoid depletion (pulmonary lymph node), multicentric sinus histiocytosis and reactive hyperplasia, chronic pericholangitis, chronic enterocolitis CD: *Brucella* sp. exposure and suspected *Brucella*-chronic infection
2	*Globicephala macrorhynchus* **Sex:** male **Age class (TBL):** juvenile (2.90 m) **History:** live-stranded (code 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	Chronic gastritis and enterocolitis, necroulcerative glossitis, splenic lymphoid hyperplasia, multicentric lymphoid depletion CD: *Brucella* sp. exposure and suspected acute *Brucella*-gastrointestinal infection
3	*G. macrorhynchus* **Sex:** female **Age class (TBL):** newborn calf (1.57 m) **History:** live-stranded (code 1), medicated and released but remained in the area. The animal was recaptured 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**, oesophagus, glandular stomach, intestine, keratinized stomach, laryngeal tonsil, laryngeal lymph node, lung, spinal cord, mesenteric lymph node, pulmonary lymph node, pyloric stomach, trachea, spleen	Blood[f], cerebrum, cerebellum, lung, spinal cord, mesenteric lymph node, pulmonary lymph node	Suppurative leptomeningitis, multicentric lymphoid reactive hyperplasia, subacute pneumonia, cryptitis (tonsil), chronic tracheitis, splenic lymphoid depletion. CD: *Brucella* sp. exposure and suspected in-utero infection. Another concomitant infectious pathogen is likely

(Continues)

TABLE 2 (Continued)

Case #	Individual data	Serology	PCR/real-time PCR	Immunohistochemistry	Culture	Pathological remarks/case definition (CD)/additional comments
4	*G. macrorhynchus* **Sex:** female **Age class (TBL):** newborn calf (1.30 m) **History:** dead-stranded (code 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 CNS (cerebrum, cerebellum, spinal cord) spleen	Adrenal, anterior stomach, cerebrum, cerebellum, oesophagus, 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, multicentric lymphoid depletion and sinus histiocytosis (lymph node) CD: *Brucella* sp. exposure and suspected in-utero infection
5	*Peponocephala electra* **Sex:** male **Age class:** adult (2.40 m) **History:** Mass live-stranded (code 1), together with case # 6. It was treated and released. Likely belonged to a group that stranded two days before on Praia de Caetanos, Amontada, state of Ceará (215 km away). Possibly stranded dead few days after release; however, the advanced autolysis of the carcass limited pathologic examination. **Body condition:** good **Stranding location:** Praia das Fontes, beberibe, state of Ceará (04°12′59.5″S, 38°02′47.1″W) **Date:** November 7, 2014	**C-ELISA**	Blood	NA	Blood	CD: *Brucella* sp. exposure
6	*Peponocephala electra* **Sex:** female **Age class:** adult (2.46 m) **History:** Mass live-stranded (code 1), together with case # 5 (see above history) **Body condition:** good **Stranding location:** Praia das Fontes, Beberibe, state of Ceará (04°12′59.5′′S, 38°02′47.1′′W) **Date:** November 7, 2014	**C-ELISA**	Blood	NA	Blood	CD: *Brucella* sp. exposure

(Continues)

TABLE 2 (Continued)

Case #	Individual data	Serology	PCR/real-time PCR	Immunohistochemistry	Culture	Pathological remarks/case definition (CD)/additional comments
7	*Pontoporia blainvillei* **Sex:** female **Age class (TBL):** newborn calf (0.62 m) **History**: by-caught (code 2) **Body condition:** good **Bycatch location:** Praia grande, state of São Paulo (24°01′S; 46°28′W) **Date:** November 9, 2011	NA	Blood, pool CNS (cerebrum, cerebellum), hepatic lymph node, kidney, liver, lung, spinal cord, **mesenteric lymph node (real-time PCR [Cq = 31.01])**, prescapular lymph node	Cerebrum, cerebellum, colon, eye, glandular stomach, heart, keratinized stomach, kidney, larynx, liver, lung, **lymph nodes (hepatic, prescapular, mediastinal, mesenteric and renal),** ovary, skin, tongue, trachea, thymus, thyroid, uterus	Blood, pool CNS (cerebrum, cerebellum), hepatic lymph node, kidney, liver, lung, spinal cord, mesenteric lymph node, pericardial fluid, prescapular lymph node	Granulomatous thymitis, lymphoid reactive hyperplasia and sinus histiocytosis, focal neutrophilic alveolitis CD: *Brucella* sp. lymphoid asymptomatic infection
8	*Sotalia guianensis* **Sex:** female **Age class:** calf (1.08 m) **History:** dead-stranded (code 2) **Body condition:** poor **Stranding location:** Guriri, São Mateus, Espírito Santo state (18°44′S; 39°44′W) **Date:** November 30, 2010	NA	Blood, cerebrum, genitalia, kidney, liver, **pulmonary lymph node (PCR/ real-time PCR [Cq = 33.13])**, spleen	Adrenal, cerebrum, cerebellum, duodenum, oesophagus, eye, optic nerve, kidney, large intestine, **lymph nodes**[b], **liver**, **lung**, spinal cord, muscle, pancreas, pituitary gland, tongue, spleen	Blood, kidney, liver, pulmonary lymph node, spleen	Neutrophilic meningoencephalitis with perivascular cuffing, acute pneumonia, subacute necrotizing hepatitis and chronic pericholangitis, chronic colitis with cryptitis, lymphoid depletion, lymph node microabscesses CD: *Brucella* sp. in-utero infection. Cetacean morbillivirus coinfection. Another concomitant infectious pathogen is likely
9[g]	*Stenella clymene* **Sex:** male **Age class:** adult (1.75 m) **History:** live-stranded (code 1), (observed the night before). Died on the same day despite veterinary treatment **Body condition:** fair **Stranding location:** Parajuru, Beberibe, state of Ceará (04°23′28.7″S, 37°49′44.5″W) **Date:** March 10, 2012	**RBT, C-ELISA**	Blood, cerebrum, heart, kidney, liver, **lung (PCR/real-time PCR [Cq = 22.64])**, 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	Chronic pneumonia, splenic and mesenteric lymphoid depletion, sinus histiocytosis (mesenteric lymph node), splenitis, chronic nephritis, lymphoplasmacytic hepatitis, chronic gastritis, enterocolitis, necroulcerative glossitis CD: Pulmonary brucellosis (*Brucella* sp.)

(Continues)

TABLE 2 (Continued)

Case #	Individual data	Serology	PCR/real-time PCR	Immunohistochemistry	Culture	Pathological remarks/case definition (CD)/additional comments
10	*S. clymene* **Sex:** male **Age class:** adult[a] (1.70 m) **History:** live-stranded (code 1) (observed the day before). Received veterinary treatment but died 4 days after **Body condition:** fair **Stranding location:** Praia do Japao, Aquiraz, state of Ceará **Date:** January 10, 2016	**C-ELISA**	Blood, heart, kidney, liver, lung, mesenteric lymph node, pool CNS (cerebrum, cerebellum, spinal cord), spleen	Adrenal, cerebrum, cerebellum, glandular stomach, intestines, keratinized stomach, kidney, liver, lung[c], spinal cord, mesenteric lymph node, pancreas, prescapular lymph node, **skin/mucocutaneous junction**, testicle, tongue, tonsil, trachea, spleen, peri-ureteral lymph node, urinary bladder	Cerebrum, cerebellum, lung, mesenteric lymph node, kidney, spinal cord	Subacute meningitis and polyneuritis, acute meningitis, subacute gastritis, vascular fibrinoid necrosis, dermal neutrophilic vasculitis, multicentric lymphoid depletion, multicentric lymph node reactive hyperplasia and sinus histiocytosis, subacute pneumonia CD: *Brucella* sp. exposure. Additional bacterial infection suspected
11	*S. clymene* **Sex:** female **Age class:** adult[a] (1.76 m) **History:** live-stranded (code 1), treated and released one day after. The animal was found dead-stranded (code 3) 4 days after first stranding **Body condition:** fair **Stranding location:** 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 away from releasing area) **Date:** May 25, 2016	**RBT, C-ELISA, SAT**	Heart, kidney, liver, lung, spinal cord, pulmonary lymph node, spleen	Anterior stomach, **cerebrum,** oesophagus, **glandular stomach**, intestines, kidney[c], lung, spinal cord, melon fat, mesenteric lymph node, pulmonary lymph node, urinary bladder	Kidney, lung, spinal cord, pulmonary lymph node	Subacute lymphocytic meningomyelitis and polirradiculoganglioneuritis, multicentric lymphoid depletion and histiocytosis, reactive hyperplasia, subacute pneumonia, chronic nephritis CD: *Brucella* sp. exposure and suspected *Brucella*-chronic infection
12	*Stenella longirostris* **Sex:** male **Age class:** juvenile (1.21 m) **History:** dead-stranded (code 2) **Body condition:** poor **Stranding location:** Praia de Quitéras, Icapuí, state of Ceará (04°43′ 39.9″ S/ 37°17′ 48.6″W) **Date:** April 2, 2015	NA	Blood, **heart (real-time PCR [Cq = 33.09]),** kidney, liver, **lung (real-time PCR [Cq = 36.4]), meninges (real-time PCR [Cq = 31.49]),** spleen	**Fibrinosuppurative atlanto-occipital osteoarthritis with right lateral abscess,** adrenal, cerebellum, **colon, keratinized and glandular stomach, heart,** kidney, liver[c], lung, lymph node[b], mandibular fat, mandibular lymph node, **mesenteric lymph node**	Abscess and blood[d,f], blood, heart, kidney, liver, lung, meninges, spleen	Fibrinosuppurative atlanto-occipital osteoarthritis with right lateral abscess, minimal lymphocytic infiltrates in leptomeninges, lymph node reactive hyperplasia and sinus histiocytosis, chronic adrenalitis, pericarditis, pneumonia, nephritis, gastritis, colitis and pericholangitis CD: Chronic systemic brucellosis (*Brucella* sp.). Coinfection with *Proteus mirabilis*

(Continues)

TABLE 2 (Continued)

Case #	Individual data	Serology	PCR/real-time PCR	Immunohistochemistry	Culture	Pathological remarks/case definition (CD)/additional comments
13	*Tursiops truncatus* **Sex:** female **Age class:** adult (2.71 m) **History:** dead-stranded (code 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, prescapular lymph node, spleen, urinary bladder	Kidney[c], **liver**, **lung**, mesenteric lymph node, muscle, prescapular lymph node, spleen, **urinary bladder**	Urinary bladder, blood, cerebrum, uterus[e,f], kidney, lung, mesenteric lymph node, prescapular lymph node, urinary bladder	Subacute pyelonephritis, chronic cystitis, colitis, pericholangitis and gastritis, splenic lymphoid depletion, sinus histiocytosis (prescapular lymph node). CD: *Brucella* sp. exposure and suspected *Brucella*-infection. Coinfection with *Edwarsiella tarda*

Note: Decomposition code: 1 = live-stranded; 2 = freshly dead; 3 = decomposed, but organs basically intact (Geraci & Lounsbury, 2005)

Positive results in each category are marked in bold.

Abbreviations: C-ELISA, Competitive Elisa; CNS, Central nervous system; Cq, quantification cycle, real-time PCR; IHC, immunolabelling in positive tissues consistently diffuse otherwise specified; NA, Not analysed; PCR, conventional PCR; RBT, Rose Bengal Test; SAT, Serum Agglutination Test; TBL: total body length (from tip of rostrum to tail notch).

[a]Sexual maturity of the individual verified by histology.

[b]Several unspecified lymph nodes tested.

[c]Inconclusive.

[d]*Proteus mirabilis* was isolated from abscess and blood.

[e]*Edwarsiella tarda* was isolated.

[f]Independent culture done by external laboratory.

[g]Case # 9 was previously published (Sánchez-Sarmiento et al., 2017).

with Mayer's hematoxylin. Slides were then mounted with Entellan® (Merck, Darmstadt, Germany). Positive control consisted of placenta of a *B. ceti* infected striped dolphin (*Stenella coeruleoalba*) (kindly supplied by R. Gonzáles-Barrientos, G. Hernandez-Mora and C. Guzman-Verri, Costa Rica National University). As negative control, nonimmune homologous serum was used instead of the primary antibody (Figure S1). Positive and negative controls were included in each analysis and run for both methods.

2.5 | Culture and molecular analysis of isolates

Available frozen tissue samples from PCR/real-time PCR- and serology-positive animals (*n* = 13, Table 2) were cultured for *Brucella* spp. following standard procedures with the simultaneous use of two selective media (Farrell's and modified Thayer-Martin) (OIE, 2012). Briefly, 1 g of tissue was 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% bovine fetal serum and the two following antibiotic mixtures: (a) polymyxin B (sulphate) (5,000 IU/L); bacitracin (25.000 IU/L); nalidixic acid (5 mg/L); nystatin (100.000 IU/L); vancomycin (20 mg/L) and cycloheximide (30 mg/L) (Farrell's medium) (OIE, 2012). Cycloheximide was used instead of Natamycin due to the highly effective antibiotic activity against mould, yeast, and phytopathogenic fungi and lower activity against bacteria. The composition of antibiotic mixture (b) was: colistin methanesulfonate (7.5 mg/L), vancomycin (3 mg/L), nitrofurantoin (10 mg/L), nystatin (100,000 IU/L) and amphotericin B (2.5 mg/L) (modified Thayer-Martin medium) (OIE, 2012). Plates were incubated at 37°C and 10% CO_2 atmosphere, for 5 weeks. To enhance the chance of isolating *Brucella* spp. present in low numbers in tissues, enrichment of 100 µl of the suspension was carried out in liquid medium consisting of *Brucella* broth supplemented with the antibiotic mixture (a). The enrichment medium was incubated at 37°C and 10% CO_2 atmosphere for 3 weeks. Blind subcultures were performed each 4 days on plates containing *Brucella* agar supplemented with 5% of bovine fetal serum and incubated at 37°C and 10% CO_2 atmosphere and growth was verified each 4 days, for 15 days. Suspect colonies (circular, convex, shiny, 1–2 mm in diameter) were examined microscopically with Gram stain for purity (Alton, Jones, Angus, & Verger, 1998), and those displaying morphology compatible with genus *Brucella* (Gram-negative coccobacilli), were cultured as described previously for additional 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 (García-Yoldi et al., 2006). In each extraction group, TE buffer was submitted to the same extraction procedures, as negative control. Conventional PCR of all suspect colonies was performed targeting highly conserved regions among *Brucella* genus, including the gene *bcsp31* as described previously (Baily et al., 1992) and primers IS-517F22 targeting the insertion sequence *IS711* (Batinga, 2017). Positive and negative controls consisted of DNA from *B. canis* reference strain RM6/66 (ATCC 23,365) and nuclease-free water respectively.

For *bcsp31* 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, 1X buffer, 1.5 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) and 0.5 µM of each primer. The PCR cycle conditions were: initial denaturation at 94°C for 2 min, followed by 35 cycles of 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, with a final extension at 72°C for 10 min.

For *IS711* 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 $MgCl_2$, 1X buffer, 1.5 U of Platinum Taq DNA Polymerase, 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 *bcsp31* and *IS711* PCRs were performed using a Veriti™ Thermal Cycler (ThermoFisher Scientific, Applied Biosystems, Waltham, MA).

PCR amplification products were analysed by standard 1.5% agarose electrophoresis, using Ethidium Bromide (Invitrogen®, Termo Fisher Scientific, Carlsbad, CA, USA) (Ausubel et al., 1995). Samples presenting a PCR product of the expected size were excised from the gel and purified using Illustra®, GFX PCR DNA and GEL Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. PCR sequencing and posterior analyses were performed as described previously. 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, Pirassununga, Brazil.

2.6 | *Brucella* case definitions

Cases definitions were established based on available results from the aforementioned analyses. We classified *Brucella*-infected animals as those with positive PCR and/or real-time PCR, in addition to IHC and/or serology and in the presence or absence of *Brucella*-type lesions (e.g. fibrinosuppurative atlanto-occipital osteoarthritis, non-suppurative meningitis and/or meningoencephalitis, necrotizing hepatitis, pneumonia and pericarditis). Asymptomatic infection was considered on those cases without *Brucella*-type lesions but confirmed positive by PCR and/or real-time PCR in addition to IHC and/or serology. Cases with positive IHC and/or serology, in which DNA amplification was not possible remained as *Brucella*-exposure with suspected *Brucella*-infection. Cases with solely evidence of antibodies remained as *Brucella*-exposure.

3 | RESULTS

Overall, molecular, serological, immunohistochemical and pathological analyses provided evidences of *Brucella*-exposure, asymptomatic

infection and acute or chronic brucellosis in cetaceans sampled along the coastline of Brazil. Test results along with main pathological findings in *Brucella*-exposed and/or infected cetaceans are summarized in Table 2. The geographical distribution of stranding events for those positive cetaceans is recorded in Figure 1. Three coinfection cases involving *Brucella* sp. and cetacean morbillivirus (CeMV), *Edwarsiella tarda* and *Proteus mirabilis* were detected (Table 2).

3.1 | Molecular and serological analysis

Thirteen of the 129 (10.1%) animals were positive by PCR and/or real-time PCR (in six different tissue samples) and/or serology (Table 2). From these, 10 (76.9%) were serologically positive (detailed results published (Sánchez-Sarmiento et al., 2018)), including one pygmy killer whale (*Feresa attenuata*), three short-finned pilot whales (*Globicephala macrorhynchus*), two melon-headed whales (*Peponocephala electra*) three Clymene dolphins and one bottlenose dolphin (*Tursiops truncatus*). Three individuals had no sera available. Four of the 13 (30.8%) animals were *Brucella*-positive by molecular analyses; two animals by PCR (*bcsp31* gene) and real-time PCR (*IS711* gene), cases # 8 and # 9 and other two animals solely by real-time PCR (*IS711* gene), cases # 7 and # 12; including one Clymene dolphin [previously published in Sánchez-Sarmiento et al., (2017), positive by PCR and real-time PCR in lung, case # 9], one spinner dolphin (*Stenella longirostris*, positive by real-time PCR in heart, lung and meninges, case # 12), one franciscana (positive by real-time PCR in mesenteric lymph node, case # 7) and one Guiana dolphin (positive by PCR and real-time PCR in pulmonary lymph node, case # 8) (Table 2). All the obtained *Brucella* sequences were identical among them and presented 100% nucleotide identity to *Brucella* spp. sequences available at GenBank (e.g. access no.

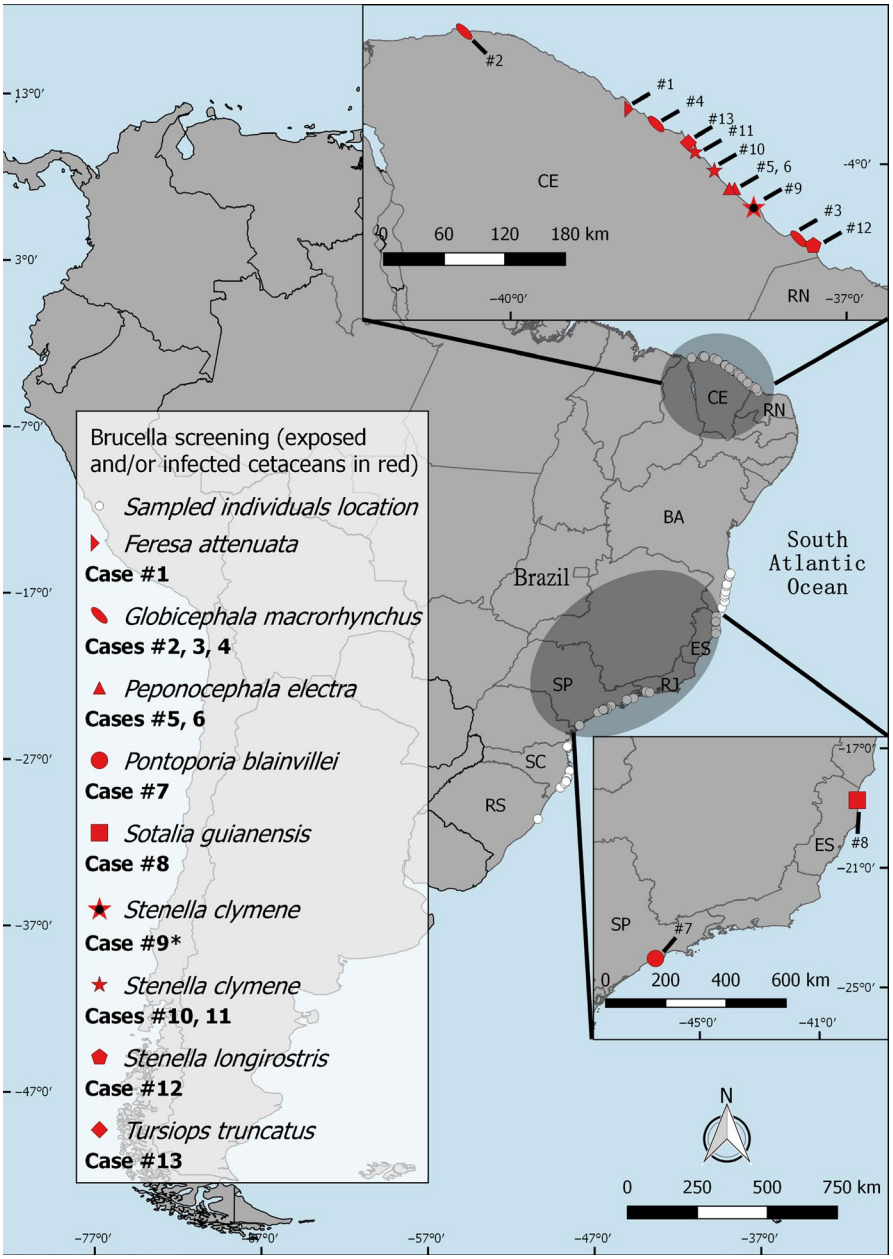

FIGURE 1 Location of individuals tested in this study (white dots) and *Brucella*-exposed and/or infected cetaceans (red symbols). Species information and case number is also provided (text box). CE, State of Ceará; BA, State of Bahia; ES, State of Espírito Santo, RJ, State of Rio de Janeiro; RN, State of Rio Grande do Norte; RS, State of Rio Grande do Sul; SC, State of Santa Catarina; SP, State of Sao Paulo. *Case # 9 was previously published (Sánchez-Sarmiento et al., 2017) [Colour figure can be viewed at wileyonlinelibrary.com]

CP006899 [*B. ceti*], CP007743 [*B. pinnipedialis*], CP007717 [*B. suis*], CP027643 [*B. canis*], and CP001578 [*B. microti*]) for the *IS711* gene and CP034103 [*B. melitensis*], CP033079 [*B. abortus*] and CP027643 [*B. canis*] for the *bcsp31* gene). The short length of the amplified fragment after excluding primers precluded the deposit of the obtained sequences in GenBank.

The positivity proportions were: 7/52 (13.5%) females, 6/74 (8.1%) males. Sex was not informed in three animals. According to age category, the results were: 3/16 (18.7%) newborn calves, 7/62 (11.3%) adults, 2/20 (10%) juveniles and 1/28 (3.6%) calves. Age class was not informed in two animals and one was a fetus. Regarding origin, 1/31 (3.2%) were bycatch and 12/92 (13%) stranding. Origin was not informed in six animals. Occurrence of *Brucella*-exposed and/or infected animals was higher in the state of Ceará (23.9%; 11/46) followed by Espírito Santo (9%; 1/11) and São Paulo (2.8%; 1/35). *Brucella* occurrence by species was: short-finned pilot whale (75%; 3/4), Clymene dolphin (50%; 3/6), pygmy killer whale (50%; 1/2), bottlenose dolphin and spinner dolphin (33.3%; 1/3), melon-headed whale (18.2%; 2/11), Guiana dolphin (4.3%; 1/23) and franciscana (2.5%; 1/40). All individuals from families Balaenopteridae, Kogiidae, Physeteridae, Ziphiidae, Balaenidae, Otariidae and Trichechidae were negative for *Brucella* using PCR and/or real-time PCR.

3.2 | Pathological and immunohistochemical analysis

A wide variety of gross and microscopic findings were observed in 11 of the 13 PCR/real-time PCR—and/or serology *Brucella*-positive cases (Table 2, Table S2). In two serologically positive animals the necropsy was not performed, thus tissues were not available for histopathology and IHC. The main gross findings included lymphadenomegaly (8/11; 72.7%), and splenomegaly (7/11; 63.4%). Grossly, case # 12 (spinner dolphin, Figure 2a) presented severe fibrinosuppurative atlanto-occipital osteoarthritis with right lateral abscess formation (considered the only gross *Brucella*-type lesion; Figure 2b,c). The main microscopic findings were: lymphoid depletion (9/11; 81.8%); lymphoplasmacytic to neutrophilic to pyogranulomatous interstitial pneumonia or bronchopneumonia (7/11; 63.3%); multicentric (oropharyngeal and laryngeal tonsils, spleen, lymph nodes) lymphoid reactive hyperplasia (7/11; 63.3%); and lymphoplasmacytic to eosinophilic enterocolitis with cryptitis (6/11; 54.5%). Non-suppurative meningitis and/or meningoencephalitis involving the spinal cord, medulla oblongata and cerebellum, necrotizing hepatitis (Figure 3a), pneumonia and pericarditis were considered *Brucella*-type lesions. Positive immunolabelling of phagocytic cells and/or free bacillary bacteria in inflammatory foci (Figure 3) for *Brucella* spp. was observed in at least one organ in 11 of the 13 PCR, real-time PCR- and/or serology-positive animals (necropsied individuals, as described previously), Table 2. Positive immunolabelling was associated with *Brucella*-type lesions in seven of these animals (case # 1, 3, 4, 8, 9, 11, 12), the last case coinfected with *P. mirabilis*. Moreover, one case of *Brucella* sp. exposure and suspected *Brucella*-infection, had coinfection with *E. tarda* (case # 13), Table 2.

3.3 | Culture and molecular analysis of isolates

Culture attempts of frozen tissue samples from PCR/real-time PCR- and/or serology-positive dolphins were unsuccessful. Thus, further characterization including genotyping of the *Brucella* strain/s involved could not be performed.

4 | DISCUSSION

To the best of our knowledge, this study represents the first long-term and large-scale survey of *Brucella* spp. in marine mammals of Brazil. We found a relatively high occurrence of *Brucella*-positivity (10.1%) in bycaught and/or stranded Delphinidae and Pontoporidae cetaceans along the coastline of Brazil. All pinnipeds and one manatee tested in this study were negative. Previous studies have documented *Brucella* infection in several pinniped species but not in manatees (Guzmán-Verri et al., 2012; Hernández-Mora et al., 2013). In this study, all *Brucella*-exposed and/or infected animals presented some pathological findings, for example, pneumonia, interstitial nephritis, enterocolitis and lymphoid depletion, that have been described before in dolphins with brucellosis (González-Barrientos et al., 2010; Sánchez-Sarmiento et al., 2017). Lymphoid depletion and systemic spread or multiorganic involvement in these cases suggests immunocompromised host responses. All cases analysed by IHC show positivity in at least one organ; however, the interpretation of *Brucella*-infection was performed carefully, taking into account that the IHC using a polyclonal antibody will react with antigenic domains of lipopolysaccharide (S-LPS) of smooth *Brucella* including marine mammals strains, but may also cross-react with other Gram-negative species (Kittelberger, Bundesen, Cloeckaert, Greiser-Wilke, & Letesson, 1998). Thus, in addition to serology and IHC, DNA amplification was used for case definition and confirmation of the status of *Brucella*-infection (acute, chronic or asymptomatic). Particular aspects of those cases and implications of the findings are discussed below.

An asymptomatic lymphoid *Brucella*-infection was determined in a bycaught newborn calf female franciscana (case # 7), based on a real-time PCR- and IHC positive mesenteric lymph node and additional IHC positive lymph nodes (hepatic, prescapular, mediastinal, renal). All lymph nodes presented diffuse lymphoid reactive hyperplasia with sinus histiocytosis. This animal also presented haemodynamic changes related to bycatch and granulomatous thymitis, an incidental finding not associated with *Brucella*-infection. Subclinical infection is commonly noted in various marine mammal species (Buckle et al., 2017; Foster et al., 2002).

Pulmonary brucellosis was reported in an adult male Clymene dolphin stranded in state of Ceará (case # 9; [Sánchez-Sarmiento et al., 2017]) with pyogranulomatous bronchopneumonia associated

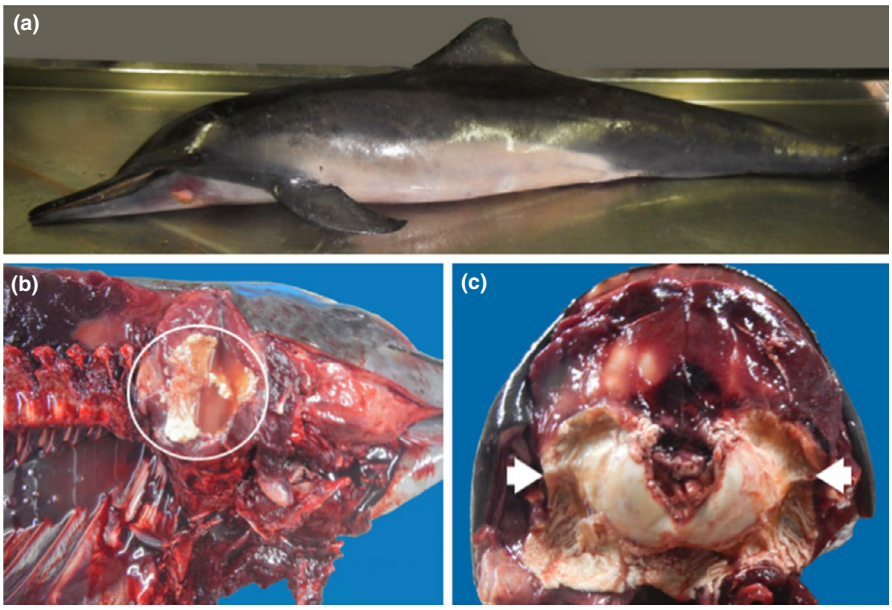

FIGURE 2 Gross findings in a spinner dolphin (*Stenella longirostris*) with chronic brucellosis (case # 12). (a) Dead-stranded animal presenting fair body condition. (b) Fibrinosuppurative osteoarthritis with right lateral abscess at the atlanto-occipital joint (circle). (c) The atlanto-occipital joint capsules are severely distended (arrows) and fibrosed, and there is remodelling of the articular cartilage [Colour figure can be viewed at wileyonlinelibrary.com]

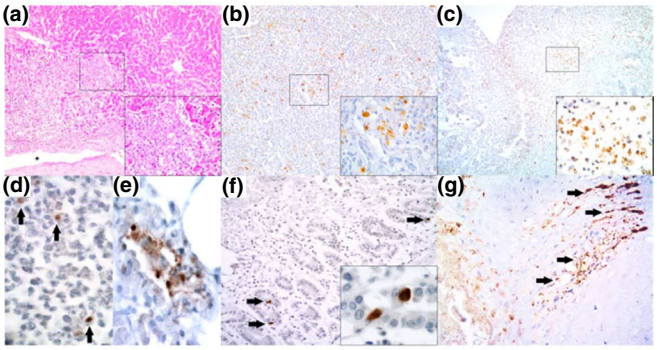

FIGURE 3 Histopathological and immunohistochemical findings of Brucella-exposed and/or infected cetaceans. Guiana dolphin (Sotalia guianensis) co-infected by Brucella spp. and cetacean morbillivirus, case # 8. (a) Focally extensive subcapsular necrotizing hepatitis, 100x. Asterisk indicates capsular lymphatic vessel. Hematoxylin and eosin (HE). Inset: higher magnification of squared area in Figure 3a, HE, 400x. (b) Multifocal strong Brucella-antigen immunolabeling in mononuclear cells (MNCs) including Kupffer cells and lymphocytes in inflamed hepatic parenchyma, DAB, 200x. Inset: higher magnification of squared area in Figure 3b, DAB, 1000x. Spinner dolphin (Stenella longirostris) with chronic brucellosis, case # 12. (c) Strong Brucella antigen immunolabeling in inflamed atlanto-occipital joint capsule, DAB, 40x. Inset: higher magnification of squared area in Figure 3c, 400x. (d) Scattered macrophages with Brucella-antigen (arrows) in the lymph node, DAB, 400x. (e) Brucella-exposed and suspected in-utero infected short-finned pilot whale, case # 4. Observe Brucella-antigen immunolabeling in alveolar macrophages and scattered in interstitium, DAB, 400x. Brucella-exposed and suspected gastrointestinal infected short-finned pilot whale, case # 2. (f) Positive immunolabeling is observed in scattered mononuclear cells (arrows) in intestinal lamina propria, DAB, 100x. Inset: Higher magnification, 400x. (g) Positive immunolabeling of bacilli in glossal lesion (arrows), DAB, 200x. [Colour figure can be viewed at wileyonlinelibrary.com]

with *Halocercus* sp. infestation. In this serologically positive case, the lung was PCR/real-time PCR *Brucella*-positive and *Brucella* antigens were detected in intraalveolar macrophages. Although previous reports documented *Brucella* spp. antigens within nematode larvae in lung of cetaceans and pinnipeds (Colegrove et al., 2016; Garner et al., 1997; Jauniaux et al., 2010; Rhyan, Garner, Spraker, Lambourn, & Cheville, 2018), suggesting a potential role for transmission of *Brucella* spp.; we did not detect immunolabelling in lungworms in the present case. Additional PCR evidence of *Brucella*-lung infections have been reported, with or without associated pulmonary lesions (Colegrove et al., 2016; Foster et al., 2002; McAloose et al., 2016; Prenger-Berninghoff et al., 2008).

Chronic systemic brucellosis was diagnosed in a spinner dolphin (case # 12), a juvenile male dead-stranded in state of Ceará, with severe, chronic fibrinosuppurative atlanto-occipital osteoarthritis and lateral abscess formation. *Brucella* DNA was detected by real-time PCR in the meninges, heart and lung and microscopic examination revealed leptomeningitis and chronic pericarditis. *Brucella* immunopositivity was noted in several tissues. Skeletal lesions associated with localized or systemic *Brucella* infection have been described in marine mammals. For instance, in a white-sided dolphin (*Lagenorhynchus acutus*) with atlanto-occipital osteoarthritis (Dagleish et al., 2007), a short-beaked common dolphin (*Delphinus delphis*) with atlanto-occipital arthritis and associated

meningoencephalitis (Davison et al., 2013), a bottlenose dolphin with discospondylitis (Isidoro-Ayza et al., 2014) and in a striped dolphin with scapulohumeral fibrinopurulent osteoarthritis also presenting meningoencephalomyelitis (González-Barrientos et al., 2010). All these cases were infected by *B. ceti*. Osteoarticular disease is the most common complication of human brucellosis (Pappas, Akritidis, Bosilkovski, & Tsianos, 2005). Moreover, the distribution pattern of *Brucella* arthritis (monoarthritis, sacroiliitis and spondylitis) may be related to differences in *Brucella* species involved (González-Gay & García-Porrúa, 2014) and host's genetic predisposition (Bravo, Colmenero, Alonso, & Caballero, 2003).

In case # 12, heart and brain inflammatory lesions differed to some extent in severity and pattern from endocarditis and pericardial fibrosis and chronic meningoencephalitis reported in *B. ceti*-infected striped dolphins (González-Barrientos et al., 2010). In humans, cardiovascular involvement is uncommon yet both endocarditis and epicarditis were described (Kaya, Eskazan, & Elaldi, 2013; Reguera et al., 2003). Central nervous system (CNS) involvement in *Brucella*-infection in man, occurs in about 5%–7% of the cases (Pappas et al., 2005) and include neurotropic presentations as meningitis, encephalitis, meningovascular disease, brain abscesses and demyelinating syndrome (Shakir et al., 1987). *Brucella*-type lesions in the CNS, recapitulating features of neurobrucellosis in humans (Shakir et al., 1987) and striped dolphins (Hernández-Mora et al., 2008) were seen in two stranded animals, an adult male pygmy killer whale (case # 1) and a juvenile male Clymene dolphin (case # 11) with positive serology and IHC in lung and lymph node (case # 1) and cerebrum and glandular stomach (case # 11), suggesting *Brucella*-chronic infection. CNS *Brucella*-type lesions may include chronic non-suppurative meningitis (more severe in the brainstem followed by the cerebral and cerebellar cortices) and periventricular encephalitis extending into the lateral, third and fourth ventricles. Perivascular cuffing, scattered foci of microgliosis and mononuclear choroiditis with lymphoid follicle formation are also common (González et al., 2002; Hernández-Mora et al., 2008). Differential aetiological diagnosis for CNS inflammation in cetaceans may include: CeMV, herpesvirus, West Nile virus, *Toxoplasma gondii*, *Sarcocystis neurona* and *Nasitrema* spp. (Barbosa et al., 2015; Di Guardo et al., 2010; Díaz-Delgado et al., 2018; Esperón, Fernández, & Sánchez-Vizcaíno, 2008; Kennedy, Lindstedt, McAliskey, McConnell, & McCullough, 1992; Resendes et al., 2002; St. Leger, 2011; Van Bressem et al., 2014). The inflammatory pattern and the visualization of the pathogen in tissue sections may allow a final diagnosis on histological examination.

We detected coinfection by marine *Brucella* and other pathogens, as reported before for several cetacean species infected by *Brucella* and CeMV (West et al., 2014), *E. tarda* (Dawson, Perrett, Young, Davison, & Monies, 2006), *T. gondii* (Alba et al., 2013) or *T. gondii* and *Listeria monocytogenes* (Grattarola et al., 2016). In this study, a stranded female calf Guiana dolphin (case # 8), previously identified as infected by a novel lineage of CeMV (Groch et al., 2014), was positive for *Brucella* sp. by PCR/real-time PCR in pulmonary lymph node and IHC in various tissues. A similar coinfection was reported in a sperm whale (*Physeter macrocephalus*) in Hawaii (West et al., 2014). Case # 12 had coinfection between *Brucella* sp. and *P. mirabilis*. The latter was isolated from blood and the atlanto-occipital abscess. *P. mirabilis* has been isolated from integumentary, respiratory and/or digestive system in bottlenose dolphin and beluga whale (*Delphinapterus leucas*) in association with other bacteria (Higgins, 2000), and is occasionally associated with pneumonia (Eo & Kwon, 2011). An adult female stranded bottlenose dolphin (case # 13), was serologically positive and presented *Brucella*-immunopositivity in several tissues (liver, lung and urinary bladder); additionally, *E. tarda* was isolated from uterus, indicating a coinfection by *Brucella* sp. and *E. tarda*. In this case, ulcerative and suppurative cystitis and pyelonephritis were attributed to *E. tarda*. Edwardsiellosis, caused by *E. tarda*, may lead to suppurative to necrotizing nephritis, hepatitis and splenitis in fish (Darwish Ahmed; Plumb, 2000; Ucko, Colorni, Dubytska, & Thune, 2016). This bacterium has been isolated from cetaceans (Buck, Overstrom, Patton, Anderson, & Gorzelany, 1991; Venn-Watson, Smith, & Jensen, 2008), and was regarded as the cause of stranding and septicemic death of a sperm whale (Cools et al., 2013). A coinfection by *E. tarda* and *Brucella* sp. was reported before in a bottlenose dolphin; however, no lesions were related to *E. tarda* (Dawson et al., 2006).

Additionally, the Guiana dolphin (case # 8) and an adult male stranded Clymene dolphin (case # 10), the latter serologically positive, presented CNS findings which diverged from *Brucella*-type lesions as previously described (González et al., 2002; Hernández-Mora et al., 2008; Muñoz et al., 2006), suggesting potential bacterial infections/coinfections as the cause of the CNS suppurative inflammation (Grattarola et al., 2016). Complete blood count (CBC) analysis in case # 10 (data not shown) supported an acute bacterial infection. This case also presented multisystemic microangiopathy characterized by fibrinoid vascular necrosis and vasculitis. However, no associated *Brucella*-antigen was detected by IHC. Similar vascular findings have been reported in dolphins (González et al., 2002) and humans (Korkmaz & Karta, 2016) with brucellosis.

Brucella-exposure was observed in two adult mass live-stranded melon-headed whales, a male and a female (cases # 5 and # 6). Interestingly, CBC indicated leukopenia and lymphocytosis (data not shown), suggestive of viral infection in these cases. *Brucella*-exposure and suspected acute *Brucella*-infection was also observed in a juvenile male short-finned pilot whale stranded in state of Ceará (case # 2) in which *Brucella*-antigen in a glossal lesion and foci of intestinal inflammation suggested gastrointestinal transmission.

Two serologically positive stranded newborn female short-finned pilot whales (case # 3 and # 4) presented pathological and IHC findings that suggested an in-utero infection and vertical transmission; acute leptomeningitis associated with positive immunolabelling and pneumonia (case # 3) and pneumonia with positive *Brucella*-antigen detected (case # 4) (Colegrove et al., 2016; Hong et al., 1991; West et al., 2014; Xavier, Paixão, Poester, Lage, & Santos, 2009). Similarly, pathological findings (acute meningoencephalitis, pneumonia, hepatitis and lymph node microabscesses), PCR and *Brucella*-antigen distribution in case # 8 (Guiana dolphin), suggested the same transmission route. Potential transmission routes of marine brucellosis

include direct transmission through social/sexual interaction, transplacental, physical trauma (violent intra- and interspecific interactions) and ingestion of contaminated prey (Foster et al., 2002). 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 (Tuon, Gondolfo, & Cerchiari, 2017). Vertical transmission of *Brucella* sp. in cetaceans was also reported in aborted (Ewalt et al., 1994), neonatal (Colegrove et al., 2016), or fetuses with no apparent lesions (González-Barrientos et al., 2010; Hernández-Mora et al., 2008).

Regarding *Brucella*-positivity and age; the highest frequency of *Brucella* sp. infection was confirmed in newborn calves (18.7%), followed by adults (11.3%), juveniles (10%) and calves (3.6%). We did not find evidence of *Brucella*-infection in a single fetus analysed in this study. This age pattern of positivity seems to be in accordance with a previous study in bottlenose dolphins stranded in South Carolina, USA, in which a relationship between age-class and *Brucella* DNA detection was found (Wu, McFee, Goldstein, Tiller, & Schwacke, 2014). By contrast, in Peru, sexually mature small cetaceans were more frequently seropositive than immature ones (Van Bressem et al., 2001). *Brucella*-type lesions such as placentitis, metritis and orchitis, are more often reported in sexually mature animals (Buckle et al., 2017; Dagleish et al., 2008; González-Barrientos et al., 2010).

The highest occurrence of *Brucella*-exposed and/or infected animals was found in the state of Ceará, suggesting a high presence of the bacterium in the area. Also, the results suggest a possible susceptibility of local Clymene dolphin and short-finned pilot whale. *Brucella*-exposure and suspected infection detected in pygmy killer whale and bottlenose dolphin and *Brucella*-exposure in melon-headed whale, corroborate previous reports indicating these species as susceptible hosts (Dawson et al., 2006; Hernández-Mora et al., 2009). We also found the first evidence of *Brucella*-exposure and suspected infection in short-finned pilot whale; *B. ceti*-associated meningoencephalitis was reported in a member of this family, the long-finned pilot whale (*Globicephala melas*) (Davison et al., 2015; Foster et al., 2015). Our findings also support the role of *Brucella* spp. in stranding and/or death, as reported before for striped dolphins (González-Barrientos et al., 2010). To the authors' knowledge, our results constitute the first report of *Brucella*-infection in the spinner dolphin, the franciscana and the Guiana dolphin, confirming those species as new *Brucella*-hosts. The franciscana and Guiana dolphin are endangered coastal species threatened by bycatch and pollution in Brazil (Barreto et al., 2011; Di Beneditto et al., 2010). This raises high concerns as previous studies in bottlenose dolphins demonstrated a possible relation between increased susceptibility to *Brucella* infection during an unusual mortality event followed by an oil spill, which negatively impacted the health of those animals (Colegrove et al., 2016). Epidemiological studies about the risks factors for *Brucella*-infection in marine mammals are lacking, and the role of environmental factors in this emerging disease remain elusive (Van Bressem et al., 2001). In general, inshore and estuarine cetaceans are at higher risks of infectious diseases than pelagic cetaceans due to anthropogenic habitat degradation (Van Bressem et al., 2001). Also, greater densities increase the chances of susceptible individuals to be exposed to the source of infection (Thrusfield, 2007). Furthermore, intra- and interspecific contact between infected and naïve cetaceans may contribute to the maintenance of the infection in the environment. Our sampling was opportunistic and thus, not adequate to further evaluate statistical associations and long-term trends. However, the results highlight the need for continuous surveillance and monitoring studies to understand how degraded and polluted ecosystems relate to occurrence of infectious diseases, for example, brucellosis, in cetaceans along the Brazilian coast. More research is needed to better understand the main pathogen-, host- and environmental factors involved in cetacean *Brucella* epidemiology, with special emphasis on Northeastern Brazil.

In summary, our results provide novel data while compelling serological, molecular, immunohistochemical and pathological evidences of *Brucella* spp. in several cetacean species in Brazil, widening the spectrum of susceptible hosts and geographical distribution range of this agent. Given the demonstrated zoonotic potential of this bacterium (Brew et al., 1999; McDonald et al., 2006; Sohn et al., 2003), proper handling of live-stranded animals and carcasses by first responders, rehabilitators, necropsy prosectors and further laboratory personnel in Brazil, particularly in the state of Ceará, is strongly encouraged. As we could not isolate *Brucella* spp., further characterization of the *Brucella* strains involved in positive cases and definitive microbiological confirmation was precluded. Decomposition and autolysis phenomena, together with postmortem bacterial overgrowth, also probably hampered our culture attempts (Buckle et al., 2017; Colegrove et al., 2016). Prolonged pre-analytical frozen time intervals might also have negatively affected bacterial viability. Future studies should focus on culture and definitive identification and characterization of circulating marine *Brucella* species, including the identification of genotype ST27, due to its public health relevance. Continuous effort on *Brucella* monitoring is necessary because of the wide range of susceptible hosts (Hernández-Mora et al., 2013) and potential endemicity of some species, such as pinnipeds (McFarlane, 2009; Tryland, Sørensen, & Godfroid, 2005).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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