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NOTE

Tuberculosis caused by *Mycobacterium pinnipedii* in a wild South American sea lion *Otaria flavescens* stranded in southern Brazil

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ABSTRACT: Tuberculosis (TB) in pinnipeds is typically caused by *Mycobacterium pinnipedii,* which has also been associated with infections in other species, such as cattle and humans. As a result, this pathogen has zoonotic potential and is a public health concern. In 2016, a female South American sea lion *Otaria flavescens* in southern Brazil presented with emaciation and severe dyspnea and died within 3 h of capture. Gross pathology identified pulmonary granulomas, and Ziehl-Neelsen stain identified acid-fast bacilli. *M. tuberculosis* complex bacteria were confirmed by a BD BACTEC™ MGIT™ 320 detection system using fibrinous exudate, lung granulomas and thoracic fluid. Molecular characterization by spoligotyping showed a hybridization pattern characteristic of *M. pinnipedii* (SIT593/PINI1). Currently, there is a paucity of data concerning the transmission and epidemiology of *M. pinnipedii* in pinniped populations in South America. The case report shows that the disease appeared in a free-ranging beached sea lion on the coast, and further surveillance is needed to determine the origin of this TB because of its potential impact on public health.

KEY WORDS: Sea lion · *Mycobacterium tuberculosis* complex · *Mycobacterium pinnipedii* · Pinnipeds · Wildlife · Spoligotyping · Zoonosis

1. INTRODUCTION

Tuberculosis (TB) occurs worldwide and infects both humans and animals. The major impacts of this disease include effects on endangered species, economic losses, trade implications and public health hazards (Thoen et al. 2009). In pinnipeds, severe respiratory compromise and weight loss may occur, although clinical signs can be nonspecific or even absent (Moser et al. 2008, Kriz et al. 2011). Therefore,

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the antemortem diagnosis of this disease can be difficult in both captive and wild animals (Kaneene et al. 2010).

TB diagnosis using serological tests such as rapid test (e.g. ElephantTB STAT-PAK, DPP® VetTB) or a multiantigen print immunoassay, accompanied by imaging studies in live pinnipeds is rarely reported (Jurczynski et al. 2011b). Most diagnoses are made at necropsy following culture of the organism or molecular confirmation of the mycobacteria in lesions. The main macroscopic findings are as follows: (1) yellowish or reddish exudate in the thoracic cavity, (2) fibrinous tissue covering the heart and/or lungs, (3) caseous nodules on the parietal and/or visceral surfaces of lungs, and (4) mesenteric lymphadenopathy (Bastida et al. 1999, Kriz et al. 2011, Amorim et al. 2014).

TB in pinnipeds is usually caused by *Mycobacterium pinnipedii,* one of the 8 members of the *M. tuberculosis* complex (MTBC), which also includes *M. tuberculosis, M. bovis, M. microti* and other less common species (Orgeur & Brosch 2018). MTBC members exhibit phenotypic differences, including different host tropisms and pathogenicities. They are characterized by 99.9% genome similarities and identical 16S and rDNA sequences (Brosch et al. 2002). These bacteria can be differentiated by evolutionary signatures using molecular methods such as spoligotyping. This technique is one of the most often used to genotype MTBC species, as it can differentiate between species and compare results to those of a global database of over 7104 different spoligotypes from 102 countries (Demay et al. 2012).

M. pinnipedii can also be associated with TB in other terrestrial species, such as seals in zoos (Jurczynski et al. 2011a), cattle (Loeffler et al. 2014) and humans that have been exposed to infected pinnipeds (Kiers et al. 2008). This bacterium has zoonotic potential and is a recognized public health concern (Kiers et al. 2008). In this paper, we describe severe TB due to *M. pinnipedii* in an adult female beached South American sea lion recovered from the southern coast of Brazil.

2. CASE DESCRIPTION

In August 2016, the staff of the Centro de Recuperação de Animais Marinhos (Marine Animals Recovery Center) of the Federal University of Rio Grande rescued an adult female South American sea lion *Otaria flavescens* on the beach of Mar Grosso, city of São José do Norte, Rio Grande do Sul, Brazil (32° 3′ 10″ S, 51° 59′ 26″ W). The emaciated animal presented with severe dyspnea and died within 3 h of rescue. A necropsy was conducted according to standard protocols (Geraci & Lounsbury 1993).

Necropsy confirmed a 142 cm long, 61.9 kg young adult female in poor physical condition with sparse subcutaneous fat. The most significant macroscopic observations were pleuritis with approximately 8 l of translucent tan-yellow serous fluid in the thoracic cavity and a white mass diffusely covering the lung surface. After this material was removed, 2 granulomas were observed in the lungs. The first was 30 mm in diameter on the parietal surface of the left lung, and the second was 70 mm in diameter in the upper lobe of the right lung. The heart was covered by fibrinous exudate, and the mesenteric lymph nodes were enlarged (Fig. 1).

Pathology was consistent with chronic inflammation and revealed nonsuppurative interstitial nephritis and foci of dystrophic calcification in the parenchyma of the kidneys with random multifocal lymphohistiocytic cholangiohepatitis. In the trachea, focal mononuclear infiltrate was interspersed between the submucosal glands and within the lungs, and there were variably extensive areas of coagulative and liquefactive necrosis with multifocal dystrophic calcification and scattered lymph histiocytic infiltrates throughout. In addition, the whitish mass adjacent to the lungs consisted of a granuloma containing a caseous center and peripheral mononuclear inflammatory cells. Morphological diagnoses included chronic interstitial nephritis, cholangiohepatitis, tracheitis and pneumonia with granuloma formation. Sections of the lymph nodes revealed reactive change and medullary histiocytosis with occasional granuloma formation. Ziehl-Neelsen stain confirmed acid-fast bacilli within the inflammation infiltrate and associated areas of necrosis.

To identify the etiological agent, samples of the fibrinous exudate, granuloma and thoracic fluid were evaluated by conventional microbiology (microscopy and bacterial isolation) and molecular characterization. Fragments of the fibrinous tissue and granuloma were separately macerated, transferred to a 50 ml conical flask and decontaminated with 5 ml of BBL™ MycoPrep™ reagent (BD Diagnostics) for 15 min. Afterwards, 45 ml of phosphate buffer (pH 6.8, 0.067 M Na_2HPO_4 + KH_2PO_4) was added, and the tubes were centrifuged for 15 min at 3660 × *g*. In parallel, 20 ml of thoracic fluid was also centrifuged for 15 min at 3660 × *g*. A 1 ml sample of the supernatant was retained to resuspend the sediment for microbiological analysis. To detect acid-fast bacilli, 100 µl of

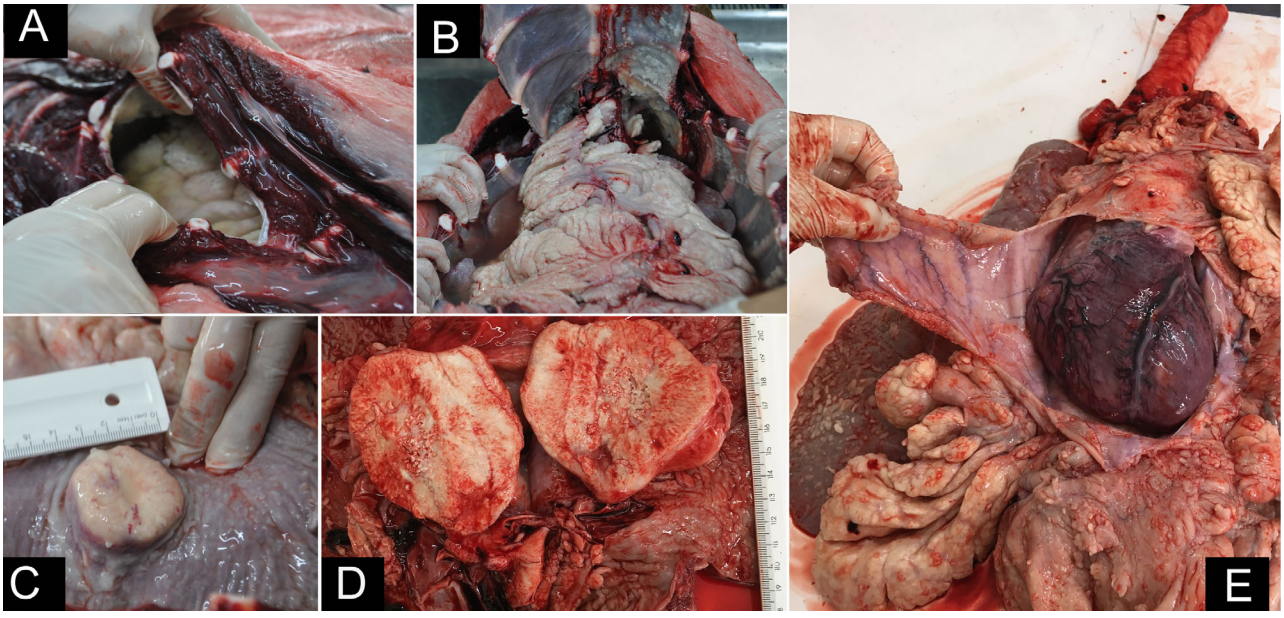

Fig. 1. Lesions found during the necropsy of a South American sea lion with tuberculosis. (A) Large quantity of yellow serous fluid in the thoracic cavity; (B) diffuse white discoloration of the visceral pleura; (C) caseous granuloma, 30 mm in diameter, on the parietal surface of the left lung; (D) caseous granuloma, 70 mm in diameter, in the upper lobe of the right lung; and (E) pericardial thickening due to fibrinous exudate and fibrosis

the suspension was smeared on slides, stained using the Ziehl-Neelsen method and evaluated at 100× magnification with immersion oil (Brazil 2008).

For mycobacterial culture, 500 µl of the suspensions were incubated in mycobacteria growth indicator tubes (MGITs) supplemented with 800 µl of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin diluted in oleic acid, bovine serum albumin, glucose and catalase according to the manufacturer's recommendations (Becton Dickinson 2002). The samples were incubated in a BD BACTEC™ MGIT™ 320 instrument.

Acid-fast bacilli were visualized by microscopy only in the granuloma smear (Fig. 2). Mycobacterial culture recovered bacteria after 15 d for the granuloma sample, 45 d for the fibrinous exudate and 46 d for the thoracic fluid (Table 1).

After culture, 1 ml of the liquid media with bacteria was transferred to a microtube and centrifuged at 12 000 × *g* for 5 min, and the supernatant was discarded. The bacterial pellet was resuspended in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.3, and 1 mM EDTA) and incubated at 80°C for 30 min. The suspension was then centrifuged at 5000 × *g* for 5 min to isolate the genomic DNA in the supernatant. The molecular identification of the genomic DNA was performed on the 3 isolates by PCR amplification of a 245 bp fragment of the IS*6110* insertion element.

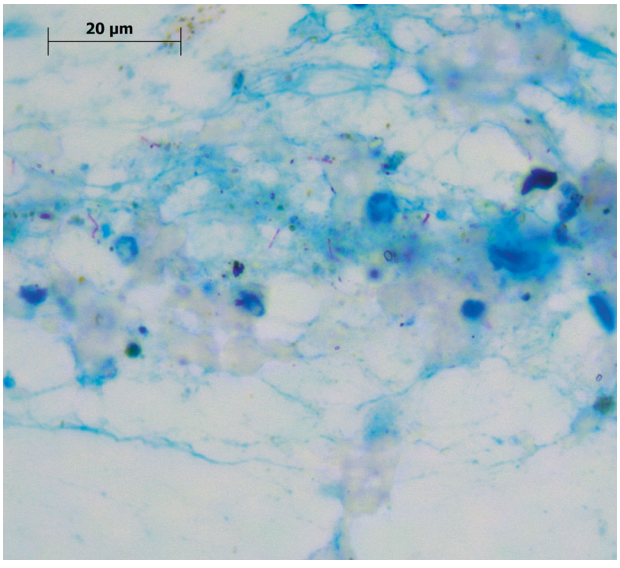

Fig. 2. Acid-fast bacilli visualized in the smear (fuchsia color; Ziehl-Neelsen stain) prepared from the lung granuloma

PCR using Ins1 and Ins2 oligonucleotides was performed according to Hermans et al. (1990).

Spoligotyping to identify the species of MTBC in the samples was performed using a commercial kit (Ocimum Biosolutions) according to the standard procedure (Kamerbeek et al. 1997). The observed

Table 1. Microbiological and molecular detection of *Mycobacterium pinnipedii* from samples of South American sea lion lesions. MGIT: mycobacteria growth indicator tube

Lesion type	Microscopy	Culture in MGITs	Time to MGIT detection (d)	Molecular detection of IS*6110*
Granuloma	Positive	Positive	15	Positive
Fibrinous tissue	Negative	Positive	45	Positive
Hydrothorax	Negative	Positive	46	Positive

spoligotyping pattern (Fig. 3) was converted to the octal format and compared to the SITVITWEB database (www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE) to identify the spoligotype international type (SIT) and clade (phylogenetic classification based on an evolutionary signature pattern). The isolated strain was identified as SIT593/PINI1, which is characteristic of *Mycobacterium pinnipedii* (Fig. 3).

3. DISCUSSION

Here, we report a case of TB caused by *Mycobacterium pinnipedii* in a beached female South American sea lion *Otaria flavescens* in the southern region of Rio Grande do Sul, Brazil. We also performed a molecular characterization of the etiological agent. Although TB has been described in seals since 1912 (Blair 1913), reports of TB in pinnipeds in Brazil are recent. The seminal case was in an adult male South American sea lion in 2014 (Amorim et al. 2014).

Nonspecific clinical signs, such as dyspnea and emaciation, as well as gross pathology were consistent with typical TB presentations found in the literature (Bastida et al. 1999, Kriz et al. 2011). The use of the liquid medium MGIT system facilitated bacterial growth, even from acid-fast negative lesions. The higher sensitivity of bacterial culture is well established for *M. tuberculosis* (Brum et al. 2016) and *M. bovis* (Hines et al. 2006). The use of spoligotyping identified the evolutionary signature common to *M. pinnipedii* and previously reported in clinical isolates from pinnipeds of Argentina, Uruguay and Australia (Cousins et al. 2003).

South American sea lions are widely distributed in South America, along both the Pacific and Atlantic coasts (Vaz-Ferreira 1981). In Brazil, there are no breeding colonies, but there are 2 haul-out sites on the coast of Rio Grande do Sul, one at Ilha dos Lobos (29° 20′ S, 52° 06′ W) and the other at Molhe Leste (32° 11′ S, 52° 04′ W). There is a large adult male cohort, probably originating from the rookeries in Uruguay in search of food (Pinedo 1990, Pavanato et al. 2013). Females show limited movement. Between foraging trips and fasting periods during nursing, females are less common than males along the Brazilian coast (Vaz-Ferreira 1981, Pinedo 1990). Recovery of this animal on a Brazilian beach and not in a haul-out could be due to the advanced stage of the disease, weakening and possible isolation from other animals.

These animals are typically gregarious, live in high-density colonies and can cooccur with other pinniped species, which poses a potential threat for cross-species transmission (Bernardelli et al. 1996, Bastida et al. 1999, Castro-Ramos et al. 2006). Furthermore, TB is zoonotic, and although rare, *M. pinnipedii* can infect humans (Kiers et al. 2008).

This case of severe TB in a sea lion on the Brazilian coast raises additional concerns. Firstly, pinnipeds are charismatic animals, and when they appear on the beach, they attract the attention of people, many of whom approach too closely (Hiicksta′dt et al. 2003). Secondly, pinnipeds can pose an occupational risk to professionals, e.g. rehabilitation staff who work with free-ranging pinnipeds and researchers who may be directly and repeatedly exposed to these animals and their body fluids. Therefore, it is extremely important that these professionals are instructed on health and safety procedures, such as the use of protective clothing, specific masks, protective goggles and gloves, to mitigate the risk of infection with TB (Thompson et al. 1993, Hunt et al. 2008, Kiers et al. 2008).

Although there are case studies of TB in captive pinnipeds (Cousins et al. 1990, Forshaw & Phelps 1991, Thompson et al. 1993, Zumárraga et al. 1999,

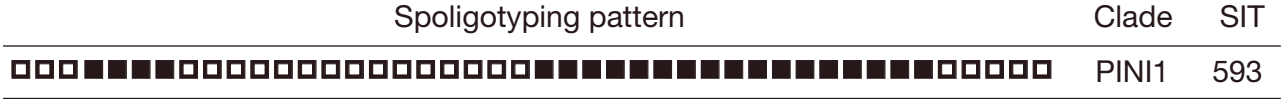

Fig. 3. Spoligotype characterization of the clinical isolate from lesions of the South American sea lion with tuberculosis. SIT: spoligotype international type

Kiers et al. 2008, Moser et al. 2008, Jurczynski et al. 2011a,b, 2012, Kriz et al. 2011), detection of this disease in free-ranging animals is significant (Cousins et al. 1993, Woods et al. 1995, Bernardelli et al. 1996, Bastida et al. 1999, Castro Ramos et al. 2006, Kaneene et al. 2010, Amorim et al. 2014, Boardman et al. 2014). This report reinforces the need for further studies on the epidemiology and pathogenesis of this disease in free-ranging pinniped populations internationally.

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