

Lacaziosis-like disease in *Tursiops truncatus* from Brazil: a histopathological and immunohistochemical approach

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ABSTRACT: Cetacean lacaziosis-like disease or lobomycosis-like disease (LLD) is a chronic skin condition caused by a non-cultivable yeast of the order Onygenales, which also includes *Lacazia loboi*, as well as *Paracoccidioides brasiliensis* and *P. lutzii*, respectively responsible for lacaziosis and paracoccidioidomycosis in humans. Complete identification and phylogenetic classification of the LLD etiological agent still needs to be elucidated, but preliminary phylogenetic analyses have shown a closer relationship of the LLD agent to *Paracoccidioides* spp. than to *L. loboi*. Cases of LLD in South American cetaceans based on photographic identification have been reported; however, to date, only 3 histologically confirmed cases of LLD have been described. We evaluated multiple tissue samples from 4 *Tursiops truncatus* stranded in the states of Santa Catarina (n = 3) and Rio Grande do Sul (n = 1), southern Brazil. Macroscopically, all animals presented lesions consistent with LLD. Hematoxylin-eosin, periodic acid-Schiff, Grocott's methenamine silver, and Mayer's mucicarmin stains were used for histological evaluation. Microscopically, numerous refractile yeasts (4–9 µm in diameter) were observed in skin samples (4/4), and for the first time in dolphins, also in a skeletal muscle abscess (1/4). Immunohistochemistry using anti-*P. brasiliensis* glycoprotein gp43 as a primary antibody, which is known to cross-react with *L. loboi* and the LLD agent, was performed and results were positive in all 4 cases. We describe 3 new cases of LLD in cetaceans based on histopathology and immunohistochemistry. This is the first report of LLD in the muscle of cetaceans.

KEY WORDS: Lobomycosis · *Paracoccidioides brasiliensis* · Cetacean · Yeast · Immunohistochemistry · *Tursiops* · Bottlenose dolphin

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INTRODUCTION

Lacaziosis-like disease or lobomycosis-like disease (LLD), an emerging disease in cetaceans (Bossart 2007, Paniz-Mondolfi et al. 2012), is caused by non-cultivable yeast of the order Onygenales. This order also includes *Lacazia loboi*, a non-cultivable fungus responsible for chronic cutaneous and subcutaneous mycoses in humans (Taborda et al. 1999, Herr et al. 2001), as well as other fungal species, such as *Paracoccidioides brasiliensis*, *P. lutzii*, *Coccidioides immitis*, *Blastomyces dermatitides*, and *Histoplasma capsulatum*, all dimorphic pathogenic fungi involved in systemic mycoses in humans (Al-Daraji et al. 2008, Teixeira et al. 2014). Lacaziosis was first described by Jorge de Oliveira Lobo in 1930, in a 52 yr old male worker of an Amazon rubber-tree plantation in Brazil (De Brito & Quaresma 2007).

In cetaceans, LLD causes chronic skin lesions, mainly located on the head, dorsal and pectoral fins, peduncle, and fluke (Reif et al. 2006). Macroscopically, lesions are well demarcated, proliferative, ulcerative or verrucous, varying from whitish to grayish colored, and even light pink (Migaki et al. 1971). Microscopically, granulomas containing occasional giant cells, as well as numerous periodic acid-Schiff (PAS) and Grocott's methenamine silver (GMS) positive, chain-forming yeasts (Rotstein et al. 2009), ranging from 4.1 to 13 μm in diameter (Haubold et al. 2000), are observed.

LLD has a worldwide distribution, with confirmed cases in the northwestern Atlantic, along the Gulf of Mexico coast (Migaki et al. 1971, Reif et al. 2006, Durden et al. 2009), northeastern Atlantic, Biscay Bay (Symmers 1983), Caribbean Sea (De Vries & Laarman 1973, Esperón et al. 2012), southwestern Atlantic (Simões-Lopes et al. 1993), Pacific coast of Japan (Ueda et al. 2013), and the Indian Ocean (Lane et al. 2014).

The first histological description of LLD in cetaceans was reported in common bottlenose dolphin *Tursiops truncatus* by Migaki et al. (1971), followed by reports in Guiana dolphin *Sotalia guianensis* (De Vries & Laarman 1973) and Indian Ocean humpback dolphin *Sousa plumbea* (Lane et al. 2014). LLD has not been observed or diagnosed in South American river dolphins such as the Amazon river dolphin *Inia geoffrensis* or the tucuxi *Sotalia fluviatilis* from the Amazon and Orinoco River basins, where human lacaziosis is endemic (Paniz-Mondolfi & Sander-Hoffmann 2009).

The first histological diagnosis of LLD in Brazil was reported in a transient *T. truncatus* from Laguna,

Santa Catarina State, in 1993 (Simões-Lopes et al. 1993). The animal was part of a threatened population, known for engaging in cooperative fishing with local fishermen (Simões-Lopes et al. 1998). A second case was histologically diagnosed in another *T. truncatus* from the Tramandaí estuary, Rio Grande do Sul State, in 2008 (Moreno et al. 2008). Two individuals, 5% of the photo-identified animals, were reported to transit between the Laguna and Tramandaí areas (Simões-Lopes & Fabian 1999). One of these individuals was diagnosed with LLD in Tramandaí 2 yr later (Moreno et al. 2008). Suggestive macroscopic lesions were observed in a *T. truncatus* from the Mampituba River (Moreno et al. 2008) inhabiting the North Bay (Flores et al. 2005), in *S. guianensis* from the Paraná estuary (Van Bresseem et al. 2009), and in several *T. truncatus* from the same Laguna population, 21 yr later (Daura-Jorge & Simões-Lopes 2011). Photographic visual assessment has been a useful tool to detect and monitor LLD (Van Bresseem et al. 2007, Murdoch et al. 2008, Daura-Jorge & Simões-Lopes 2011). However, Tajima et al. (2015) recently reported a case of suggestive LLD lesions in *T. aduncus* presenting no histological or immunohistochemical evidence of yeast, showing that diagnoses based on photographic visual assessment should be considered carefully.

It is believed that infection in cetaceans occurs through injured skin, as observed in shark bites that became granulomatous (Murdoch et al. 2008, Van Bresseem et al. 2009, Paniz-Mondolfi et al. 2012, Ueda et al. 2013). At least 2 cases of mother and calf with suggestive LLD lesions have been described (Kiszka et al. 2009, Van Bresseem et al. 2009); however, horizontal or vertical transmission has not been established. To date, only a single case of dolphin-to-human transmission based solely on histopathology has been reported (Symmers 1983) and until recently, it was believed that the same agent species (*L. loboi*) was involved, although this hypothesis was partially discredited by Esperón et al. (2012) through molecular analysis. Interestingly, serological cross-reactivity has been demonstrated among serum from human patients with lacaziosis, and *P. brasiliensis* (Camargo et al. 1998), and positive immune-staining with *P. brasiliensis* antisera has previously been reported in *T. truncatus* with LLD (Ueda et al. 2013). Nevertheless, all 3 agents present significant differences in size (Haubold et al. 2000), potential differences in host/organism interaction (Haubold et al. 2000), and phylogeny. Molecular studies show a greater homology between the LLD agent and *P. brasiliensis* than *L.*

Table 1. Identification, date and place of stranding in Brazil, and size of the studied adult male *Tursiops truncatus*. SC: Santa Catarina, RS: Rio Grande do Sul

Identification	Date	Location	Size (cm)
MM625	21 Oct 2014	Baía Sul, Florianópolis, SC 27.61°S, 48.62°W	317
MM626	30 Oct 2013	Laguna estuary, SC 28.39°S, 48.88°W	301
MM509	04 Oct 2011	Florianópolis, SC 27.57°S, 48.42°W	320
Lobisomem (GEMARS 1259)	03 Nov 2005 ^a	Tramandaí estuary, RS 29.98°S, 50.13°W	339

^aSpecimen previously diagnosed with lacaziosis-like disease (Moreno et al. 2008)

loboi (Rotstein et al. 2009, Esperón et al. 2012, Ueda et al. 2013). Our goal is to further characterize the agent and pathogenesis of 4 cases of LLD in dolphins from southern Brazil with the aid of histological and immunohistochemical techniques.

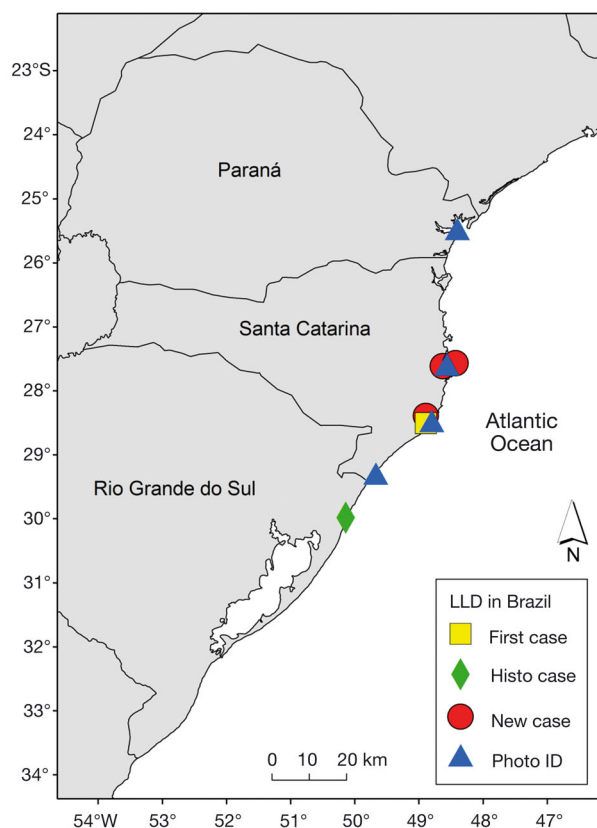


Fig. 1. Lacaziosis-like disease (LLD) cases in adult male *Tursiops truncatus* in Brazil. Symbols represent first case reported, photo-identified cases, old case confirmed by histopathology, and new cases confirmed by histo-pathology (2011–2014)

MATERIALS AND METHODS

Samples

We evaluated 4 adult male *Tursiops truncatus* found dead along the southern coast of Brazil, in the states of Santa Catarina (n = 3) and Rio Grande do Sul (n = 1) (Table 1, Fig. 1). The specimen from Rio Grande do Sul had been previously diagnosed with LLD based on histological evaluation by Moreno et al. (2008). Necropsies were performed following the standard procedures established by the institution of origin.

Animal MM509 was necropsied according to the protocol described by Geraci & Lounsbury (2005), while only skin samples were collected from animals MM625, MM626 and Lobisomem. Tissue samples were collected and fixed in 10% neutral buffered formalin or 70% alcohol, and frozen samples were stored at -80°C .

Histological examination

Histological evaluation was performed on formalin-fixed tissues embedded in paraffin wax, sectioned at 5 μm , and stained with hematoxylin-eosin (HE), PAS, GMS, and Mayer's mucicarmin stains.

Immunohistochemistry

Immunohistochemistry (IHC) was performed in 3–4 μm formalin-fixed paraffin sections of suspected lesions incubated with a rabbit polyclonal antibody against *Paracoccidioides brasiliensis* glycoprotein gp43 diluted at 1:50 000, modified from Ueda et al. (2013). The polyclonal antibody used in this study was kindly provided by the Laboratory of Clinical Mycology, College of Pharmaceutical Sciences, São Paulo State University, campi Araraquara (Brazil), and synthesized by inoculation of 1.0 mg ml^{-1} of *P. brasiliensis* 14-3-3 protein in a rabbit. IHC antigen retrieval was achieved with 10 mM of citric acid solution, cooked under pressure, and a horseradish peroxidase polymer system with 3,3'-diaminobenzidine chromogen (HRP/DAB) for signal detection and amplification. Positive human *P. brasiliensis* slides were used as controls.

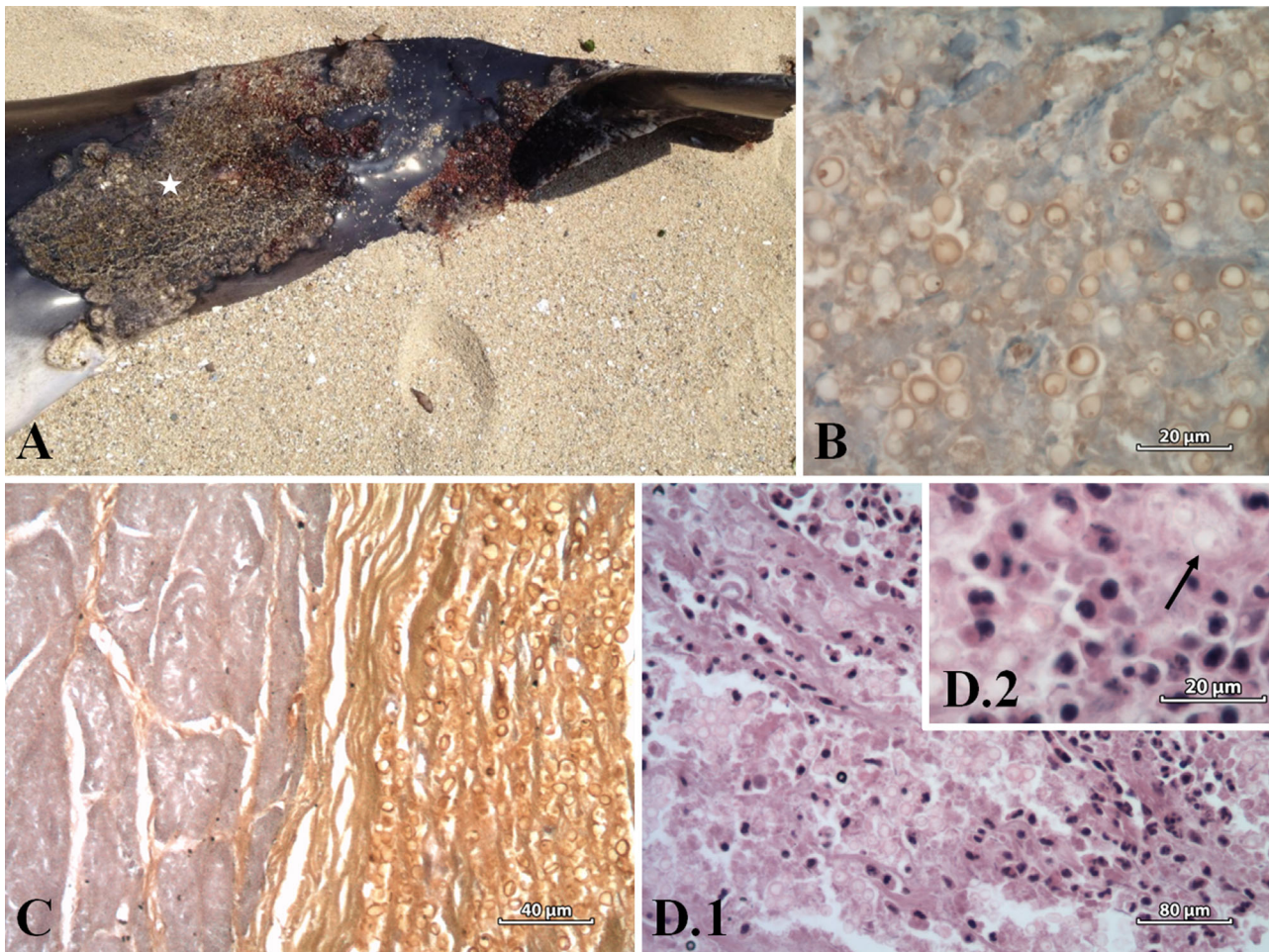


Fig. 2. *Tursiops truncatus* affected by lacaziosis-like disease (LLD). (A) Specimen MM625. Macroscopic cutaneous LLD lesions on the peduncle (star). (B) MM625, skin. Spherical yeast cells stained immunohistochemically. (C) MM509, muscle: presence of yeast in the muscle abscess, stained with Grocott's methenamine silver. (D.1) Center of the abscess shown in (C), hematoxylin-eosin stain. Note the presence of yeast and moderate number of necrotic poly-morphonuclear cells. (D.2) Detailed view of yeasts (arrow) and suppurative response

RESULTS

The affected specimens of *Tursiops truncatus* were found in 3 different locations along the southern coast of Brazil: Tramandaí estuary (n = 1), Laguna estuary (n = 1), and Florianópolis island (n = 2) (Fig. 1).

Macroscopic evaluation showed multiple, elevated, nodular to verrucous, crusty, whitish skin lesions, ranging from 5 to 40 cm in diameter, mainly located on the rostrum, peduncle, and fins, and occasionally distributed throughout the body (Fig. 2). Cut surfaces exhibited irregular circumscribed nodules extending into the deep dermis and epidermis, suggestive of LLD. Microscopically, the dermis exhibited extensive mild granulomatous lesions and necrosis, especially at the dermal-epidermal junction, characterized by

the presence of macrophages and multinucleated giant cells occasionally filled with yeast (Table 2). The epidermis was multifocally irregular and hyperplastic. Numerous round, oval, or elliptical yeasts were observed, ranging from 4 to 9 μm in diameter, with doubly contoured, birefringent, and approximately 1 μm thick walls. Yeasts were poorly stained by HE and were negative for Mayer's mucicarmin and positive for GMS and PAS (Fig. 2). Occasionally, yeasts formed chains connected by a tubular isthmus, resembling a string of pearls. Due to the availability of material for an IHC assay, the case diagnosed by Moreno et al. (2008) was also included in our study. All tested samples were positive for IHC performed with antibodies against *Paracoccidioides brasiliensis* gp43 protein in all 4 of our investigated cases.

Table 2. Location and histopathological findings of the new studied cases of lacaziosis-like disease in adult male *Tursiops truncatus* from Brazil

Identification	Location of the lesions	Histopathological description
MM509	Medium dorsal region of the body, peduncle, and fluke	Perivascular granulocytic diffuse dermatitis, acanthosis, focal areas of atrophy of the dermis and multifocal presence of yeast, polymorphonuclear cells and giant cells. Necrotic muscle layer abscess, characterized by a suppurative exudation composed of necrotic polymorphonuclear cells, cellular debris and numerous yeasts, surrounded by a thin fibrous capsule.
MM625	Jaw, beside the pectoral fin, peduncle, and fluke	Perivascular dermatitis, mixed infiltrate, acanthosis. Multifocal presence of yeast and giant cells.
MM626	Medial part of the body	Presence of numerous yeasts, without inflammatory cells. Loss of epidermis.

DISCUSSION

Microscopic findings were consistent with previous histological descriptions of LLD in cetaceans (Migaki et al. 1971, Haubold et al. 2000). In 1 individual, a necrotic muscle layer abscess was observed, filled by numerous yeasts and mixed inflammatory infiltrate, surrounded by a thin fibrous capsule. This finding has not been described in any other LLD cases before, and may be considered an indication of the invasive ability of this subcutaneous mycosis. The presence of the yeast in the muscle could occur through lympho-hematogenous spread, as observed in secondary lesions in other Onygenales members, such as *Paracoccidioides brasiliensis* (Brummer et al. 1993). Nevertheless, given the proximity of the skin lesion, it is more plausible that it was a contiguous infection rather than a systemic infection.

All studied skin and muscle samples were positive for IHC against *P. brasiliensis*. This result was consistent with the IHC findings reported by Ueda et al. (2013) in LLD-affected dolphins in Japan, in which positive immune-staining for *P. brasiliensis* antisera was observed in skin samples from dolphins with LLD. The evidence observed in our study places the LLD yeast into the order Onygenales.

The exact location where the individuals were infected is still unknown, but contact between animals inhabiting the estuaries of Tramandaí and Laguna, which are separated by only 219 km (Simões-Lopes & Fabian 1999), has been reported. These areas are close to large ports and cities and are heavily impacted by human activities, such as direct discharge of untreated wastewater and consequent chemical pollution and biological contamination (Fabricio 1989, SDM 1998, Andrade 2004, Eichler et al. 2012, Moresco et al. 2012). All of these factors may

lead to immunosuppression, increasing animals' susceptibility to infections (Wilson et al. 1999, Reif et al. 2006, Van Bresseem et al. 2007, 2009, Murdoch et al. 2008).

The individual from the Tramandaí estuary belonged to an inshore population actively involved in local dolphin-human cooperative fishing (Moreno et al. 2008). The social organization in which the other 3 animals lived remains unclear, although some animals in Laguna were also involved in such activities (Simões-Lopes et al. 1998). Despite a recent report of LLD in an offshore *Tursiops truncatus* (Rotstein et al. 2009), cases of LLD (Cowan 1993, Durden et al. 2009) and suggestive LLD (Van Bresseem et al. 2007, 2009, Kiszka et al. 2009) are more frequently reported in individuals of inshore populations. This could be related to influx of terrestrial pollutants (Woodward-Clyde Consultants 1994) in ecosystems sustaining enclosed bodies of water with freshwater influx, leading to variations in temperature, tides anaerobic sediment conditions, and salinity levels. Salinity could also be involved in dermal lesions in resident inshore cetaceans (Wilson et al. 1999, Murdoch et al. 2008, Burdett Hart et al. 2011). Another aspect to be considered is the fact that offshore animals are rarely examined in comparison to inshore animals.

Even though some resident dolphins had LLD, there were no cases of human lacaziosis registered in areas of cooperative fishing between dolphins and fishermen studied by Siciliano et al. (2008). Reif et al. (2006) drew a similar conclusion regarding dolphins with LLD from Florida (USA), where LLD is considered endemic and human lacaziosis has never been reported. On the other hand, in the Amazon basin endemic areas of human lacaziosis, river dolphins failed to present any suggestive lesions of infection (Da Silva et al. 2008).

Direct molecular and phylogenetic studies evaluating LLD agents affecting dolphins from different locations have never been performed. The possibility of a remote zoonotic origin or a common ancestor cannot be excluded, and may indicate posterior diverging evolution in order to adapt to different hosts and environments (Paniz-Mondolfi et al. 2012).

The present study describes the histopathology of LLD in *T. truncatus* and the first use of IHC techniques to characterize the agent in 4 cases in the Americas. It is also the first time LLD yeast has been described in a skeletal muscle abscess. Nevertheless, additional genetic research is needed in order to establish the taxonomy of the LLD agent in dolphins.

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