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Short Communication

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Toward the identification, characterization and experimental culture of *Lacazia loboi* from Atlantic bottlenose dolphin (*Tursiops truncatus*)

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Abstract

Lobomycosis (lacaziosis) is a chronic, granulomatous, fungal infection of the skin and subcutaneous tissues of humans and dolphins. To date, the causative agent, the yeast-like organism *Lacazia loboi,* has not been grown in the laboratory, and there have been no recent reports describing attempts to culture the organism. As a result, studies on the efficacy of therapeutics and potential environmental reservoirs have not been conducted. Therefore, the objective of the current study was to utilize both classical and novel microbiological methods in order to stimulate growth of *Lacazia* cells collected from dolphin lesions. This included the experimental inoculation of novel media, cell culture, and the use of artificial skin matrices. Although unsuccessful, the methods and results of this study provide important insight into new approaches that could be utilized in future investigations of this elusive organism.

Key words: Lobomycosis, Lacazia loboi, bottlenose dolphin.

Introduction

Lobomycosis (lacaziosis) is a chronic fungal disease of the skin of humans and dolphins caused by the yeast-like organism *Lacazia loboi*.[1] Phylogenetic analysis places this agent in the order Onygenales along with other dimorphic fungi.[2] Human infection is endemic in tropical rural areas of South and Central America.[3] Occupationally associated exposures in rubber workers, farmers, fishermen, miners, hunters, and other outdoor workers have been reported.[3,4] Based on the diversity of occupational exposures that lead

to infections, reservoirs of the causative agent could include soil, vegetation, and aquatic environments.[4] Inoculation of the organism into the dermis appears to occur through wounds, abrasions, insect bites, or other traumatic events on the exposed surfaces of the body.[3,4]

Lobomycosis in bottlenose dolphins (*Tursiops truncatus)* was originally described along the Gulf of Mexico[5] and Atlantic coasts of Florida.[6] Cases were subsequently reported from Europe,[7] the Brazilian coast,[8] the Japanese coast,[9] and the western Pacific,[10] suggesting that the ecological niche for the organism may be expanding. Lobomycosis is endemic in the Atlantic bottlenose dolphin population of the Indian River Lagoon, Florida (IRL) with a reported prevalence of 10%.[11]

There is evidence to suggest that the organisms which cause lobomycosis in humans and dolphins are not identical,[12] despite the clinical appearances and histological characteristics are similar.[11] Morphologically, *Lacazia* cells in dolphin tissues are approximately 30 percent smaller and shorter in their main axis than those found in lesions from humans, suggesting that the organisms from these two species may represent phylogenetic divergence.[4] Further, the results of molecular sequencing of ribosomal DNA from an infected dolphin were more similar to *Paracoccidiodes brasiliensis* than to *Lacazia loboi* of human origin.[13] However, sera from infected dolphins cross react with antigens from human *L. loboi* more strongly than with antigens from *P. brasiliensis* in western blotting analyses.[14] Direct molecular and phylogenetic comparison of *Lacazia* of dolphin and human origin has not been reported. Zoonotic transmission of lobomycosis occurs extremely rarely.[15]

Regardless of the host, attempts to culture *L. loboi in vitro* using standard microbiological approaches have been uniformly unsuccessful, despite the abundance of organisms in lesions.[1,2] The original isolation attempts were described in 1940[16] and subsequently in 1971.[17] Although some growth was noted, the organism isolated was later shown not to be *Lacazia*.[1] Thirty years later, attempts to culture *Lacazia* from two Brazilian patients were reported.[2] Despite a more than one month incubation on Sabouraud agar at 25 and 37°C, no growth was obtained. In the ensuing 15 years, we found no further reports of attempts to culture this elusive organism.

Attempts to characterize the pathogenesis of the disease have relied on direct inoculation of tissue extracts or live fungal cells into mice, hamsters, tortoises, and armadillos.[4] As a result, development of diagnostic tools (e.g., serological assays) and efficacy studies on potential therapeutics have been delayed, and definitive identification of environmental reservoirs for the disease remains impossible. The objectives of this study were to purify *Lacazia* cells obtained from lesions in dolphins, identify them microscopically, and attempt to cultivate *Lacazia* using both classical and novel media, including an artificial matrix.

Figure 1. Characteristic nodular lesions from a bottlenose dolphin with lobomycosis.

Materials and methods

Sample collection

Four histologically confirmed lesions from wild bottlenose dolphins (*Tursiops truncatus)* from the IRL were collected during capture-release health assessment studies from the Bottlenose Dolphin Health and Environmental Risk Assessment Project (HERA) between 2010 and 2012. Methods used in HERA were approved under National Marine Fisheries Service Scientific Research Permit No. 998–1678 issued to G. Bossart and by the Florida Atlantic University Institutional Animal Care and Use Committee. Three histologically confirmed lesions were collected from deceased stranded animals along the east coast of Florida from the Sebastian inlet (27.85°N, 80.44°W) to the Jupiter inlet (26.95°N, 80.07°W) between 2010 and 2013. Grossly, the lesions in all animals had the classical appearance of lobomycosis as previously described[11] (Figure 1). Affected dolphins typically had multiple firm white raised cutaneous nodules and plaques on the leading edges of the dorsal and pectoral fins, head, fluke and caudal peduncle. Tissue samples from live dolphins were collected by biopsying the nodular lesions under local anesthesia using 3% mepivacaine. A 3 mm section of tissue from the leading edge of the lesion was removed using sterile technique, subsectioned into three pieces, and placed directly into KM 50:50 media [222.5 ml RPMI 1640 (HyClone, GE, USA); 222.5 ml keratinocyte SFM (Genlantis, CA, USA) containing 5 ml penicillin-streptomycin (MP Biomedicals, USA) and 50 ml fetal bovine serum (Sigma-Aldrich, USA)]. Fresh tissues were transported on ice to the laboratory within 4 hours of collection.

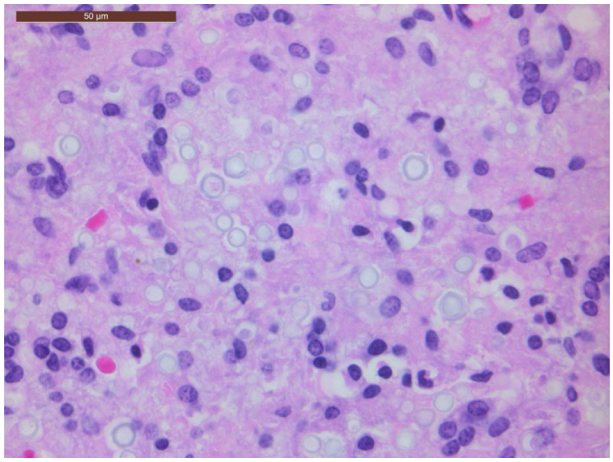

Figure 2. Photomicrograph of a granuloma from an affected dolphin showing multiple round yeasts arranged containing a thick double wall and birefringent capsule with a folded basophilic nucleus. Hematoxylin and eosin X600.

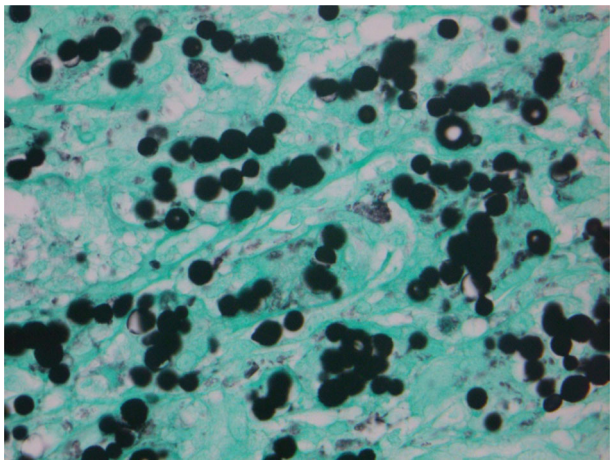

Figure 3. Photomicrograph of a granuloma from an affected dolphin showing multiple round yeasts arranged individually and in short chains connected by tube-like bridges characteristic of *Lacazia loboi*. Gomori methenamine silver X600.

Organism identification

A portion of each biopsied lesion was stored in 10% buffered formalin, processed and sectioned at 5 μm thickness. The sections were stained with hematoxylin and eosin (Figure 2) and Gomori methenamine silver (Figure 3), and examined microscopically for fungal cells consistent with *Lacazia* morphology. All seven tissue samples were shown to contain *Lacazia* based on the characteristic morphologic appearance of the organism. Microscopically, abundant 6 to 12 μm, round to lemon-shaped yeast-like bodies with thick, refractile walls were seen within inflammatory infiltrates, occurring singly or in chains connected by tubular projections. These cells stained with methenamine silver and had the typical histopathological appearance of *Lacazia loboi* described in humans and dolphins.[11] Vital staining was performed using methods previously described for visualization of cells.[18] Briefly, 200 μl of cells were mixed with 100 μl of fluorescein diacetate (50 μg/ml; Sigma-Aldrich) and 100 μl of ethidium bromide (50 μg/ml; Sigma-Aldrich) and then incubated at 37°C for 15 min prior to microscopic examination.

Preparation of cell suspensions

Tissues were prepared for further study by finely mincing with sterile scissors. The lesion material was then suspended in 2 ml of phosphate buffered saline (PBS) containing BioSpec Mini-Beadbeater beads. The suspension was shaken vigorously in a bead beater (BioSpec) for 30 seconds and transferred to a fresh tube and centrifuged for 20 s at 13,000 rpm. Half of the lesion material was treated only by mechanical disassociation of cells. The remaining supernatant was then removed, and transferred to a new vial containing a 1 ml solution of dispase in 100 ml PBS (Dispase, Roche Diagnostics) to dissociate the mammalian tissue as described previously.[19] The tube was incubated at 37°C for 24 h. The cells were then washed twice with PBS and pelleted by centrifugation resulting in a cell suspension. The cells were observed using Olympus BX-53 microscope with phase contrast optics (Olympus, Tokyo, Japan) and quantified using a 1/25 sq. mm counting chamber.

Growth media

Twelve-well plates were prepared with one of the 18 media in each well (Table 1) and inoculated with approximately 2.5×10^3 *L. loboi* cells per well. Two replicates of each media were used. This concentration was chosen based on the overall availability of cells. Plates were incubated at 37°C and at 30°C in a chamber containing 5% CO_2. Cell growth was assessed daily and, when observed, fungal colonies were subsampled on to both the original culture medium and Sabouraud Dextrose Agar (SDA). Subcultures were then incubated at the temperature at which growth had been observed.

Fungal identification

When possible, fungi were identified based on microscopic morphology and confirmed by amplification of the ITS region of the ribosomal RNA gene. Briefly, DNA was extracted from cells by bead beating using the method of Haugland et al.[20] and the ITS region amplified using the method of White et al.[21] with the ITS1 and ITS4 primer set. The resulting amplicons were sequenced by MCLab (South San Francisco, CA) and analyzed using the BLASTN tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Dolphin epidermal cells and artificial skin matrix

An artificial matrix was developed by combining an immortalized epidermal cell line derived from bottlenose

Table 1. Preparation of experimental media.

Medium	Method/Manufacturer
Sabouraud Dextrose Agar (SDA)	Prep per manufacturer (Difco)
SDA with Olive Oil	100 μL of sterilized Oil to well
Emmon's SDA	Prep per manufacturer (Difco)
Emmon's SDA with Olive Oil	100 μl of sterilized Oil to well
Brain Heart Infusion Agar	Prep per manufacturer (Difco)
Orange Serum Agar	Prep per manufacturer (BBL)
Littman Agar	Prep per manufacturer (US Biological)
MycoPhil Agar low pH	Prep per manufacturer (BBL)
Rose Bengal Agar	Prep per manufacturer (Difco)
Malt Extract Agar	Prep per manufacturer (Difco)
Potato Dextrose Agar	Prep per manufacturer (Difco)
$\frac{1}{2}$ Strength Potato Dextrose Agar	$\frac{1}{2}$ weight PDA & 7.5 g/l agar
Corn Meal Agar	Prep per manufacturer (Difco)
Bird Seed Agar	50 g ground seed,1 g glucose, 1 g H_2PO_4, 1 g creatinine, 15 g agar, 1000 ml distilled water
Malassezia Agar	12 g agar, 10 g dextrose, 10 g yeast extract, 3 g peptone, 2 g NaCl, 2 g ox-bile, 2 g thioglycolate, 2 g L-asperagine, 2 g palm oil, 10 ml Tween-80
Leeming and Notman Agar	10 g peptone, 5 g glucose, 0.1 g yeast extract, 4 g ox-bile, 1 mL glycerol, 0.5 g glycerol monostearate, 0.5 ml Tween 40, 10 ml whole fat cow's milk, 8 g agar
Dixon's Agar	18 g Malt extract,18 g peptone, 7.25 g agar, 10 g ox-bile, 5 ml Tween 40, 2.5 ml Glycerol Mono-oleate, 50 ml distilled water
RPMI-Serum-MOPS Agar	90 mL RPMI, 10 mL fetal bovine serum, 1.5 g/50 ml agar

***Note*:** All media were supplemented 250 μl/50 ml penicillin-sreptomycin (MP Biomedicals).

dolphins[22] with an acellular matrix composed of 90% collagen. MatriStem MicroMatrix® (ACell, Columbia, MD) is a sterile, porcine-derived, naturally occurring lyophilized extracellular matrix that maintains and supports a healing environment for wound management. By combining this 3-dimensional matrix with dolphin-derived cells we attempted to provide an environment that could mimic the suspected natural circumstances of infection and thereby promote the growth of *L. loboi*.

Two experimental approaches were applied. In the first, fungal cells were introduced directly onto the MatriStem matrix as a suspension in PBS. In the second treatment, both the matrix material and dolphin epidermal cells were used. Briefly, epidermal cells were placed in a 75 ml culture flask with warmed 50:50 media (225.5 ml RPMI, 225.5 ml Keratinocyte SFF, 50 ml PBS, 5 ml Pen/Strep) and incubated at 37°C with 5% CO_2 at a calculated density of 1.7×10^5 cells/ml. Once established, the dermal matrix was introduced to provide a structure for cellular adherence. Each flask containing dolphin epidermal cells and MatriStem was incubated at 37°C at 5% CO_2 for 72 h prior to the introduction of 10 μl of *L. loboi* cells and 10 ml of PBS. The incubation conditions were maintained at 37°C and 5% CO_2 to ensure survival, adherence and replication of dolphin epidermal cells. Aliquots of 25 μl were removed from each flask and examined under phase contrast microscopy every 24 h for signs of adherence and fungal cell growth. Vital stains including trypan blue were applied to the cellular materials as previously described.[18]

Results

Isolation of L. loboi from dolphin tissue

The isolation of fungal cells from the dolphin lesions was most successful using mechanical dissociation, which yielded a larger number of cells for further experimentation than the enzymatic digestion method. Yeast-like cells were clearly visible and morphologically identical to *L. loboi* with a typical lemon-shape ranging from 6 to 12μm in length and organized into chains connected by tubular projections. Attempts to determine the numbers of viable cells by vital staining were inconclusive; varied degrees of staining by both ethidium bromide and fluorescein acetate and completely unstained cells were seen in suspensions. Vital staining was not pursued further due to the limited quantity of material available for study.

Classic and novel media

After 72 hrs of incubation, wells containing SDA with Olive Oil, Emmon's SDA, Potato Dextrose and Corn meal agar and maintained at 37°C at 5% CO_2 (Table 1) demonstrated growth of fungal colonies. Wells containing SDA maintained at 30°C at 5% CO_2 had visible growth as well. The individual colonies were subcultured on the original culture

medium and on SDA and held for 15 days. Growth was then observed using standard light microscopy and fungi were identified based on morphology and the identities confirmed by PCR through the amplification of the ITS1-ITS4 region. In all cases, the growth was identified as the common fungi *Penicillium* spp. and *Bipolaris* spp. No colonies contained cells that matched the classic histological morphology of *L. loboi*.

Dolphin epidermal cells and artificial skin matrix

No growth was noted on the artificial skin matrix, skin matrix with epidermal cells nor was growth noted in any of the flasks containing only dolphin epidermal cells. Small chains of 3 to 6 *Lacazia* cells with visible organelles were visible for 4 days; the cell counts then gradually decreased. *Lacazia* cells were no longer visible after 15 days in culture at which time significant bacterial growth was observed. No replication was observed during the 15-day culture period.

Discussion

Several novel approaches were used in an attempt to isolate and grow *L. loboi* in the laboratory. Despite these efforts, the fungus from dolphins remains uncultivated.[1] The proteolytic enzyme dispase was used successfully by Salgado et al.[19] to isolate *Lacazia* from skin biopsies of human patients. This method for the isolation of *L. loboi* cells from dolphin lesions was attempted, in addition to mechanical means of separation. However, mechanical separation of cells proved to be the most efficient method for obtaining cells for further characterization. As anticipated, the cells were morphologically similar in size and shape to previous descriptions of *L. loboi* isolated from dolphins.[5, 11]

We also utilized culture conditions under which *P. brasiliensis* is grown *in vitro* due to the similarities in molecular phylogeny,[2] serological cross-reactivity in Western blot analyses,[14] and geographic distribution between the organisms. Inoculation of Sabouraud Dextrose Agar and Blood Agar at 37°C will elicit colony growth for *P. brasiliensis*.[23] In addition, Malassezia, Leeming, and Notman agars all contained yeast extract, which has been reported to increase the recovery of *P. brasiliensis*.[24] Despite the similarities between *L. loboi* and *P. brasiliensis*, these methods were not successful in promoting growth of *Lacazia* from dolphin tissues. The only growth identified was *Penicillium* spp. and *Bipolaris* spp., which could indicate contamination during collection or laboratory processing. Further investigation into the optimal field collection techniques may be required to eliminate contamination from *Bipolaris* spp., while laboratory protocol and refinement of sterile procedure is needed to limit the growth of *Penicillium*.

To our knowledge, the use of an artificial tissue matrix with or without the addition of epidermal cells has not been reported previously in attempts to isolate *Lacazia*. Live cells were maintained using standard cell culture media and methodology for 4 days post isolation with cells derived from fresh biopsy material but cell replication was not observed. Similarly, the use of an artificial skin matrix with or without epidermal cells failed to promote growth of the organism.

The lack of *L. loboi* growth could be attributed to multiple factors. *Lacazia* growth *in vitro* may be temperature dependent. In nature, the organism grows on the exposed surfaces of the body such as the ears and extremities in humans, and the dorsal fin and peduncle in dolphins. Therefore, the required temperature for cultivation may be lower than the 30°C used in our initial isolation efforts. However, a previous attempt to culture *Lacazia* at 25°C was unsuccessful.[2] Other members of the Onygenales including the related *Paracoccidiodes* are culturable at 37°C.[2]

Plausibly, growth of *L. loboi* may be dependent on a host interaction with the immune system or participation with host cellular defense mechanisms. Human patients with lobomycosis have a partial loss of cellular immunity and normal humoral immunity.[25] Similarly, affected dolphins have significant impairment in adaptive immunity.[26] Depressed lymphocyte proliferation in response to T and B cell mitogens, reduced antibody titers to common marine micro-organisms and multiple alterations in lymphocyte phenotypes and other immune markers were described. Additionally, dolphins with lobomycosis had significant decreases in CD4+ helper T cells, and CD19+ and CD21+ B cells as well as lymphocytes expressing MHC class II molecules.[26] It is unclear whether the immunologic perturbations found in dolphins with lobomycosis represent the host response to infection or whether the disease is manifested in dolphins with a compromised immune system.

The lesions found in dolphins with lobomycosis are characterized by chronic, granuloma formation containing numerous histiocytes and multinucleated giant cells.[11] Abundant yeast-like organisms, occurring singly and in chains, are found within the granulomas. These cells may be contained within macrophages and giant cells as shown with special stains.[11] Therefore, the inflammatory response to *L. loboi* in the dolphin appears to be ineffective in destroying the organism and preventing progression of the lesions. Similarly, the nodular skin lesions in human patients with lobomycosis are characterized by granuloma formation and infiltration of $CD68^+$ histiocytes, CD4+ T cells, Langhans-type multinucleated giant cells, and to a lesser extent B cells.[27] The cytokines observed are consistent with a Th2 response, with increases in IL-10 and TGF-β in peripheral blood mononuclear cells and in cells obtained from the

inflammatory infiltrate.[28] The infiltration of macrophages in lesions suggests that growth may not be initiated until after phagocytosis by the host cells.[27] Cytokines in the microenvironment may facilitate the growth of the fungus and prevent an effective immune response by switching to a Th2 pattern, which would be expected to be less effective in containing and eliminating the agent. Similarly, complement (C-3) and immunoglobulins (IgG) appear to play a role in the host defense against *L. loboi*.[29] These conditions have not been replicated in previous isolation attempts. Therefore, we hypothesize that success in culturing *L. loboi* might be obtained by mimicking the molecular microenvironment of the lesions and should be considered in future laboratory experiments. The current results represent a novel approach to the cultivation of an enigmatic fungus of significance and the first report on culture attempts in 15 years. While unsuccessful to date, these efforts provide methodology and insights that could be used as a starting point in future research efforts.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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