

Case Report

Two Cases of Lacaziosis in Bottlenose Dolphins (*Tursiops truncatus*) in Japan

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Lacaziosis, formerly called lobomycosis, caused by *Lacazia loboi*, is a zoonotic mycosis found in humans and dolphins and is endemic in the countries on the Atlantic Ocean. Although the Japanese coast is not considered an endemic area, photographic records of lacaziosis-like skin lesions were found in bottlenose dolphins (*Tursiops truncatus*) that were migrating in the Goto Islands (Nagasaki Prefecture, Japan). We diagnosed 2 cases of lacaziosis in bottlenose dolphins captured simultaneously at the same coast within Japanese territory on the basis of clinical characteristics, cytology, histopathology, immunological tests, and detection of partial sequences of a 43 kDa glycoprotein coding gene (*gp43*) with a nested-PCR system. The granulomatous skin lesions from the present cases were similar to those found in animals from endemic areas, containing multiple budding and chains of round yeast cells and positive in the immune-staining with anti-*Paracoccidioides brasiliensis* serum which is a fungal species related to *L. loboi*; however, the *gp43* gene sequences derived from the present cases showed 94.1% homology to *P. brasiliensis* and 84.1% to *L. loboi*. We confirmed that the causative agent at the present cases was different genotype of *L. loboi* from Amazon area.

1. Introduction

Lacaziosis is a granulomatous chronic skin infection caused by the fungus *Lacazia loboi* and is endemic in the Atlantic coastal waters of Latin American countries [1–3]. Interestingly, *L. loboi* is a sister taxon with a close phylogenetic relationship to *Paracoccidioides brasiliensis*, which is a highly pathogenic fungal species also endemic in Latin American countries [4]. The disease it causes, lacaziosis, is formerly

known as Jorge Lobo's disease [5, 6] or lobomycosis until 2005 [7].

The characteristics of the disease are chronic keloidal skin lesions accompanied by pruritus, sensations of burning, and pain [2]. The hosts include humans and 3 species of dolphins: the bottlenose dolphin (*Tursiops truncatus*), the Indian Ocean bottlenose dolphin (*Tursiops aduncus*), and the estuarine dolphin, "costero" (*Sotalia guianensis*) [8]. Only one case of dolphin-to-human infection has been reported in a dolphin



FIGURE 1: The skin lesion (approximately 25 × 25 cm) in Case 1 in August 2010 (a, b).

trainer in The Netherlands who contacted the infected animal, suggesting that lacaziosis should be considered a zoonotic fungal infection [9, 10].

Lacaziosis is usually found at altitudes above 200 m in tropical, humid, or subtropical forests with an average temperature of 24°C and more than 2,000 mm of annual rainfall [1–3, 8]. The natural reservoir of *L. lobo* is unknown; however, its habitat is likely to be in rural environments on the basis of the observed distribution of the disease, and soil and vegetation seem to be probable sources of infection. Lacaziosis in dolphins suggests that some aquatic reservoir also exists [2, 3].

Human cases have been reported in Brazil, Costa Rica, Panama, Venezuela, Colombia, Guiana, Surinam, French Guiana, Ecuador, Peru, Bolivia, Honduras, and Mexico. Some cases have also been reported in the United States, but those patients thought to have been infected in Venezuela, Canada, The Netherlands, Surinam, France, South Africa [1–3, 8, 10], and Greece [11] bordering countries to the Atlantic Ocean. Interestingly, an unconfirmed case was reported from Bangladesh [12] which is not an endemic area.

On the other hand, cetacean cases have been reported in Brazil [13], Surinam [14], Florida and North Carolina (United States) [15–18], Mexico [19], the Bay of Biscay (Spain) [9], Spain in a dolphin that originated in Cuba [20], and Hawaii in a bottlenose dolphin transported from Florida [21]. Furthermore, lacaziosis-like cases have been observed in Indo-Pacific bottlenose dolphins in Mayotte waters located close to Madagascar, in the Indian Ocean [22], Venezuela, Colombia, Ecuador, Peru, Chile, Brazil [23], and in the Goto islands, Nagasaki Prefecture at the Kyushu area of the Japanese Sea [24]. Therefore, lacaziosis and lacaziosis-like diseases are endemic in the Atlantic, Pacific, and Indian Oceans [23]. According to Kiszka et al. [22], this lacaziosis-like disease is very similar to lobomycosis but lacks a histologic diagnosis [24]. Except for one case from Spain, most cases were diagnosed by photography and macroscopy without histopathologic, serologic, or molecular biological data [20].

The causative fungal species *L. lobo*, is difficult to culture except by animal passages [2, 3, 8, 10]. Therefore, the diagnosis of lacaziosis is based on clinical symptoms, histopathologic observations, and immunologic tests by cross-reaction to *Paracoccidioides brasiliensis*, a closely related fungal species of *Onygenales* [1–4, 7, 8, 10]. Furthermore, the diagnosis seems to be very difficult to confirm outside of endemic areas, where no doctor or veterinarian would consider lacaziosis when they encountered chronic granulomatous keloidal skin lesions either in humans or dolphins.

The present study aims to establish a diagnosis in two cases of lacaziosis in bottlenose dolphins captured simultaneously on the same coast of Japan and cared for in individual aquariums, on the basis of clinical characteristics, cytology, histopathology, immunological testing, and detection of partial sequences of the 43 kDa glycoprotein coding gene (*gp43*) corresponding to those of *P. brasiliensis* [4, 7].

2. Cases

2.1. Case 1. A male bottlenose dolphin (*Tursiops truncatus*), estimated to be 17 years old, was captured in 2007 in a coastal area of Japan and cared for under outdoor conditions at an aquarium. The body weight and body length at the time of death were 280 kg and 303 cm, respectively.

The dolphin's testosterone level decreased to less than 1 ng/mL beginning in March 2010. The skin of the back of the dolphin became swollen in July 2010. In August 2010, the skin developed a cauliflower-like eczema approximately 20 × 25 cm in size (Figures 1(a) and 1(b)). The animal received antibiotics without effect. The blood β -glucan level was 22.4 pg/mL (>6 pg/mL is regarded as positive for fungal infection according to a commercial laboratory). The biopsied skin lesions contained yeast-like structures, as determined by a commercial pathology laboratory. The dolphin's condition was diagnosed as fungal infection, and terbinafine hydrochloride was administered orally at a dose of 2 mg/kg SID. However, no improvement was noted.



FIGURE 2: The margin of the skin lesion with abundant small fistulae appeared in November 2010. The arrow indicates the biopsy site.

In October 2010, the lesion had expanded, and pain was detected by palpation. Administration of fluconazole orally at a dose of 800 mg (2.7 mg/kg) was initiated; however, no effect was seen. The animal's body temperature was elevated slightly at 37°C or more. According to Hampton et al., the deep body temperature normally ranges from 37°C to 37.5°C during the active time; however, temperatures decrease during sleeping and after feeding times [25].

In November 2010, the body temperature decreased (35°C), and the number of blood white cells increased to 33,000 cells/ μL , while the normal number varies from 5,000 to 9,000/ μL in captive dolphins [26]. The size of the lesion was approximately 30 × 35 cm. There were abundant small fistulae at the margin of the lesion (Figure 2). The biopsy was also done. The lesion had a topical temperature of approximately 40°C, as detected by thermography (Figure 3(a)). The echo image of the lesion suggested that the invasion was limited to the subcutaneous connective tissue and did not extend to the muscle (Figure 3(b)). Round or multiple budding yeast cells consisting of a spherical to piriform mother cell (4–12 μm in diameter) with some small daughter cells (less than 0.5–6 μm mm in diameter) connected by a narrow base (less than 0.5 μm in width and 0.5 μm in length) were detected in the smear of the biopsied skin samples of the dermis stained with Giemsa, periodic acid-Schiff reaction (PAS), and Gomori methenamine method (GMS) (Figures 4(a)–4(c)).

On 19 December 2010, the animal showed a spontaneous increased respiratory ratio indicating lower vital signs. We speculated that the dolphin was suffering from sepsis and tried to administrate fluid replacement; however, there was no response. By the next day, the animal had died.

Purulent pneumonia and hepatic failure were detected at the autopsy. *Staphylococcus aureus* and *Morganella morganii* were isolated both from the lung and the blood. We concluded that the cause of the death was sepsis. In addition, the autopsied skin samples cultured on potato dextrose agar (Difco potato dextrose agar, Becton, Dickinson and Company Japan, Tokyo, Japan) supplemented with 100 mg/L of chloramphenicol and Mycosel agar (BBL Mycosel agar, Becton, Dickinson and Company Japan) plates at 25 and 35°C for 4 weeks produced negative results.

Abundant yeast-like cells were detected in the skin lesions stained with hematoxylin and eosin, PAS, and GMS (Figures 5(a)–5(c)). Most of the yeast cells appeared as chains, while multiple budding yeast cells connected by narrow bases to the mother cells were also detected.

For immunohistochemistry, sections from skin lesions were incubated with a rabbit polyclonal antibody against *P. brasiliensis* which is a related species to *L. loboi* for 16 hrs at 4°C. The bounded antibodies were detected with horse radish peroxidase conjugated to anti-rabbit secondary antibody (Histofine Simple Stain MAXPO; Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories Inc., Burlingame, CA, USA) as chromogen. The yeast cells reacted positively to immune-staining by anti-*P. brasiliensis* rabbit serum (Figure 5(d)).

The serum obtained at death showed a slight precipitation line in the immunodiffusion test against a fungal cell antigen of *P. brasiliensis* detected by macroscopical observation on a slit lighting system.

Detection of the 43 kDa glycoprotein coding gene (*gp43*) with reference to the sequences of *P. brasiliensis*, *P. lutzi*, and *L. loboi* was tried because of adequate sequence data in the GenBank database and its high homology [7].

The biopsied or paraffin-embedded specimens were examined. The fresh samples were fixed with 70% ethanol at least overnight, cut into approximately 5 × 5 × 5 cm³ sized pieces, and placed into a 1.5 mL sized sterile microtube. The samples were washed 3 times with sterile water by centrifugation at 13,201 g for 5 min, 0.5 mL of DEXPAT (TaKaRa, Otsu, Japan) solution for DNA elution was added, and they were heated at 100°C for 10 min. Then the microtubes containing the tissue samples and DNA eluting solution were cooled on ice and centrifuged at 13,201 g for 10 min. The supernatant was collected and stored as a crude DNA solution. For paraffin-embedded tissue samples, 3 sections of 10 μm thick paraffin-embedded tissues were placed into micro tubes (1.5 mL), and 0.5 mL of DEXPAT solution was added. After being boiled for 10 min., the tubes were centrifuged at 13,201 g for 10 min, and the supernatants were stored as crude DNA solutions. After being purified by an ethanol precipitation method, the DNA solutions were processed to amplify *gp43* using a primer set for *gp43* of *P. brasiliensis* [27]. It is because that both *P. brasiliensis* and *L. loboi* are closely related fungal species [7] and showed higher homology in the gene sequences [4].

We placed 2.5 μL of the sample, 2.5 μL of 20 pM primers MAE (5'-TGCTGCGGCGGGTTAAACCATGTC-3') and ATO (5'-GTTGTGGTATGTGTCGATGTAGACG-3') [27], and 17.5 μL of distilled water in a 0.2 mL PCR tube with one Ready-to-Go bead (Amersham Pharmacia, Tokyo, Japan). The reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 40 cycles of amplification at 94°C for 1 min, 50°C for 1.5 min, 72°C for 2 min, and then a final extension step at 72°C for 10 min in a PCR Thermal Cycler MP (TaKaRa, Otsu, Japan). For amplification in the second-round PCR, the first PCR product was processed by the ethanol precipitation method, and the same PCR reaction was repeated.

The PCR products were approximately 550-base pair sized bands amplified from both fresh and paraffin-embedded tissue samples. The sequences were determined by

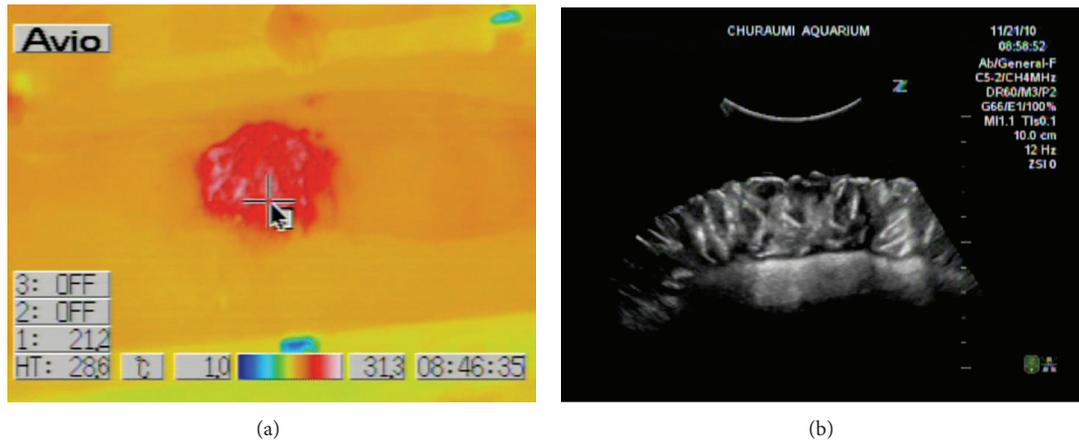


FIGURE 3: A topical temperature of approximately 40°C (arrow) was determined by a thermographic image of the lesion (a), and the depth of invasion was detected by an echo system (b).

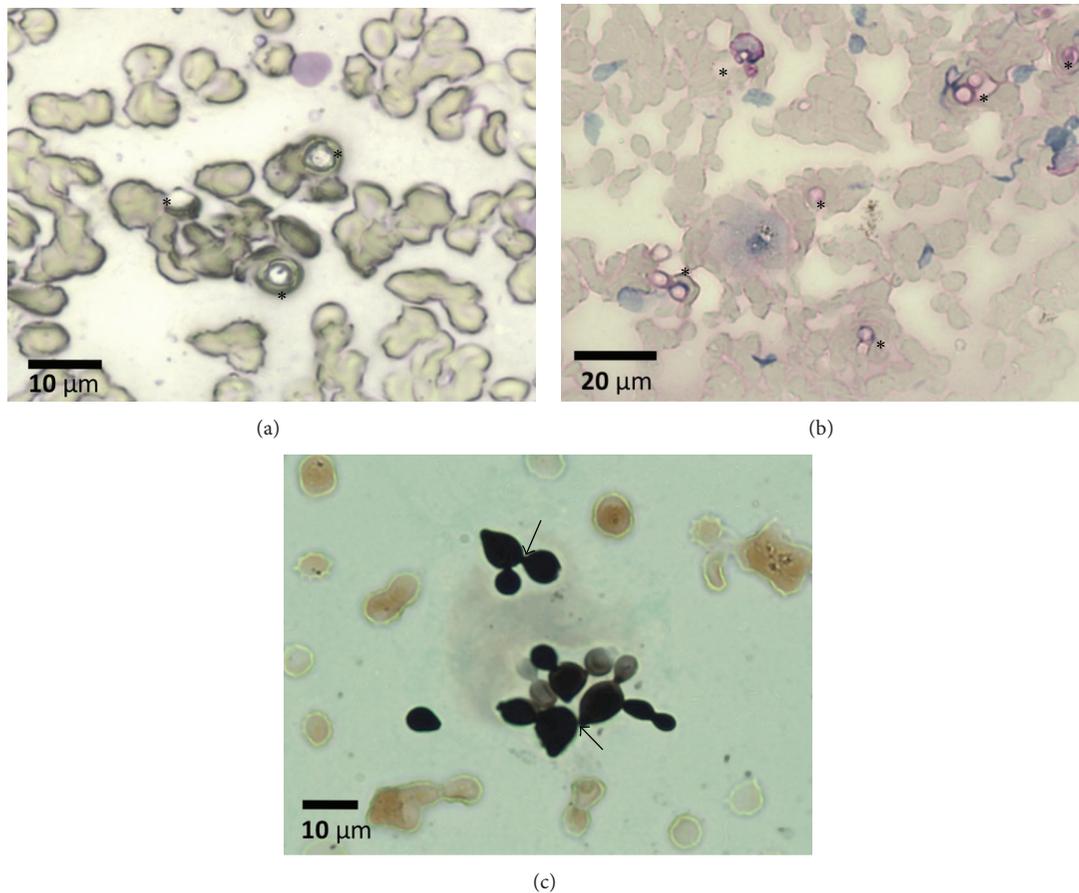


FIGURE 4: Spherical yeast cells (*) stained with Giemsa ($\times 200$, (a)), with PAS ($\times 200$, (b)), and spherical to piriform mother cells with some small daughter cells connected by a narrow base (arrows) stained with GMS ($\times 400$, (c)).

a direct sequencing method using ABI PRISM 3100 sequencer (Applied Biosystems) labeled with the primers MAE and ATO [28]. DNA sequences were aligned with the GENETEX-MAC genetic information processing software (Software Development Co., Ltd., Tokyo, Japan). The reliable sequence

comprised 471 bases obtained from the paraffin-embedded tissue sample; however, the biopsied sample failed to confirm the sequence. The accession numbers of this sequence were registered as AB811031 in the GenBank database. The sequence was localized in the cluster containing *P. brasiliensis*,

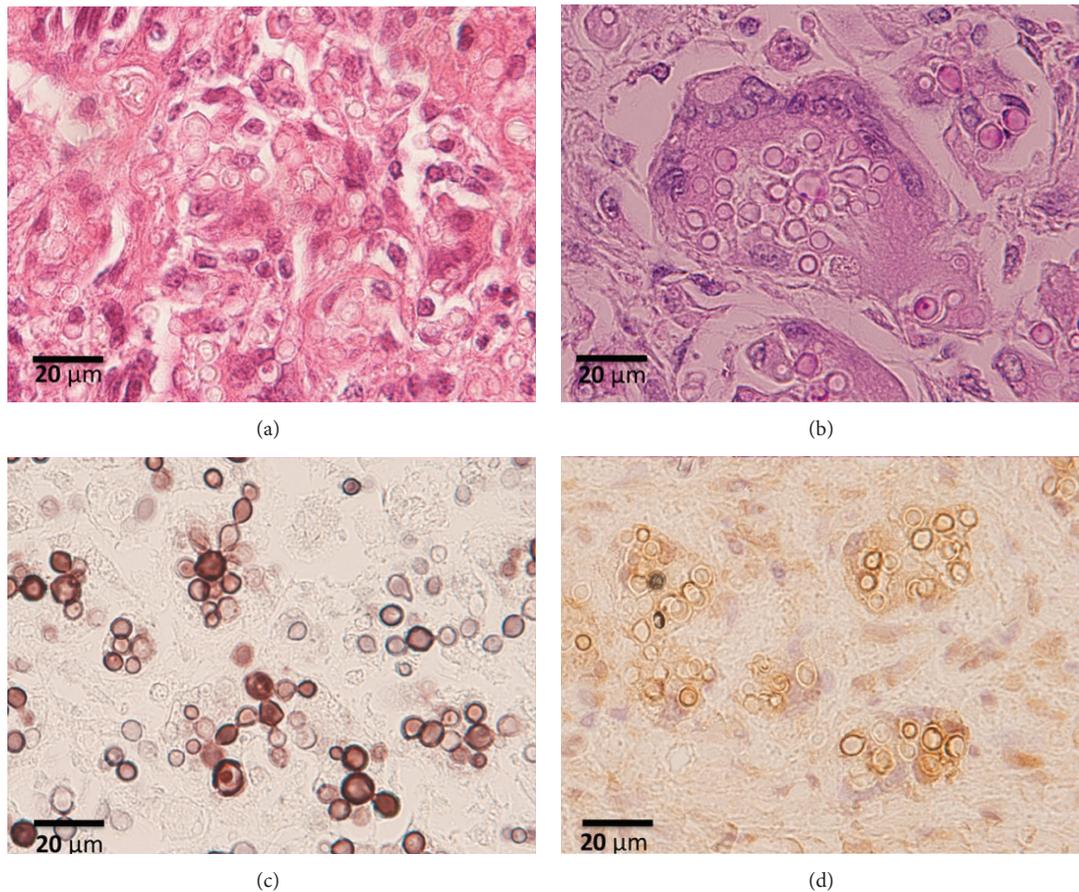


FIGURE 5: Yeast-like cells appeared as abundant round hyaline cells stained with hematoxylin and eosin ($\times 200$, (a)), PAS positive cells arranged in a multiple budding detected in a giant cell stained with PAS ($\times 200$, (b)), well-defined chains or multiple buddings stained with GMS ($\times 200$, (c)), and positive in immune-staining with anti-*P. brasiliensis* rabbit serum ($\times 200$, (d)) in the dermis.

P. lutzii, and *L. loboi* through a BLAST search (<http://blast.ncbi.nlm.nih.gov/>).

The 471 base pairs sequence showed 94.9% homology to *P. brasiliensis* (PBU26160) [29], 87.7% to *P. lutzii* (XM00279244), and 84.1% to *L. loboi* (EU109947) [4] (Table 1). The homologies to fungal species related to *Paracoccidioides* spp., such as *Ajellomyces dermatitidis* (XM_002624715) and *A. capsulatus* (XM_001540694) retrieved from the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>), were 66.3 and 63.6%, respectively (Table 1).

We diagnosed the dolphin as lacaziosis on the basis of the clinical characteristics of the skin lesion, cytologic and histopathologic findings, immunostaining and immunodiffusion test, and the molecular biological study.

2.2. Case 2. A female bottlenose dolphin (*Tursiops truncatus*), estimated to be 5 years of age, was captured in 2007 in the coastal region of Japan simultaneously with the Case 1 dolphin and cared for under outdoor conditions in an aquarium at the same institution as Case 1 for 2 years. Then the animal was transported to the present aquarium where it was cared for until its death. The body weight and body length at the time of death were 175 kg and 240 cm.

The dolphin had two granulomatous lesions in the skin of both upper eye lids in February 2011 (Figures 6(a) and 6(b)). The lesions expanded by March 2011. We hypothesized that the animal might suffer from lacaziosis and performed a biopsy. There were some yeast-like components on the smears of the lesions stained by Giemsa solution (data not shown); however, yeast cells were not detected by histopathology, and *gp43* was not amplified. In addition, *Candida glabrata* was detected from the breath.

In May 2011, we biopsied and cauterized the lesions. *C. glabrata* and *Aspergillus niger* were detected on the breath. The animal received topical applications of ketoconazole cream and oral administration of itraconazole 1,000 mg/body BID followed by antibiotics and hydrocortisone. In addition, a granulomatous mass at the oral cavity appeared (Figure 7(a)).

We started to measure the blood β -glucan levels in June 2011 and found 8–41 pg/mL during July and August 2011, which indicated a fungal infection.

In September 2011, a new skin lesion appeared on the back fin (data not shown). Several scars caused by shark bites also became granulomatous (Figure 7(b)). We biopsied these lesions and detected multiple budding yeast-like cells

TABLE 1: Homologies of the partial sequence of *gp43* to *Paracoccidioides brasiliensis*, *P. lutzii*, *Lacazia loboi*, *Ajellomyces dermatitidis*, and *A. capsulatus*.

GenBank accession no.	bps	Position (total bases)	Identity (%)	Isolate or ID [ref]
Present case	471	1–471 (471)	—	SUM
<i>Paracoccidioides brasiliensis</i>				
PBU26160	466	2603–3068 (3702)	94.9	B339 [29]
<i>P. lutzii</i>				
XM.002792442*	466	1136–1601 (2016)	87.7	Pb01
<i>Lacazia loboi</i>				
EU109947	463	1–463 (483)	84.1	10-RMS [4]
<i>Ajellomyces dermatitidis</i>				
XM.002624715*	469	484–952 (1260)	66.5	SLH14081
<i>A. capsulatus</i>				
XM.001540694*	469	475–943 (1251)	63.3	Nam1

*The sequence was retrieved from the GenBank database.



FIGURE 6: Granulomatous skin lesions of Case 2 at the left upper eye-lid in February 2011 (a, b).

by cytologic observation of samples stained by Giemsa (Figure 8(a)), mounted with 5% KOH (Figure 8(c)), and added with lactophenol cotton blue (Figure 8(c)) and GMS (Figure 8(d)). All biopsied skin samples from May, June, and September 2011 cultured on potato dextrose agar supplemented with 100 mg/L of chloramphenicol and Mycosel agar plates at 25 and 35°C for 4 weeks were negative.

The DNAs derived from Case 2 were not amplified through the PCR conditions used in Case 1. Therefore, we designed an inner primer set: SUM F1 (5'-GTCATC-GATCTCCATGGTGTAAAG-3') and SUM R2 (5'-GGC-AGARAAGCATCCGAAA-3') with reference to the *gp43* sequences from *P. brasiliensis* (PBU26160, AY005408, and AB304681) and *L. loboi* (AY697436 and EU109947); the sequence determined in the Case 1-derived DNA was aligned by GENETYX-MAC ver. 12.0 genetic information processing software (GENETYX CORPORATION, Tokyo, Japan). The PCR condition was as the same as the first PCR.

We detected 382 base pairs of a partial sequence of *gp43* showing 100% identity to those from Case 1 by the nested-PCR system from the biopsied sample collected at the above times.

The serum also showed a slight precipitation line in the immunodiffusion test against a fungal cell antigen of *P. brasiliensis* (data not shown).

On the basis of these clinical characteristics and cytologic and molecular biological observations, we made a diagnosis of lacaziosis.

The dolphin died suddenly in December 2012. The animal had shown a higher respiratory ratio at 7 times per min since the summer of 2012. The macroscopic findings were intestinal occlusion caused by cardiac disorder, pulmonary chronic inflammation, and one purulent cyst at the scar from cauterization of the animal's lesion without yeast-like cells.

3. Discussion

The present 2 dolphins showing chronic granulomatous skin lesions represent the first examples of lacaziosis from Pacific Ocean diagnosed on the basis of clinical, cytologic, histologic, serologic, and molecular biological data. Although many cases of lacaziosis-like diseases in dolphins from Ecuador, Colombia, Peru, and Chile along the coast of the Pacific Ocean have been recorded, they were diagnosed by

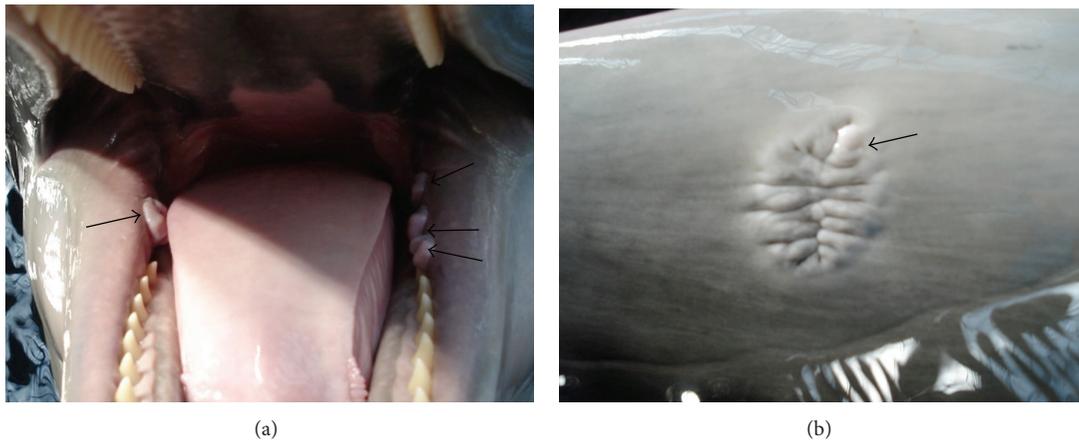


FIGURE 7: Granulomatous masses at the oral cavity appeared in May 2011 (arrows, (a)). One of the newly appeared skin lesions on the scars caused by a shark bite in September 2011 (arrow, (b)).

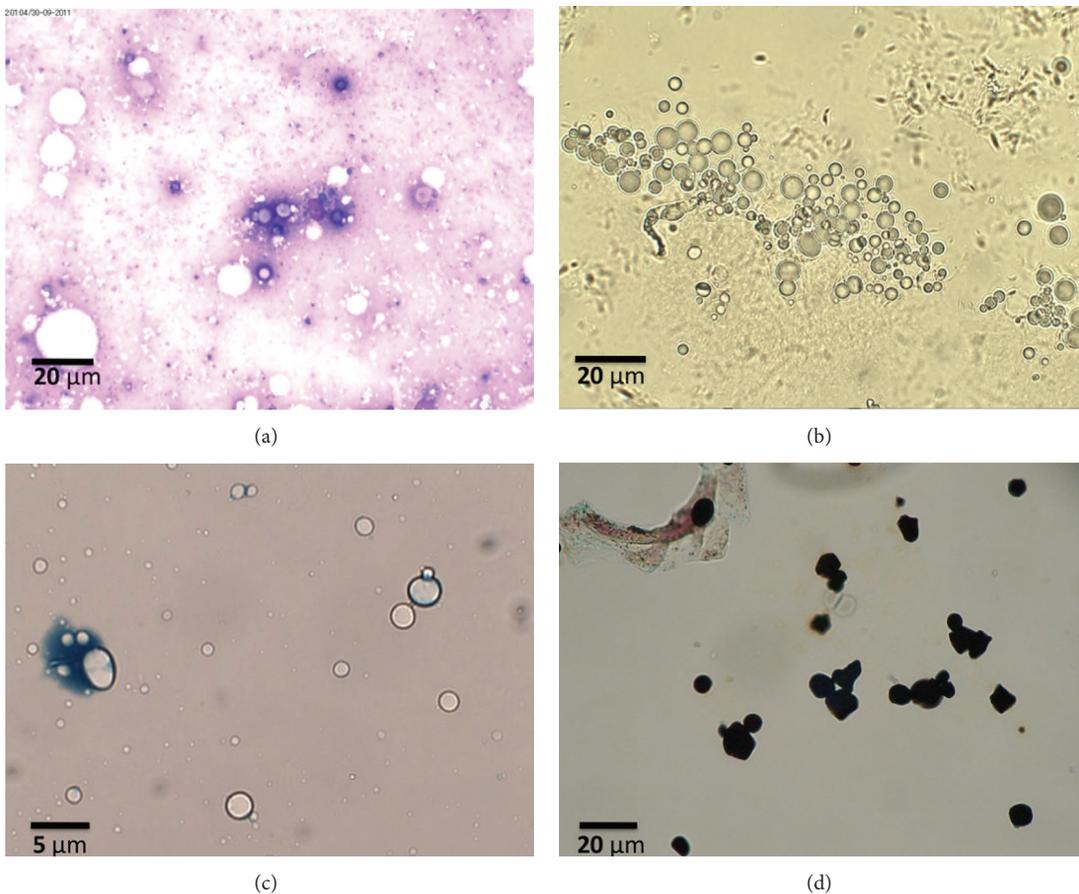


FIGURE 8: Multiple budding yeast-like cells derived from Case 2 stained by Giemsa ($\times 200$, (a)), in 5% KOH mount ($\times 200$, (b)), in 5% KOH plus lactophenol cotton blue ($\times 400$, (c)), and stained with GMS ($\times 200$, (d)).

macroscopy and photography without histopathologic and molecular biological data [23]. Furthermore, some lacaziosis-like diseases have been recorded in dolphins in Japanese waters [24]; however, those cases were also diagnosed by photographic images. Therefore, the present cases are the first of lacaziosis from the Pacific Ocean to be diagnosed

according to the definition recommended by Kiszka et al. [22] as follows: lacaziosis-like disease is very similar to lobomycosis but lacking a histologic diagnosis.

The serum cross-reaction in the immunodiffusion test with *P. brasiliensis* antigen and positive immune-staining with *P. brasiliensis* antisera have previously been reported as

characteristics of lacaziosis [30]. The slightly positive reaction in the immunodiffusion test with *P. brasiliensis* antigen and the positive results upon immune-staining were matched to Brazilian human lacaziosis.

Establishing a diagnosis of lacaziosis requires clinical data, cytologic observation, histopathologic techniques, immunological methods, and detection of species-specific genes because of the difficulty of culture [2, 3, 8, 10]. Furthermore, it seems very unlikely that cutaneous lesions of cauliflower-like eczema in dolphins would be recognized as lacaziosis outside of endemic areas. In such cases, molecular biological techniques are useful.

In general, identification of fungal species is made on the basis of the internal transcribed spacer (ITS)-1-5.8S-ITS-2 regions of ribosomal RNA (ITS rRNA) gene sequences with more than 98 to 99% diversity between species or at least 95% even in a fungal species with higher intraspecies diversity [31]. However, we failed to detect the ITS rRNA gene from the dolphins' samples in the present study and could not compare to those from the Atlantic Ocean [20].

Interestingly, Esperón et al. reported that the ITS region of the ribosomal RNA sequence derived from dolphins living in the Atlantic Ocean was more related to *P. brasiliensis* than to *L. loboi* [20]. The present cases also showed a close relationship of the genotype of the *gp43* to *P. brasiliensis*. Further study may confirm that the genotypes of lacaziosis out of Amazon areas.

The virulence of lacaziosis endemic in Japanese waters remains unknown; however, the disease seems to be virulent among dolphins since the animals that have spent a period in the same tribe suffered lacaziosis caused by identical genotype. They might be infected by contact or receive the pathogen simultaneously, and/or endemic in the tribe.

Lacaziosis sometimes causes immune disorders in dolphins [32]. Although we could not evaluate immune markers, the present animals suffered fatal outcomes caused by systemic bacterial infections or cardiac and respiratory problems, indicating that they were suffering from severe immune disorders.

Japan is a maritime nation. Many people in Japan have contact with the sea and marine products: not only fishermen but also the general public through swimming, fishing, boating, and visiting the marine aquarium. Therefore, we speculate that some latent human cases of lacaziosis may exist in our country.

4. Conclusions

We diagnosed 2 cases of lacaziosis in bottlenose dolphins on the basis of clinical, cytologic, histologic, serologic, and molecular biological data and confirmed that the causative agent at the present cases was a different genotype of *L. loboi* from Amazon area.

Conflict of Interests

The corresponding author, Ayako Sano, declared that there is no conflict of interests submitted to the University of the Ryukyus no. H24-763.

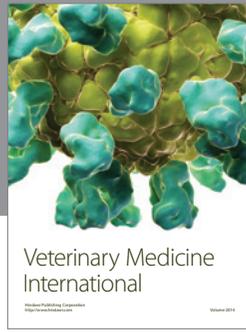
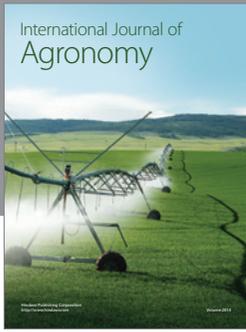
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