(*EUBALAENA AUSTRALIS*) NEONATE

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ABSTRACT: A female southern right whale (*Eubalaena australis*) neonate was found stranded on the Western Cape coast of southern Africa. Skin samples were taken the same day from three different locations on the animal's body and stored at −20 C. Isolation through repetitive culture of these skin sections yielded a single yeast species, *Candida zeylanoides*. Total genomic DNA also was isolated directly from skin samples. Polymerase chain reaction analysis of the internal transcribed spacer region of the fungal ribosomal gene cluster revealed the presence of *Filobasidiella neoformans* var. *neoformans*, the teleomorphic state of *Cryptococcus neoformans*. Fungal infections in cetaceans seem to be limited when compared to infections caused by bacteria, viruses and parasites. However, *Candida* species appear to be the most common type of fungal infection associated with cetaceans. To our knowledge this is the first report of a *C. zeylanoides* infection in a mysticete, as well as the first report of a dual infection involving two opportunistic pathogenic yeast species in a cetacean.

Key words: Candida zeylanoides, *Eubalaena australis*, *Filobasidiella neoformans* var. *neoformans*, skin biopsies, southern right whale, yeast infection.

INTRODUCTION

Every year between June and November, southern right whales (*Eubalaena australis*) migrate from the Southern Ocean into the coastal waters of the Western Cape of South Africa to calve and nurse their young. The calves are born in these coastal waters around mid-August and remain there with their mothers until old enough to travel south again (Best, 1990). Occasionally, some of these cetaceans beach or wash ashore along this coast and skin lesions resembling fungal infections have been noticed on their bodies.

The results of a 4-yr year study of bowhead whales (*Balaena mysticetus*) near Barrow, Alaska that were exposed to petrochemical industrial activities, were presented in 1990 (Schotts et al., 1990). These whales were characterized by dozens of roughened areas on their skin surface. Swabs from the lesions, as well as nonlesioned skin, were cultivated, and 80 species bacteria and yeasts were isolated and identified. The dominant yeast species belonged to the genera *Candida*, *Cryptococcus*, and *Rhodotorula*

and it was pointed out that these taxa have the ability to degrade hydrocarbons (Schotts et al., 1990).

Fungal infections in marine mammals are relatively rare when compared to infectious diseases caused by viruses, mycoplasmas, bacteria, and parasites (Migaki and Jones, 1983). Fungi can however, be the cause of severe and systemic infections that are most often not treated successfully. As far as diagnosis is involved, mycotic infections can be grouped into systemic infections that cause severe pyogranulomatous lesions, as well as superficial infections causing less severe symptoms (Migaki and Jones, 1983). The first type normally occurs by inhalation of spores with the respiratory system being the primary affected site. However, cases of severe mycotic dermatitis caused by *Fusarium* have been described in cetaceans (Frasca et al., 1996). Animals at risk generally are immune (Moeller, 1997) or integumentarily compromised (Miller et al., 2002), suffer from malnutrition, have been on cortisone or antibiotic treatment for extended periods of time, or had pre-existing diseases (Migaki and Jones, 1983).

Infections caused by *Candida* seem to be the most prevalent type of fungal infection in cetaceans, with *Candida albicans* being the most common species, especially in captive cetaceans (Dunn et al., 1982; Migaki and Jones, 1983; Higgins, 2000). Their wild counterparts seem to be less affected or unaffected by yeast infections (Higgins, 2000). *Candida* species generally are associated with mucocutaneous areas, and lesions associated with this yeast occur primarily around the blowhole, esophagus, vagina or anal area (Moeller, 1997; Higgins, 2000). As in humans, these infections usually are observed in animals subjected to long-term antibiotic therapy (Henk and Mullan, 1996; Moeller, 1997), but also can be associated with corticosteroid treatment and overtreatment of water (Higgins, 2000). These infections are characterized by white or yellow creamy plaques in the area of the infected organs and focal areas of necrosis also can be visible (Dunn et al., 1982; Moeller, 1997; Gaydos et al., 2004). Other species isolated from captive cetaceans include *Candida guilliermondii*, *Candida lambica*, *Candida ciferrii*, *Candida pseudotropicalis*, *Torulopsis candida*, and *Trichosporon cataneum* (Higgins, 2000).

On 22 August 2001, a southern right whale neonate (#01/06) was found stranded at Dwarskersbos, St. Helena Bay in Southern Africa. This was a 5.44 m female calf of that year. It appeared to be freshly dead, not bloated, with the skin still intact and a heavy infestation of cyamids (whale lice) over the head region and some scattered over the rest of the body. The carcass was examined the same day and, using photographs taken at the time of examination, its condition was assessed post hoc as code 2 of Pugliares et al. (2007). Upon dissection, the bladder was found to be grossly distended and the pericardial sac contained 2 l of a straw-colored fluid. The lungs contained air and the umbilicus was unhealed but granulating, indicative of an animal that had been alive for some time. With the first possible birth recorded being on 29 June (Best, 1994), this calf was unlikely to have been more than 2 mo old. Tissue samples of the heart and kidney were collected and immediately fixed in 10% formalin, and a urine sample was refrigerated. Histologic examination of the heart revealed mild interstitial edema, although the muscle fibers were well-developed. The kidney was well developed although the renal cortex appeared to be very narrow (suggesting an embryonic condition) with severe congestion. No microbial growth could be observed in any of the sections. The urine from this young whale was a bright light yellow color with a pH of 6.0 and a specific gravity of 1.030. The urine also contained traces of protein, although tests for glucose and nitrate were negative and microscopic studies revealed no leukocytes, red blood cells, or epithelial cells. Cultured urine from this young whale produced a mixed growth of *Proteus*, a recognized cause of urinary infections, as well as unidentified species of yeasts and filamentous fungi. Skin samples were collected and stored at −20 C, and in this study, we describe the subsequent examination of these skin samples for the presence of opportunistic fungal pathogens.

MATERIALS AND METHODS

Skin samples were taken from the back, sides and belly at three different locations ca 1 m apart along the animal's body (V3=Ventral surface, D5=Dorsal surface, and L3=Lateral surface). Shortly after collection, all samples were stored in foil at −20 C. This work was carried out under permits issued to PBB according to the Sea Fishery Act, 1988 (Act no. 12 of 1988) of South Africa, dated 10 March 1998, and the Marine Living Resources Act, 1998 (Act no. 18 of 1998) of South Africa, dated 29 January 1999 and 27 January 2000, respectively.

Isolation of yeasts/filamentous fungi from the whale skin

Frozen skin samples (blocks of approximately 3×3 cm) were dissected under sterile conditions into smaller blocks to represent

different depths: the outer skin layer, the inner skin layer, and the deepest inner skin layer with blubber. Samples were placed on a series of different growth media to facilitate the cultivation of diverse fungal taxa and incubated at 20 C. Growth media used included half strength Difco Potato Dextrose Agar (Becton Dickinson, Sparks, Maryland, USA), Biolab Malt Extract Agar (Biolab Diagnostics [Pty] Ltd., Wadeville, Republic of South Africa), Blood Agar Base, Sabouraud Glucose Agar, Sabouraud Glucose Agar with Olive Oil (Ronald, 1993), and Bird Seed Agar (Kurtzman and Fell, 2000). After 7 days of incubation, morphologically similar butyrous yeast colonies were observed on the plates. Representative colonies were selected and purified by repetitive culturing on Yeast Extract Peptone Dextrose (YEPD) Medium (Ronald, 1993) at 20 C.

Physiologic tests

Isolates were tested for their ability to ferment glucose in test tubes containing 10 ml Biolab Yeast extract broth, amended with 50 mM D(+) Glucose (anhydrous, Merck, Wadeville, Republic of South Africa), as well as Durham tubes. The ability to grow at 37 C was tested on YEPD Medium (Ronald, 1993). YEPD Medium (without agar) containing cycloheximide (0.1% and 0.01%) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) was used to test the ability of these isolates to grow in the presence of cycloheximide. Screening for protease activity among the isolated yeasts was conducted by growing them on solid medium containing skimmed milk and a pH indicator (0.1% yeast extract, 1% skim milk, 2% bacteriological agar, and 0.01% Bromocresol purple Indicator (Riedel De Haën AG Zeelze, Hannover, Germany, pH 5.2) and then observing the plates for the formation of clearing zones (St Leger et al., 1999).

Molecular identification of yeast isolates

Cells from each pure yeast culture were inoculated into Yeast Malt Extract (YM Broth) (Ronald, 1993) and incubated overnight at 28 C on a culture roll drum (10 rpm). A volume of 2 ml culture was extracted and centrifuged (Biofuge fresco, Heraeus Instruments Inc., S. Plainfield, New Jersey, USA) in 2 ml Eppendorf tubes (Seabreeze, Cape Town, South Africa) for 5 min at 13,793 relative centrifugal force (RCF). Pelleted cells were resuspended in 500 μl DNA lysis buffer containing 20% (v/v) Tris-HCl (1 M, pH 8.0); 10% (v/v) EDTA (0.5 M); 5% (v/v) SDS 20%,

and 1,300 μl sterile distilled water. Acid-washed glass beads (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany, Cat no: G4649-100G) were added and the tubes were vortexed for 4 min (Vortex Genie 2; set at 8, Scientific Industries, Bohemia, New York, USA) and placed on ice for 5 min. A volume of 275 μl ammonium acetate (7 M) was added and the tubes were incubated at 65 C (5 min) and cooled down on ice for 5 min. A volume of 500 μl chloroform was added and the tubes were vortexed and centrifuged (Biofuge fresco, Heraeus Instruments Inc., S. Plainfield, New Jersey, USA) for 5 min at 13,793 RCF (4 C). The supernatant was removed and precipitated with 1 volume isopropanol at 22 C for 5 min. The precipitate was centrifuged (Biofuge fresco, Heraeus Instruments Inc.) for 5 min at 13,793 RCF (4 C). The pellet was washed with 70% (v/v) alcohol, dried and dissolved in 50 μl TE buffer containing 0.5% (v/v) RNase A (Roche Diagnostics GmbH, Mannheim, Germany, Cat no: 109169), and incubated at 37 C for 20 min. Extracted DNA was stored at −20 C.

The D1/D2 (600–650 bp) region of the large subunit (26S) rDNA was amplified by the polymerase chain reaction (PCR) employing the universal forward primer F63 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse primer LR3 (5′-GGT CCG TGT TTC AAG ACG G-3′; Fell et al., 2000). The PCR was performed in a 50 μl reaction containing 10 ng DNA, 2.5 mM MgCl$_2$, 0.25 mM deoxynucleotide triphosphates (dNTPs), 0.1 μM of each primer, and 0.5 U Fermentas *Taq*. Amplification was in a GeneAmp PCR system (Model 2400, Applied Biosystems Inc., Foster City, California, USA) with the following cycling parameters for 30 cycles: initial denaturation for 3 min at 94 C, denaturation for 1 min at 94 C, followed by annealing for 1 min at 61 C, elongation for 2 min at 72 C, followed by a final elongation of 7 min at 72 C. The amplified product was separated on an agarose gel (0.8%, w/v) and visualized by ethidium bromide staining (Chory and Pollard, 1999).

Three of the isolates were also identified using the sequence of the ITS1/5.8S/ITS2 regions of the fungal ribosomal gene cluster. The same gDNA isolation procedure was used as for the D1/D2 method. However, in the PCR reaction, the universal fungal primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) was used in combination with the reverse primer ITS4 (5′-TCCTCCGCTTATTGA-TATGC-3′) (White et al., 1990). The cycling parameters were the same except for the annealing temperature which was set at 56 C.

All PCR products were purified with the

High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) and sequenced using a Hitachi 3730xl DNA Analyzer (Applied Biosystems Inc.). Data from the forward and reverse sequences were compared and aligned by using DNA-MAN for WINDOWS Version 4.13 (Lynnon Biosoft, Lynnon Corporation, Quebec City, Quebec, Canada). Each isolate was identified by comparing known sequences using the BLAST program of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov./BLAST).

Extraction and purification of genomic DNA from skin samples

Genomic DNA (gDNA) was extracted from the L3 skin sample from the neonate by grinding a small block of skin (1.5 cm^3), that was frozen in liquid nitrogen, into a fine powder using a precooled (-20 C) mortar and pestle. An aliquot (0.3 g) of the powder was transferred to a 2 ml Eppendorf tube, which was kept on ice, and suspended in 500 μl extraction buffer (200 mM Tris-HCl, pH 8; 250 mM NaCl; 25 mM EDTA; and 0.5% SDS) and briefly vortexed. Phenol (350 μl) was added, vortexed, followed by 150 μl chloroform:isoamyl alcohol (24:1) and vortexed again. The mixture was centrifuged at 13,793 RCF for 1 hr at 8 C. The aqueous phase was immediately transferred to a sterile 1.5 ml Eppendorf tube containing 25 μl RNAse A and incubated at 37 C for 5–10 min. One volume chloroform:isoamyl alcohol (24:1) was added and gently mixed by inverting. The mixture was centrifuged once more at 13,793 RCF for 10 min. The aqueous phase was transferred to a fresh 1.5 ml Eppendorf tube and the DNA precipitated by adding 0.54 volumes of isopropanol, followed by gentle mixing. The supernatant was discarded after 1 min centrifuging at 13,793 RCF. The pellet was washed with 70% alcohol and dried in an oven for 10 min. The DNA was resuspended in 50 μl TE buffer. To assess the degree of purity and degradation, samples containing the isolated DNA were examined by electrophoresis on 0.8% (w/v) agarose gels containing ethidium bromide (1 μg/ml; Chory and Pollard, 1999).

Amplification and sequence analysis of fungal rDNA within skin samples

Fragments of the nuclear rRNA gene complex, including a section of the small subunit (SSU) or 18S rRNA gene, the internal transcribed spacer region (ITS1, ca. 113 to 121 bp), 5.8S rRNA gene (ca. 161 bp), and ITS2 region (ca. 222 to 230 bp) (Lloyd-MacGilp et al., 1996) were amplified using Takara EX *Taq* DNA polymerase (Takara BioCo., Shiga, Japan). Amplification of the abovementioned nuclear rRNA genes, followed by purification and sequencing, was performed using the same method as described earlier. The fungal DNA was identified by comparing known sequences using the BLAST program of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov./BLAST).

RESULTS

A number of plated skin sections, representing the full range of different growth media, yielded similar butyrous yeast colonies from 5 days postincubation. After repetitive culturing of these yeast colonies, 12 representative isolates were chosen based on characteristics of colonies, and isolation media, as well as sampling sites on the animal's body and depth of the skin section (outer, inner, or inner skin with blubber) (Table 1). Colonies of the 12 isolates were similarly smooth, cream colored, and butyrous. Microscopic evaluation of these isolates grown on Yeast Peptone Dextrose agar showed many similarities, such as pseudohyphae and ovoid blastoconidia. No sexual reproduction could be observed.

Physiologic characterization of the isolates included tests on the ability to grow at 37 C, fermentation of carbohydrates, as well as ability to grow on media containing cycloheximide. The ability to ferment sugars was determined in test tubes containing 10 ml Yeast Extract Broth amended with 50 mM glucose, as well as Durham tubes. No formation of gas could be observed in the Durham tubes, and our results, therefore, indicated that the yeast isolates were unable to ferment glucose. All the isolates were unable to grow at 37 C on a solid medium and the optimum temperature for these yeast isolates appears to be lower than 37 C. All the yeast isolates were able to grow in yeast extract medium containing two concentrations of cycloheximide (0.1% and 0.01%). The

TABLE 1.   Isolates from the different skin sections of the southern right whale (*Eubalaena australis*) neonate (SRW 01/06) cultured on different growth media (PDA = Potato Dextrose Agar, SGA = Sabouraud Glucose Agar, MEA = Malt Extract Agar, STA = Sabouraud Glucose Agar with Olive Oil, BSA = Bird Seed Agar) and subsequently identified using sequencing of the D1/D2 region of the large subunit (26S) and ITS1/ITS2* regions of ribosomal gene cluster.

Reference no. of whale and sampling site	Reference no. of the isolate	Medium on which skin section was plated out	Identification of fungus from sequencing data	GenBank Accession number
SRW 01/06 V3	W21	Outer skin on half strength PDA	*Candida zeylanoides*	EU131527
SRW 01/06 V3	W28	Outer skin on half strength PDA	*Candida zeylanoides*	EU131528
SRW 01/06 V3	W29	Whole skin with blubber on half strength PDA	*Candida zeylanoides*	EU131529 EU131530*
SRW 01/06 V3	W30	Inner skin on SGA	*Candida zeylanoides*	EU131531
SRW 01/06 V3	W31	Outer skin on SGA	*Candida zeylanoides*	EU131532
SRW 01/06 V3	W32	Whole skin on MEA	*Candida zeylanoides*	EU131533
SRW 01/06 V3	W33	Inner skin with blubber on STA	*Candida zeylanoides*	EU131534
SRW 01/06 V3	W34	Inner skin with blubber on STA	*Candida zeylanoides*	EU131535
SRW 01/06 D5	W35	Inner skin with blubber on STA	*Candida zeylanoides*	EU131536
SRW 01/06 D5	W36	Inner skin with blubber on SGA	*Candida zeylanoides*	EU131537*
SRW 01/06 D5	W37	Whole skin on BSA	*Candida zeylanoides*	EU131538
SRW 01/06 D5	W38	Whole skin on MEA	*Candida zeylanoides*	EU131539*

ability of the isolates to produce protease was tested on a medium containing skimmed milk as well as a pH indicator. It was found that the plates changed color significantly, from yellowish-green to clear purple zones after 2 days of incubation.

The Blast search identified 10 of the isolates as *Candida zeylanoides* (Table 1) with a 98–100% match, using the sequencing results of the D1/D2 and partial 26S rRNA region. These sequences were then aligned with the D1/D2 region of the type strain of *C. zeylanoides* (CBS 619, Genbank accession nr. Y15471 and AY497688) resulting in a 99–100% match. Our isolates also aligned 100% with other *C. zeylanoides* isolates, including two isolates from a bottlenose dolphin (*Tursiops truncates*) (AB278159 and AB278160; only in NCBI Database, 2006) and another from the marine environment (EU034630; unpublished).

Isolate W29 also was identified using the sequencing results of the ITS1, 5.8S and ITS2 regions, and isolates W36 and W38 were only identified using this region, as a cross reference (marked with * in Table 1). This BLAST search also identified these yeast isolates as *C. zeylanoides*, with a 99–100% match. These three sequences were then aligned with the Genbank sequence of the type strain of *C. zeylanoides* (CBS 619, Genbank accession nr. AY542871). The alignment revealed a 99–100% match to the ITS1/5.8S/ITS2 regions of the type strain.

Total genomic DNA also was isolated directly from skin samples of the SRW 01/06 neonate, after which PCR analysis of the internal transcribed spacer (ITS) regions of the fungal ribosomal gene cluster revealed the presence of another opportunistic fungal pathogen, *Filobasidiella neoformans* var. *neoformans*. The

relevant sequence was deposited in Genbank (Accession number: EU131540).

DISCUSSION

The study by Schotts et al. (1990) found that *Candida* species were the dominant yeasts found on bowhead whales subjected to oil pollution and that these yeast species were found on both normal, as well as lesional skin. Species isolated and identified included *Candida krusei*, *C. parapsilosis*, *C. rugosa*, *C. humicola*, *C. intermedia*, *C. guilliermondii*, *C. viswanathii*, *C. lipolytica*, *C. stellatoidea*, and *C. utilis*. Species of *Cryptococcus* and *Rhodotorula* also were found to be associated prominently with lesional, as well as normal, skin. The authors speculated that these whales were subjected to spills from oil and gas exploration sites and the oil might adhere to the animals' skins, causing damage to the epidermal layers. The damaged skin would then allow resident organisms access to the blood.

Candida zeylanoides was isolated from two of three sampling sites (D5 and V3) on the body of the southern right whale neonate (SRW 01/06) found stranded at Dwarskersbos. To our knowledge, this is the first report of *C. zeylanoides* isolated from a mysticete species. This fungus previously has been reported from the skin of a "blue" dolphin from the Atlantic Ocean (CBS 5262) and a bottlenose dolphin in Japan (NCBI Database, 2006). This yeast is relatively rare in humans but is included in the Atlas of Clinical Fungi (De Hoog et al., 2000) and has been reported from skin, nails, and blood as an opportunistic yeast pathogen (Levenson et al., 1991; Crozier, 1993; Hazen, 1995; Lee et al., 2001) and also in a report of endocarditis in a HIV-positive patient. Hazen (1995) described *C. zeylanoides* as a new and emerging pathogen and observed that these yeasts could, in many cases, be isolated from blood cultures repeatedly, indicating a constant shedding into the bloodstream.

Studies on bacterial pathogens, as well as *C. albicans* have shown that virulence is regulated by genes that are directed by signals from the host niche, including pH. It was found that these signals are essential to the virulence and survival of the pathogen during infection. The virulence factors of these organisms also include secretory hydrolases, particularly proteases, which could facilitate penetration into host tissue (St Leger et al., 1999). We tested for the production of protease on a medium containing milk powder and bromocresol purple as pH indicator. Medium color changed from yellowish green to purple and the purple zones were clear after only 2 days of incubation. We therefore can conclude that *C. zeylanoides* produces proteases and the fact that the pH changed so rapidly might be indicative of the potential virulence of this organism.

The extraction of gDNA directly from skin of SRW 01/06 and subsequent sequencing of the PCR product, originating from the ITS region of the nuclear rRNA gene complex, revealed the presence of *Filobasidiella neoformans* var. *neoformans*. This represents the teleomorph of an encapsulated yeast that has a worldwide distribution (Migaki and Jones, 1983; Higgins, 2000). Although it occurs as a saprophyte in nature (Higgins, 2000), *C. neoformans* is a well-known pathogen for a host of mammals, including dolphins (Migaki et al., 1978; Migaki and Jones, 1983; Henk and Mullan, 1996; Miller et al., 2002). There seems to be a high correlation between *C. neoformans* and the dry excrements in bird shelters, e.g., pigeons, suggesting that the fecal material of the birds supply is a suitable substrate for growth of this fungus (De Hoog et al., 2000). It is suspected that inhalation of the *Cryptococcus* spores can lead to the development of cryptococcal pneumonia, which can spread to other organs, particularly the central nervous system (Migaki and Jones, 1983).

In our study, we were able to isolate the gDNA of *Filobasidiella neoformans* var. *neoformans* directly from skin, although

we were not able to culture it from the skin samples. Only *Candida zeylanoides* could be cultured from the skin samples. It is possible that *C. zeylanoides* is a more opportunistic colonizer than the *Cryptococcus* species, and this could result in *C. neoformans* colonies being obscured by *C. zeylanoides* colonies. Denning et al. (1990) described *C. albicans* obscuring *C. neoformans* colonies on plates from sputum samples of patients with Acquired Immunodeficiency Syndrome (AIDS), and another study described the isolation of both *C. zeylanoides* and *Cryptococcus laurentii* causing fungenemia from the central venous catheter of a girl with myelogenous leukemia (Lee et al., 2001).

To our knowledge, this is the first report of a dual infection by *C. zeylanoides* and *F. neoformans* var. *neoformans* in a cetacean. However, further research on the fungi associated with diseases of these animals along the Western Cape coast of South Africa is needed to evaluate the frequency of these fungal infections.

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