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NOTE

Phaeohyphomycosis resulting in obstructive tracheitis in three green sea turtles *Chelonia mydas* stranded along the Florida coast

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ABSTRACT: Three wild immature green sea turtles *Chelonia mydas* were found alive but lethargic on the shores of the Indian River Lagoon and Gulf of Mexico in Florida, USA, and subsequently died. Necropsy findings in all 3 turtles included partial occlusion of the trachea by a mass comprised of granulomatous inflammation. Pigmented fungal hyphae were observed within the lesion by histology and were characterized by culture and sequencing of the internal transcribed spacer 2 domain of the rRNA gene and D1/D2 region of the fungal 28s gene. The dematiaceous fungus species *Veronaea botryosa* was isolated from the tracheal mass in 2 cases, and genetic sequence of *V. botryosa* was detected by polymerase chain reaction in all 3 cases. Genetic sequencing and fungal cultures also detected other dematiaceous fungi, including a *Cladosporium* sp., an *Ochroconis* sp., and a *Cochliobolus* sp. These cases are the first report of phaeohyphomycosis caused by *V. botryosa* in wild marine animals.

KEY WORDS: Fungus · Reptile · Sea turtle · Pigmented · Respiratory · *Veronaea botryosa*

INTRODUCTION

Most fungal infections in chelonians are considered opportunistic and secondary to underlying conditions or immunosuppression (Jacobson et al. 2000, Schumacher 2003). Factors that may predispose an individual to fungal infection include suboptimal environmental temperatures and humidity, nutritional imbalances, infection by other pathogens, and trauma (Jacobson et al. 2000, Schumacher 2003). Much of the information on mycotic disease in reptiles is derived from captive animals, which likely reflects the importance and frequency of captivity-related disorders as predisposing factors, as well as the greater number of captive animals examined by diagnosticians. Infections by pigmented or dematiaceous fungi comprise a minority of reported infections, and most involve the skin or underlying tissues (Paré & Jacobson 2007). Infection is often attributed to traumatic inoculation (Paré & Jacobson 2007).

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Veronaea botryosa is a dematiaceous ascomycete fungus in the family Herpotrichiellaceae. It belongs to a small genus of widely distributed but poorly understood saprobic fungi that are typically found in soil and on plant materials (de Hoog et al. 2011). *Veronaea* clusters within the paraphyletic genus *Exophiala*, in a clade known as the *salmonis*-clade, along with significant animal pathogens including *E. pisciphila* and *E. equina* (de Hoog et al. 2011). Reported cases of *V. botryosa* infection in humans include examples of deep, chronic infections and infections in apparently immunocompetent individuals (Matsushita et al. 2003, Sang et al. 2011, Bonifaz et al. 2013). To our knowledge, *V. botryosa* has not been previously described in reptiles or the marine environment, although closely related species have been documented as a cause of phaeohyphomycosis in individual captive and free-ranging terrestrial chelonians (Joyner et al. 2006, Stringer et al. 2009). This case series describes *V. botryosa* infection associated with obstructive granulomatous tracheitis in 3 wild green sea turtles *Chelonia mydas* that were found stranded in Florida, USA.

MATERIALS AND METHODS

Clinical history and necropsy

All 3 sea turtles were collected in Florida by the Sea Turtle Stranding and Salvage Network between 2006 and 2010. Straight carapace length (SCL) was measured from the nuchal notch to the caudal tip of the supracaudal scute. One turtle that survived for a short period was admitted to the Mote Marine Laboratory and Aquarium for care. The carcasses were transported to the University of Florida (Gainesville, Florida) for necropsy. Tissues were fixed in 10% neutral buffered formalin and processed for histology by routine methods. Sections were stained with hematoxylin and eosin and Grocott's method for fungi (GMS; Grocott 1955). Samples of tracheal lesions were collected and stored at −80°C. All rehabilitation, necropsy, and diagnostic testing were authorized under permits MTP-081, MTP-086, and MTP-126 from the Florida Fish and Wildlife Conservation Commission.

Fungal culture

After thawing, samples of the tracheal masses from each turtle were collected as cleanly as possible for fungal culture by searing the outer surface and excising an internal area of the lesions using a new scalpel blade. The tissue was inoculated onto inhibitory mold agar and potato flake agar (Hardy Diagnostics) and incubated at room temperature (22 to 25°C). Cultures were examined grossly every day. At 2 wk of growth, morphologically distinct fungi were subcultured onto Sabouraud dextrose (Sabdex) agar and Columbia agar with 5% sheep blood, colistin, and nalidixic acid (CNA) agar for selective culture (Hardy Diagnostics). Four weeks after subculture, isolates were examined using phase-contrast light microscopy with tape preparations and lactophenoblue stain, performed according to the manufacturer's instructions (Hardy Diagnostics).

Molecular studies

DNA was extracted from frozen tissue of the tracheal lesion and fungal cultures with a commercial extraction kit (DNeasy® Blood and Tissue Kit, Qiagen) following the manufacturer's instructions for whole tissue. PCR amplification of the internal transcribed spacer 2 (ITS2) domain of the rRNA gene was undertaken using previously described methods (Sugita et al. 1999, Turenne et al. 1999). In addition, the D1/D2 region of the fungal 28S gene was also sequenced for *Veronaea botryosa* isolates (White et al. 1990).

Half of the PCR product (15 µl) was electrophoresed through a 1% agarose gel and then examined for bands. Samples with multiple bands were extracted from the gel using a QIAquick gel extraction kit (Qiagen), and samples with 1 band only were extracted using the remaining PCR product via the QIAquick PCR purification kit. The purified DNA was then submitted to the University of Florida Interdisciplinary Center for Biotechnology Research for bidirectional sequencing on ABI 3130 DNA sequencers (Applied Biosystems). Sequences were compared using the BLASTN algorithm to identify similarity to sequences from known fungal species in GenBank (National Center for Biotechnology Information, Bethesda, Maryland), European Molecular Biology Laboratory (EMBL-EBI, Cambridge, UK), Data Bank of Japan (Mishima, Shizuoka, Japan), and Centraalbureau voor Schimmelcultures (Fungal Biodiversity Centre, Utrecht, the Netherlands) (Altschul et al. 1990).

In addition to fungal PCR, the DNA extracted from the tracheal lesions was tested for the presence of herpesviruses using degenerate primers targeting

herpesviral DNA polymerase in a nested reaction, as previously described (VanDevanter et al. 1996). The PCR product was electrophoresed and sequenced as described above.

RESULTS

Clinical history and necropsy

Three green sea turtles were found alive but lethargic on the coast of Florida. The location of stranding, date of discovery, and size are shown in Table 1. Cases 1 and 2 had loud upper airway respiratory noise coupled with slow, labored inspiration and exhalation. Both animals died on the same day prior to clinical evaluation. Case 3 had severe fibropapillomatosis and multiple hematological and biochemical abnormities, including mild leukocytosis (16 900 μl^{-1}) characterized by a heterophilia (14 703 μl^{-1}) with toxic change, profound hypoglycemia (18 mg dl^{-1}), hypocalcemia (5.3 mg dl^{-1}), and elevated aspartate aminotransferase (818 IU l^{-1}). No additional clinical diagnostic evaluation was attempted due to its moribund state. Death occurred 3 d after stranding during attempted stabilization with parenteral fluids, calcium, glucose, and antibiotic therapy. This turtle had stranded 9 mo previously during a mass cold-stunning event in the Indian River Lagoon and was held in captivity for approximately 5 d at a temporary facility prior to release.

All 3 turtles were immature juvenile females based on SCL and gonadal examination. Upon necropsy, all 3 turtles had a multifocally ulcerated, firm, broad-based mass that almost completely occluded the trachea (Fig. 1A). The lesion was consistently located 2.0 to 3.0 cm caudal to the glottis. Case 1 was emaciated and had an acute, non-lethal injury involving the shoulder. Case 2 was in fair nutritional condition and also had a 1.0 cm (diameter) granuloma within the left lung. Case 3 was underweight and had large numbers of fibropapillomas arising from multiple areas, including the eyes.

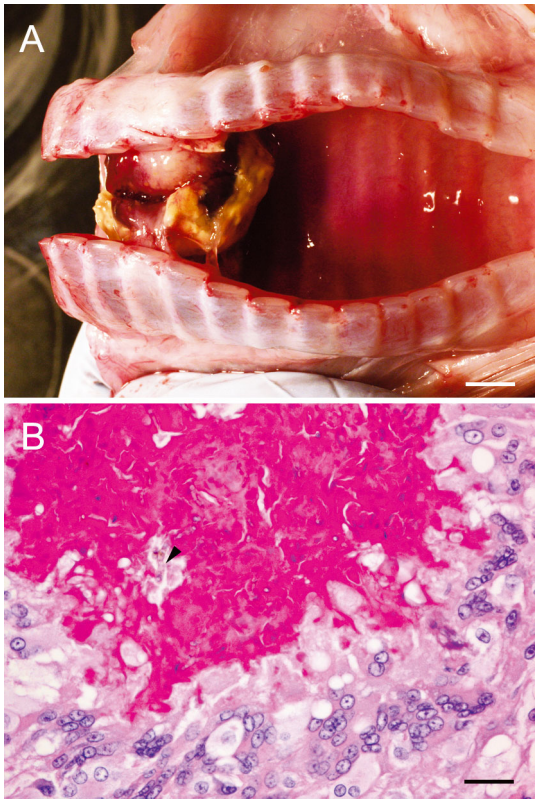

Fig. 1. Green turtle *Chelonia mydas* found stranded along the Indian River Lagoon, Florida, USA, on 3 December 2006. (A) The cranial trachea is obstructed by a locally extensive, ulcerated mass. Scale bar = approximately 0.4 cm. (B) The mass is composed of granulomatous inflammation characterized by central zones of necrotic cellular debris surrounded by multinucleated giant cells. Septate, branching, lightly pigmented fungal hyphae with irregularly parallel walls are seen within the center of the granuloma (arrowhead). Hematoxylin and eosin. Scale bar = 25 µm

All tracheal lesions were chronic and characterized by granulomatous inflammation, fibrous connective tissue, and heterophilic exudate that projected into and filled the tracheal lumen. Granulomas included central aggregates of eosinophilic cellular debris surrounded by multinucleated giant cells. Occasional lightly pigmented, septate fungal hyphae with irregularly parallel walls and dichotomous branching were present within the centers of the granulomas and were numerous within the surface exudate of case 3 (Fig. 1B). Fungal morphology was consistent among

Table 1. Summary of date and location of stranding within Florida, USA, and straight carapace length (SCL) for green sea turtles *Chelonia mydas* with granulomatous tracheitis. SCL was measured from the nuchal notch to the caudal tip of the supracaudal scute

Case	Date	Location	Coordinates	SCL (cm)
1	3 Dec 2006	Indian River Lagoon	27° 50.91′ N, 80° 27.24′ W	60.4
2	22 Mar 2010	Gulf of Mexico	26° 55.76′ N, 82° 21.88′ W	37.6
3	8 Oct 2010	Indian River Lagoon	28° 43.14′ N, 80° 44.60′ W[a]	42.6

[a]Coordinates are approximate and are based on a physical description of the stranding location

cases, and only pigmented fungi were observed within tracheal granulomas. Fibrovascular connective tissue surrounded the granulomas and was infiltrated by lymphocytes and granulocytes. The mucosa was ulcerated with various degrees of necrosis of the underlying submucosa and reactive connective tissue, and intense infiltration by heterophils. Bacteria also were present within the surface exudate. The pulmonary granuloma in case 2 contained non-pigmented, septate fungal hyphae. This lesion was not sequenced as it was beyond the scope of this study.

Fungal culture

Dematiaceous fungi were isolated from all 3 cases. Growth from case 1 was a single morphology, case 2 grew 4 morphologically distinct colonies, and case 3 produced 2 distinct types. A representative isolate genetically identified as *Veronaea botryosa* was forwarded to the University of Texas Health Science Center in San Antonio for morphological characterization and accessioned as UTHSCSA DI 14-348. Colonies were olivaceous and velvety, with a black reverse. Pale 2-celled, clavate conidia with rounded apices and truncate bases were produced from dark, geniculate conidiophores, consistent with *V. botryosa* (Fig. 2). The isolate has been deposited into the University of Alberta Microfungus Collection and Herbarium as [UAMH 11861].

Molecular studies

In all cases, PCR amplification of the fungal ITS2 from DNA extracted from the tracheal lesions yielded 284 to 286 nucleotide products of the expected size when primer sequences were edited out. The sequences (deposited into GenBank as accessions KJ641601–KJ64603) were all 100% identical to *V. botryosa* sequences (GenBank accession JX566723 for the 284 bp amplicons and KF772207 for the 286 bp amplicon). The sequence of an additional 240 bp product (GenBank accession KJ64604) from case 2 was 99% identical to a *Cladosporium* sp. (GenBank accession KF561894).

For cases 1 and 3, ITS2 and D1/D2 sequences obtained from fungal isolates were 100% identical to *V. botryosa* reference sequences (GenBank accessions KJ64609 and KP217187, respectively). A second isolate from case 3 produced a 286 nucleotide amplicon for ITS2 (GenBank accession KJ64610) that was 99% identical to *Cochliobolus* sp. (GenBank accession JX868697). None of the isolates from Case 2 were identified as *V. botryosa*. Isolates from this case yielded *Cladosporium* sp. ITS2 sequences homologous to those obtained from tissue (GenBank accession numbers KJ64605–KJ64607) and an additional ITS2 sequence (GenBank accession KJ64608) that was 94% identical to *Ochroconis* sp. (GenBank accession HQ667538) across 231 nucleotides.

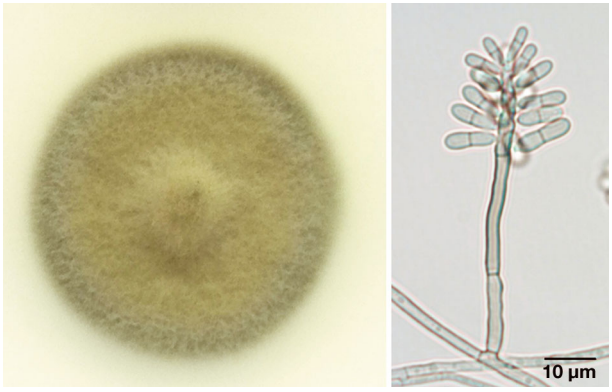

Fig. 2. *Veronaea botryosa* isolated from an intratracheal granuloma from a green turtle *Chelonia mydas*. The colony (left) is olivaceous and velvety with a black reverse. Pale 2-celled, clavate conidia with rounded apices and truncate bases arise from dark, geniculate conidiophores (right)

DISCUSSION

Veronaea botryosa was detected by PCR in all cases and by culture in 2 turtles. Although all of the dematiaceous fungi detected in this study may cause infection, *V. botryosa* was the only organism consistently found in all lesions. Our inability to isolate *V. botryosa* from one turtle may reflect overgrowth by *Cladosporium*, which grows readily and could have easily outcompeted other organisms (Revankar & Sutton 2010). In addition to mixed infection, there are multiple possible explanations for the additional isolates obtained by culture in 2 cases. Larger samples of the lesions are used for culture as compared to sampling for DNA extraction, thus there is the potential that isolates reflect fungi present in the contaminated surface zones. In addition, conditions of culture may allow selective overgrowth of fungi that are not necessarily reflective of relative abundance within the lesions. Last, pigmented fungi are common laboratory contaminants (Revankar & Sutton 2010). Although additional techniques, such as *in situ* hybridization, may be used to identify specific species

within histological lesions, the detection of dematiaceous fungi in all diagnostics performed (histology, culture, and PCR) is sufficient for diagnosis of phaeohyphomycosis, and *V. botryosa* was a consistent finding among all 3 cases. Medical reports of *V. botryosa* have largely been limited to humans, although Steckler et al. (2014) recently described systemic *V. botryosa* infection in hatchery-reared sturgeon.

The size of the affected turtles is typical of juvenile green turtles found in coastal waters of Florida (Ehrhart et al. 2007, Foley et al. 2007). Exposure to cold temperatures and concurrent disease may have contributed to infection given the time of year in which two of the turtles stranded, and a history of cold-stunning and severe fibropapillomatosis likely contributed in one case. Fungal pneumonia is a relatively common problem in cold-stunned Kemp's ridley sea turtles *Lepidochelys kempii* undergoing rehabilitation and is attributable to immunosuppressive effects of hypothermia (Manire et al. 2002, Innis et al. 2009, Williams et al. 2012). Similarly, fungal infections in sea turtles that strand in Florida tend to occur during or following winter months or are often concurrent with poor nutritional condition or other health problems (B. A. Stacy unpubl. data). Infections most frequently are caused by non-dematiaceous species and result in isolated pulmonary granulomas or more extensive pulmonary or disseminated infections. There was no evidence of underlying herpesvirus infection in the present cases based on histology or PCR, although prior viral tracheitis cannot be completely ruled out.

The similar tracheal lesions in the 3 cases of this report represent a distinct presentation encountered in stranded green turtles in Florida. The cause for localization of infection within the cranial trachea is unknown. If airflow turbulence was a contributing factor, the lesions are expected to occur at the carina, where turbulence is increased as the radius of the airways suddenly decreases (Johnson & Byrne 2003). Infection may occur where fungal spores first contact the airway, although nasal passages and the glottis seem equally susceptible. Unfortunately, the skulls were not sectioned to completely examine the olfactory sacs, although the discrete nature of the tracheal granuloma does not suggest a more widespread infection.

Another consideration is localized injury, given that phaeohyphomycosis is often attributed to inoculation. The affected region of the trachea is just caudal to the hyoid skeleton, and consequently is the first anatomically unprotected area. If this area was previously traumatized, then subsequent fungal exposure could result in localized infection. There was no evidence of trauma to provide further insight into any initiating injury, which is not necessarily unexpected given the chronicity of infection. In general, common injuries involving the neck region and trachea include penetrating foreign bodies originating from the esophagus and ligature wounds caused by entanglement in fishing line and other material.

Phaeohyphomycosis appears to be relatively uncommon in wild green turtles in the southeastern US, based on infrequent detection in stranded animals. Nonetheless, information on baseline or historical occurrence is essential to disease surveillance and monitoring of long-term trends. These cases contribute to greater ecological understanding of these fungi and to our collective knowledge of diseases of wild sea turtles.

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