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Concurrent Phaeohyphomycosis and *Ranavirus* Infection in an Eastern Box Turtle (*Terrapene carolina*) in Athens, Georgia, USA

David Perpiñán,[1,7] Uriel Blas-Machado,[2,3] Susan Sánchez,[4] and Debra L. Miller[5,6] [1]Naturavets Consultancy, 16 Firth Crescent, Auchendinny, EH26 0RA, Scotland, United Kingdom; [2]Athens Veterinary Diagnostic Laboratory, College of Veterinary Medicine, The University of Georgia, 501 D. W. Brooks Dr., Athens, Georgia 30602, USA; [3]Department of Pathology, College of Veterinary Medicine, The University of Georgia, 501 D. W. Brooks Dr., Athens, Georgia 30602, USA; [4]Department of Infectious Diseases, College of Veterinary Medicine, The University of Georgia, 501 D. W. Brooks Dr., Athens, Georgia 30602, USA; [5]Tifton Veterinary and Investigational Laboratory, PO Box 1389, 43 Brighton Rd., Tifton, Georgia 31793, USA; [6]Current address: Center for Wildlife Health and College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37996, USA; [7]Corresponding author (email: dperpinan@yahoo.es)

ABSTRACT: An eastern box turtle (*Terrapene carolina*) was found in a stream in the southeastern US, with a mass affecting the distal right forelimb. The turtle developed complications during hospitalization, including lethargy and oral caseous plaques and eventually died. Postmortem analyses diagnosed a mixed infection of phaeohyphomycosis and *Ranavirus*.

An adult male eastern box turtle (*Terrapene carolina*) was found in a shallow stream in Athens, Georgia, US (33°57′N, 83°22′W) in August 2009 with an ulcerated mass on the distal right forelimb. The turtle was transported to a veterinary hospital for diagnostic testing and therapeutic procedures. On physical examination the turtle was quiet but alert and responsive with a good mass (398.3 g). There was a red, round, ulcerated, 21×16-mm soft tissue mass on the distal right forelimb that extended from the dorsal to the palmar surface between toes 3 and 4 (Fig. 1A). An additional 12×9-mm subcutaneous mass was located on the caudal aspect of the right tibiotarsal joint. The remainder of the physical examination and diagnostic tests (radiographs, hematology, and biochemistry) were unremarkable. Fine needle aspirates were nondiagnostic due to blood contamination. Surgery to remove both masses was performed 5 d after admission; however, the patient deteriorated after surgery, with progressive lethargy and the development (9 d after admission) of malodorous, caseous plaques covering the oral palate, which eventually led to euthanasia 12 d after admission.

Necropsy was performed immediately after death, and significant gross findings were limited to the oral cavity. There were scattered foci of erosion and hyperemia at the commissure of the mouth and the rostral half of the tongue. The base of the tongue and palatine mucosa were covered with a light tan, 1- to 2-mm-thick layer of cobblestonelike caseous material mixed with mucus and debris (Fig. 1B). Representative tissues from masses removed at surgery and from tissues collected during necropsy were fixed in neutral buffered 10% formalin, processed routinely, sectioned at 4 μm, and stained with H&E.

Microscopically, the lesions seen in the biopsy and in the tissues collected at necropsy were similar. The dermis contained nodular collections of epithelioid macrophages and scattered heterophils with widely spaced, 60-μm-diameter clusters of pigmented, pleomorphic fungal hyphae. Individual hyphae were branched, septated, and 1–6 μm across (Fig. 2A). In the oral cavity, the mucosal surfaces were sloughed and covered with a thick layer of hypereosinophilic granular material, mixed with fibrin, necrotic cellular debris, bacteria, and occasional fungal hyphae, which predominantly occurred as single pigmented, septated fungal hyphae, 4–5 μm across (Fig. 2B). The lung had a single 600-μm-diameter granuloma, which consisted of a collection of multinucleated giant cells centered about a cluster of filamentous pigmented fungi rimmed by a layer of fibroblasts (Fig. 2C). Individual hyphae were similar to those described previously. The histologic diagnosis

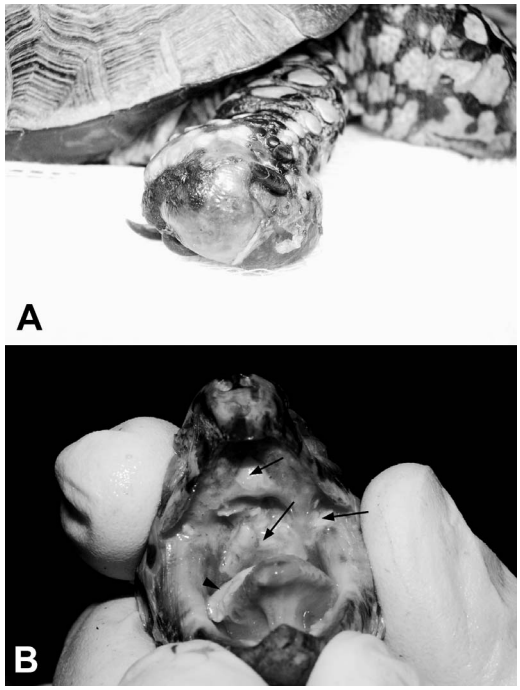

FIGURE 1. Clinical signs in a box turtle (*Terrapene carolina*) diagnosed with *Ranavirus* infection and phaeohyphomycosis in Athens, Georgia, USA. (A) Marked swelling on the forelimb. (B) Caseous material covering the palatine mucosa (arrows) and the base of the tongue (arrowhead).

was caseous stomatitis with cutaneous and pulmonary phaeohyphomycosis.

Ancillary tests were performed to identify etiologic agents. Culture of fresh tissues failed to produce growth of pigmented fungal isolates consistent with histopathology from the lungs, oral lesions, or skin but resulted in heavy growth of *Candida glabrata* and *Candida albicans* from the lungs and oral lesions, respectively. Tissue samples after homogenization were plated on Mycosel, Sabouraud dextrose agar (SDA), and SDA-chloramphenicol (BBL prepared plates, BD Diagnostics, Franklin Lakes, New Jersey, USA) and incubated at room temperature. Culture plates were held for 30 d prior to cultures determined to be negative for filamentous fungi. Yeast growth was further characterized by using API 20C AUX (bioMérieux, Durham, North Carolina, USA). Later attempts to determine the fungal agents involved in the

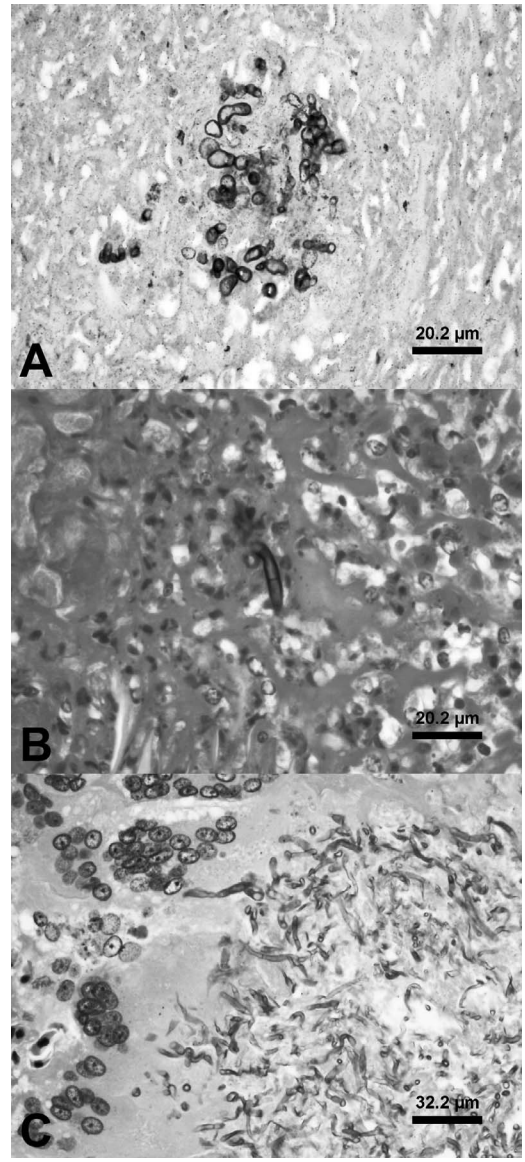

FIGURE 2. Clinical pathology in a box turtle (*Terrapene carolina*) from Athens, Georgia, USA, with *Ranavirus* infection and phaeohyphomycosis. Histologic images show pigmented, pleomorphic, fungal hyphae from (A) skin, (B) oral cavity, and (C) lung. H&E stain. Scale: ×400 original magnification.

lesions were performed by using two sets of panfungal primers in the internal transcribed spacer region covering approximately 550 and 280 base pairs (bp) and the D2/D2 region (Kwiatkowski et al. 2012). Amplification was successful, but sequencing failed multiple

times (Seqwright, Houston, Texas, USA; Ferrer et al. 2001). Additionally, conventional PCR was performed targeting an approximately 50-bp region of the major capsid protein gene for *Ranavirus*. The DNA was extracted from paraffin-embedded tissues by using a QIAamp DNA Mini Kit (Qiagen, Valencia, California, USA). The PCR was then performed on the DNA by using the MCP 4 and MCP 5 primers reported by Mao et al. (1997). Conventional PCR for *Ranavirus* was positive on liver, kidney, and oral cavity. The PCR product was sequenced (Seqwright) and was 99% identical to frog virus 3.

Ranavirus infection has been reported in free-ranging and captive chelonians over the last two decades (Marschang et al. 1999; Johnson et al. 2007). More specifically, *Ranavirus* has been associated with significant mortality events in free-ranging box turtles from several states in the eastern US (Johnson et al. 2007). Phaeohyphomycosis is an infection caused by pigmented fungi with dark-walled, septated hyphae, usually involving genera, such as *Alternaria*, *Exophiala*, *Phialophora*, and others (Hargis and Ginn 2007). These fungi are plant pathogens, soil saprophytes, or, in some instances, normal flora that enters the skin at sites of trauma (Hargis and Ginn 2007; Revankar 2007). There has been a report of phaeohyphomycosis in a box turtle also producing swelling of a limb (Joyner et al. 2006).

In our case, it is likely that the turtle was infected with *Ranavirus* previous to day 0, as no contact (direct or indirect) happened between this turtle and any other lower vertebrate during hospitalization. Intensive treatment (including anesthesia, surgery, and daily handling) likely accelerated the development of clinical signs typical of *Ranavirus* infection, such as generalized illness and the development of oral plaques (Johnson et al. 2008).

The subcutaneous lesions of phaeohyphomycosis may have been the result of localized trauma and invasion by saprophytic pigmented fungi. However, the observation of pigmented fungi in the lungs and the isolation of two species of *Candida* in the lung and mouth were indicative of secondary infections. Pigmented fungi in animals and humans can be found in internal organs when there is immune suppression (Hargis and Ginn 2007). The immune-suppressing effect of *Ranavirus* in turtles has been previously suggested as bacterial and fungal infections are commonly associated with chelonians infected by the virus (De Voe et al. 2004), and differences have been seen in clinical signs and pathologic lesions between animals infected with the same virus (De Voe et al. 2004; Johnson et al. 2007).

We described the association of *Ranavirus* and phaeohyphomycosis in a free-ranging box turtle. The significant worsening of the turtle's health after intensive treatment indicates that stress may facilitate or accelerate the development of severe clinical signs. Veterinarians should remember to collect and maintain frozen tissues until histopathology has been reviewed to optimize the identification of etiologic agents.

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