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NOTE

First case of ranavirus and associated morbidity and mortality in an eastern mud turtle *Kinosternon subrubrum* in South Carolina

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ABSTRACT: Ranaviruses are double-stranded DNA viruses that infect amphibians, fish, and reptiles, causing global epidemics in some amphibian populations. It is important to identify new species that may be susceptible to the disease, particularly if they reside in the same habitat as other at-risk species. On the Savannah River Site (SRS) in Aiken, South Carolina, USA, ranaviruses are present in several amphibian populations, but information is lacking on the presence, prevalence, and morbidity of the virus in reptile species. An eastern mud turtle *Kinosternon subrubrum* captured on the SRS in April 2014 exhibited clinical signs of a ranaviral infection, including oral plaque and conjunctivitis. Quantitative PCR analyses of DNA from liver tissue, ocular, oral, nasal, and cloacal swabs were all positive for ranavirus, and sequencing of the template confirmed infection with a FV3-like ranavirus. Histopathologic examination of postmortem tissue samples revealed ulceration of the oral and tracheal mucosa, intracytoplasmic epithelial inclusions in the oral mucosa and tongue sections, individualized and clusters of melanomacrophages in the liver, and bacterial rods located in the liver, kidney, heart, stomach, and small intestine. This is the first report of morbidity and mortality of a mud turtle with a systemic ranaviral infection.

KEY WORDS: Chelonian · Amphibian · Frog virus 3 · FV3 · Iridovirus · Systemic infection · Zoonosis · Savannah River Site

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INTRODUCTION

Amphibian and reptile populations are in the midst of a sixth mass extinction, threatening global biodiversity (Daszak et al. 1999, Gibbons et al. 2000). Many factors have been attributed to this massive decline, including emerging infectious diseases (Green et al. 2002, Collins & Storfer 2003, Stuart et al. 2004). While much research has focused on understanding the effects of ranaviruses on amphibian populations (Brunner et al. 2007, Blaustein et al. 2012, Haislip et al. 2012), few studies have focused on reptilian populations (Sutherland et al. 2014). Viruses in the genus *Ranavirus* (family *Iridoviridae*) have the ability to infect fish, amphibians, and reptiles (Brenes et al. 2014). Amphibian mortality and morbidity events have been attributed to these pathogens across 5 continents (Speare & Smith 1992, Green et al. 2002, Fox et al. 2006, Ariel et al. 2009, Geng et al. 2011), but less is understood about their impacts on reptilian populations, particularly chelonians (Allender et al. 2006).

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Eastern box turtles *Terrapene carolina* are among the most commonly reported turtle species associated with ranaviral die-off events (De Voe et al. 2004, Allender et al. 2006, 2011, Currylow et al. 2014). However, surveillance and experimental challenge studies have demonstrated that semi-aquatic turtles, such as red-eared sliders *Trachemys scripta elegans*, can also become infected with ranaviruses (Allender et al. 2013, Goodman et al. 2013). The eastern mud turtle *Kinosternon subrubrum* is a lentic wetland, bottom-dwelling species endemic to the southeastern USA that uses terrestrial ecosystems for dispersal and aestivation (Gibbons 1983, Buhlmann & Gibbons 2001). Rare, cryptic, or secretive host species often have poorly understood life histories and small population sizes (Cecala et al. 2013). Eastern mud turtles reside in similar wetland habitats as species of amphibians that are susceptible to ranaviruses, such as pond-breeding salamander species, American bullfrogs *Lithobates catesbeiana* (Hoverman et al. 2012), and green frogs *Lithobates clamitans* (Gray et al. 2007), which are known carriers of ranaviruses. It is important to understand the potential impact of ranaviruses on the entire wetland community, including new host species or potential reservoirs for the virus.

MATERIALS AND METHODS

Background

The Savannah River Site (SRS) is an 80 000 ha Department of Energy installation located in west-central South Carolina. Established in 1951, the SRS encompasses a variety of contaminated and reference (i.e. uncontaminated) wetland ecosystems including a network of reference wetlands known as Risher Pond sloughs. These heavily vegetated, temporary wetlands within the Savannah River floodplain are often inundated with water from nearby streams during years of heavy rainfall (Willson et al. 2005). Association with nearby streams creates the opportunity for amphibians, fish, crayfish, aquatic snakes, turtles, and a variety of other species to colonize and inhabit these wetlands.

On 12 April 2014, during a herpetology class field trip, we opportunistically hand-captured an eastern mud turtle that appeared to have clinical signs of a ranaviral infection. It was found resting along the water's edge of one of the Risher sloughs. Mud turtles are common residents in this area, along with spotted turtles *Clemmys guttata* and common snapping turtles *Chelydra serpentina*. Periodic aquatic trapping in the Risher Slough system (primarily between 2002 and 2007) yielded >150 mud turtle captures, but none exhibited clinical symptoms of disease or infection. We kept the individual in captivity for 17 d due to its pronounced lethargy, observable oral plaque (Fig. 1), conjunctivitis and mucus discharge from both eyes (Fig. 2) and cloaca. On 25 April, we took oral, cloacal, ocular, and nasal swabs and stored them in a −20°C freezer. The turtle died during the evening of 28 April or early the following morning and the necropsy was performed on 29 April.

Molecular methods

We extracted DNA from the swabs and liver tissue using a DNeasy Blood and Tissue Kit (Qiagen) fol-

Fig. 1. *Kinosternon subrubrum* with oral plaque and ulcerations located on the dorsal plate of the oral cavity due to ranavirual infection

Fig. 2. Conjunctivitis in the *Kinosternon subrubrum* individual found with clinical signs of a ranaviral infection, with severe ocular discharge and a thick crust around both eyes

lowing the manufacturer's instructions. We used a quantitative PCR TaqMan assay (Taqman® primers, FAM dye labeled, Applied Biosystems) following the methods of Allender et al. (2013) and using an iCycler (Bio-Rad Laboratories). We ran each sample 4 times on the same plate and included a serial dilution of ranavirus standard from 10^6 to 10^1 viral copies μl^{-1}. The standard was frog virus 3 (FV3) isolate ID#061405 from leopard frog tissue. To confirm viral identity using sequencing, we then ran a second PCR with extracted DNA using primers 4 and 5 from Mao et al. (1997), purified the PCR products with Exonuclease I and Shrimp Alkaline Phosphatase enzymes (New England Biolabs) and bidirectionally sequenced them using BigDye v.3.1 (Life Technologies) and following manufacturers protocols. We ran the sequencing reactions on an ABI 3130xl (Life Technologies) and assembled, aligned, and edited the sequences using the program Sequencher v.5.2.4 (Gene Codes Corporation). No tests for other pathogens were performed.

Histopathology

We performed a postmortem necropsy to collect samples including major viscera (liver, testes, kidneys, spleen, stomach, intestines, lung, and trachea), eyes, and brain. Following collection, we fixed samples in 10% buffered neutral formalin and submitted them to the University of Georgia's College of Veterinary Medicine Infectious Disease Laboratory, where they were dehydrated in graded alcohols and embedded in paraffin wax. Cassettes of paraffin-embedded sections were prepared and stained with hematoxylin and eosin (H&E) stains.

RESULTS

Molecular

Based on quantitative PCR (qPCR) results, all samples (oral, ocular, nasal, liver, and cloacal) were definitively positive for FV3-like ranavirus. The viral loads per nanogram of extracted DNA were highest in the oral (82 828 ± 12 094) and ocular swabs (73 071 ± 5893) and lowest in the cloacal swabs (5389 ± 303). Our sequence analysis resulted in a 510 bp sequence (Genbank Accession #KM114262) that, based on the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool, most closely matched the *Ranavirus* type species FV3. The 510 bp fragment we sequenced corresponded to bases 56 to 565 of the FV3 complete major capsid protein coding sequence (Accession #FJ459783) and differed by 3 base pairs. These nucleotide differences result in 2 synonymous changes (G to C at position 150, and C to T at position 354) and one non-synonymous change (C to A, Leu to Met, at position 400) to the capsid domain sequence.

Histopathology

The renal tubules in the kidney were lined by degenerate epithelial cells with vacuolation and necrosis. Intravascular bacterial rods were also present in examined liver, kidney, heart, vessels in the mesentery attached to the small intestine, and spleen sections. Sections of the trachea contained complete ulceration of the mucosa covered with intraluminal necrotic cellular debris and bacteria. The adjacent esophagus was deeply ulcerated and covered by caseous exudate. Oral mucosa and tongue sections had multifocal necrotic to ulcerated foci with hemorrhage, basal necrosis, and degeneration within the lamina propria with multifocal superficial bacterial basophilic inclusion bodies. Several epithelial cells in and around these foci contained small round intracytoplasmic basophilic inclusion bodies. Within the conjunctiva, there were locally extensive areas of ulceration and necrosis covered with septic caseous exudate (Fig. 3). There was also intraluminal caseous exudate and intravascular bacteria within the nasal cavity.

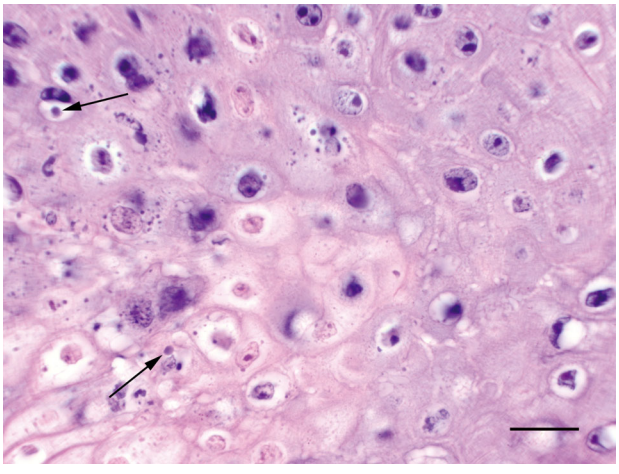

Fig. 3. Photomicrograph of inclusions (arrows) located in the oral tissue of the *Kinosternon subrubrum* individual exhibiting clinical signs of a ranaviral infection. Scale bar = 20 µm

DISCUSSION

Ranavirues are a group of emerging pathogens that affect chelonian populations throughout the USA (Johnson et al. 2008). Ranaviruses, specifically FV3 or FV3-like viruses, have been associated with mortality events in species such as the leopard tortoise *Geochelone pardalis pardalis* (Benetka et al. 2007), eastern box turtle (De Voe et al. 2004), and soft-shelled turtle *Trionyx sinesis* (Huang et al. 2009, Chinchar & Waltzek 2014) in captivity. To our knowledge, this is the first case of morbidity and mortality and subsequent detection of FV3-like ranavirus from a wild eastern mud turtle.

Clinical, histological, and molecular results were all consistent with a FV3-like ranavirus infection contributing to the morbidity and mortality of this mud turtle. The turtle displayed severe clinical signs and symptoms of a ranaviral infection upon capture, with noted progression of infection while being held in captivity. Symptoms included pronounced weakness and lethargy in combination with clinical signs including swollen eyes, discharge from the nose and cloaca, and presence of white and yellow plaque on the palate and tongue. Histological analyses detected lesions in almost every tissue examined, with intracytoplasmic inclusions in the oral cavity (Fig. 3) and tongue epithelium that have been recorded in previous ranaviral cases (De Voe et al. 2004). Finally, the detection of FV3-like ranavirus from systemic lesions and liver samples also supports the diagnosis of ranaviral infection as a contributing causal factor. The mud turtle may have been compromised due to a secondary bacterial infection, causing acute septicemia and contributing to the mortality of the animal. Further investigation by culturing would be necessary to determine the cause of sepsis recorded in this turtle and to identify the associated enterobacteria.

Amphibians in Risher sloughs (the location of capture) were tested for ranaviruses in 2011 and 2012; at that time, 17 of 63 individuals tested positive (27% prevalence) for the disease (authors' unpubl. data). However, no tests have been conducted in this area since 2012, thus the current presence and prevalence of ranaviruses in this amphibian population is unknown. Although the mud turtle in this case study is the first turtle from the SRS tested for ranavirus, we have not previously observed clinical signs in any turtle species in any wetland system on the SRS.

The results reported in this note expand the list of turtle species susceptible to ranaviruses and highlight the need to investigate cryptic and secretive species when studying disease dynamics. Species such as mud turtles may be exposed to ranaviral infections at an increased rate due to their life history strategies. Eastern mud turtles often live in lentic wetland habitats that serve as breeding areas for amphibians. In addition, eastern mud turtles will scavenge dead fish and other types of carrion, potentially increasing their exposure to ranaviral infections (Buhlmann et al. 2008). Future studies should survey a wide array of potential hosts to better inform conservation efforts.

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