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PCR Prevalence of *Ranavirus* in Free-Ranging Eastern Box Turtles (*Terrapene carolina carolina*) at Rehabilitation Centers in Three Southeastern US States

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ABSTRACT: Ranaviruses (genus *Ranavirus*) have been observed in disease epidemics and mass mortality events in free-ranging amphibian, turtle, and tortoise populations worldwide. Infection is highly fatal in turtles, and the potential impact on endangered populations could be devastating. Our objectives were to determine the prevalence of ranavirus DNA in blood and oral swabs, report associated clinical signs of infection, and determine spatial distribution of infected turtles. Blood and oral swabs were taken from 140 eastern box turtles (*Terrapene carolina carolina*) that were presented to the wildlife centers at the University of Tennessee (UT; $n=39$), Wildlife Center of Virginia (WCV; $n=34$), and North Carolina State University (NCSU; $n=36$), as well as a free-ranging nonrehabilitation population near Oak Ridge, Tennessee (OR; $n=39$) March–November 2007. Samples were evaluated for ranavirus infection using polymerase chain reaction (PCR) targeting a conserved portion of the major capsid protein. Two turtles, one from UT and one from NCSU, had evidence of ranavirus infection; sequences of PCR products were 100% homologous to Frog Virus 3. Prevalence of ranavirus DNA in blood was 3, 0, 3, and 0% for UT, WCV, NCSU, and OR, respectively. Prevalence in oral swab samples was 3, 0, and 0% for UT, WCV, and NCSU, respectively. Wildlife centers may be useful in detection of *Ranavirus* infection and may serve as a useful early monitoring point for regional disease outbreaks.

Key words: Eastern box turtle, PCR, *Ranavirus*, *Terrapene carolina*.

The eastern box turtle (*Terrapene carolina carolina*) is listed as an Appendix II species by the Convention on the International Trade of Endangered Species, near threatened by the International Union for Conservation of Nature red list (IUCN, 2009), and is threatened or a species of concern in several US states (Swarth and Hagood, 2004). Recently, significant declines have been observed in numerous areas throughout its range (Swarth and Hagood, 2004). Causes for the decline have been attributed to human-induced factors including road and mowing mortality, collection, nest depredation, prescribed burning, disturbance of nest sites by off-road vehicles, and habitat loss (Swarth and Hagood, 2004). While a combination of factors is likely involved in the population declines, disease outbreaks attributed to ranavirus infection have been emerging in chelonians across the eastern United States (DeVoe et al., 2004; Allender et al., 2006; Johnson et al., 2008).

Our aim was to determine the prevalence of ranavirus infection in populations of eastern box turtles in the southeastern United States using polymerase chain reaction (PCR). Our hypotheses were 1) molecular and viral isolation evidence of ranavirus infection exists in turtles presented to rehabilitation centers in the southeastern United States; 2) prevalence of ranavirus DNA in turtles (as measured by PCR) will be greater than 0; and 3) there will be no difference in the ability to detect molecular evidence of ranavirus infection between oral swabs and blood. We also aimed to determine the spatial distribution of ranavirus-positive turtles.

TABLE 1. Population parameters of eastern box turtles (*Terrapene carolina carolina*) presented to three wildlife medical facilities in the eastern United States, 2007.

	N	Adult	Juvenile	Unknown age
University of Tennessee		35		
Total	39		4	0
Female	14	14	0	0
Male	16	16	0	0
Unknown sex	9	0	0	9
Wildlife Center of Virginia		19	3	12
Total	34			
Female	7	4	3	0
Male	15	15	0	0
Unknown sex	12	0	0	12
North Carolina State University		33	2	1
Total	36			
Female	21	21	0	0
Male	11	11	0	0
Unknown sex	4	0	4	0
Oak Ridge		35	4	0
Total	39			
Female	10	10	0	0
Male	17	17	0	0
Unknown sex	12	0	12	0

Eastern box turtles were sampled March–November 2007. There were 39, 34, and 36 box turtles presented to wildlife centers at the University of Tennessee (UT), Wildlife Center of Virginia (WCV), and North Carolina State University (NCSU), respectively. We sampled 39 turtles from a free-ranging nonrehabilitation population near Oak Ridge, Tennessee (OR). Population parameters are shown in Table 1. Some turtles did not show clear secondary sex characteristics and were recorded as unknown sex. These were not included in the analysis between sexes.

On initial examination at participating institutions, blood samples, collected from the subcarapacial sinus during the initial examination at participating institutions, were placed in microtainers coated with lithium and heparin (Becton Dickinson, Franklin Lakes, New Jersey, USA). Oral swabs using wooden-handled cotton-tipped applicators were collected in duplicate. All samples were stored at −20 C until shipping or analysis.

Blood samples from 29, 34, 36, and 39 turtles were collected at the UT, WCV, NCSU, and OR study sites, respectively, and oral swabs from 30, 34, and 36 turtles were collected from UT, WCV, and NCSU, respectively. No oral swabs were collected from the OR population. We extracted DNA from swabs and whole blood using the DNA mini kit following the manufacturer's protocols (Qiagen, Valencia, California, USA). Sense (5′-GACTTGGCCACTTATGAC-3′) and antisense primers (5′-GTCTCTGGAGAAGAAGAA-3′) were used as described (Mao et al., 1997) to amplify approximately 500 base pairs of a highly conserved portion of the *Ranavirus* major capsid protein gene. Products were sequenced in both directions directly at the Molecular Biology Resource Facility at UT and compared to known sequences in GenBank using the Basic Local Alignment Search Tool (TBLASTX).

The prevalence of ranavirus infection was determined for turtles from each

institution based on the PCR results. Exact binomial confidence intervals were determined for all proportions. Age category, sex, habitat, and location-specific prevalences were computed and compared using Pearson's chi-square and Fisher's exact test. Agreement between PCR and virus isolation were compared using kappa statistic.

Two turtles, one from UT and one from NCSU, were ranavirus PCR-positive. Prevalences (95% CI) of ranavirus DNA in blood were 3% (0.2–19.6%), 0% (0–8.8%), 3% (0.1–16.2%), and 0% (0–11.1%) for turtles presented to UT, WCV, NCSU, and OR, respectively. Prevalences of ranavirus DNA in oral swab samples were 3% (0.2–19.0%), 0% (0–8.8%), and 0% (0–8.3%) for turtles presented to UT, WCV, and NCSU, respectively. The same turtle from UT was PCR positive in both blood and swab. Sequence analysis of all three PCR-positive samples demonstrated 100% homology to Frog Virus 3.

We attempted viral isolation on 29 blood samples and 29 oral swabs (all UT rehab turtles). Cell cultures (Terrapene Heart, TH-1; ATCC, Rockville, Maryland, USA) were maintained at 30 C with Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 IU/ml penicillin, 100 g/ml streptomycin, and amphotericin (0.025 μg/ml) in 25 cm^2 flasks. Cytopathic effect (CPE) was observed in both the blood and oral swab inoculated cell cultures from the PCR-positive UT turtle; no CPE was seen in any of the 28 PCR-negative samples (kappa=1). The PCR on the isolate was positive and the sequence was 100% homologous to Frog Virus 3.

Clinical signs were evaluated by the admitting clinician and recorded on a standard data sheet. The ranavirus-positive turtle was a male with conjunctivitis, nasal discharge, ocular discharge, oral plaques, and respiratory distress. No clinical sign was significantly associated with ranavirus status ($P>0.05$).

TABLE 2. Distribution of turtles (*Terrapene carolina carolina*) by county and ranavirus polymerase chain reaction assay result presented to the University of Tennessee and a free-ranging, nonrehabilitation population near Oak Ridge, Tennessee, USA, in 2007.

County	Total *N*	Positive	Negative
Campbell	1	0	1
Roane	1	0	1
Anderson	37[a]	0	37
Blount	2	0	2
Sevier	3	0	3
Loudon	5	0	3
Knox	22	1	21
Sullivan	2	0	2

[a] Includes 36 animals in the Oak Ridge population.

We mapped a subset of the population (36 UT, 37 OR) including the ranavirus-positive turtle from UT. Locations were recorded from capture information directly attained or provided by the individual who found the turtle and then global positioning system (GPS) coordinates were obtained through commercial software (Google Earth© Mountain View, California, USA) or a handheld unit (eTrex Vista, Garmin International, Olathe, Kansas, USA). The GPS coordinates of turtle locations were mapped using ArcGIS (ArcGIS 9.3.1, ESRI, Redlands, California, USA). The distribution of turtles by county and disease status is presented in Table 2. Spatial relationship of the ranavirus-positive turtle to negative turtles is shown in Figure 1.

Box turtles are often presented to rehabilitation centers in significant numbers because of their habitat overlap in urban and suburban landscapes, and have, for that reason, been proposed as sentinels for emerging pathogens (Brown and Sleeman, 2002; Schrader et al., 2010). The traditional role of these centers has been to triage and release animals into suitable habitat, ideally close to the capture location. However, with an increased ability to survey for emerging and zoonotic diseases, wildlife species presented to these centers have been proposed as biomonitors of ecosystem health (Slee-

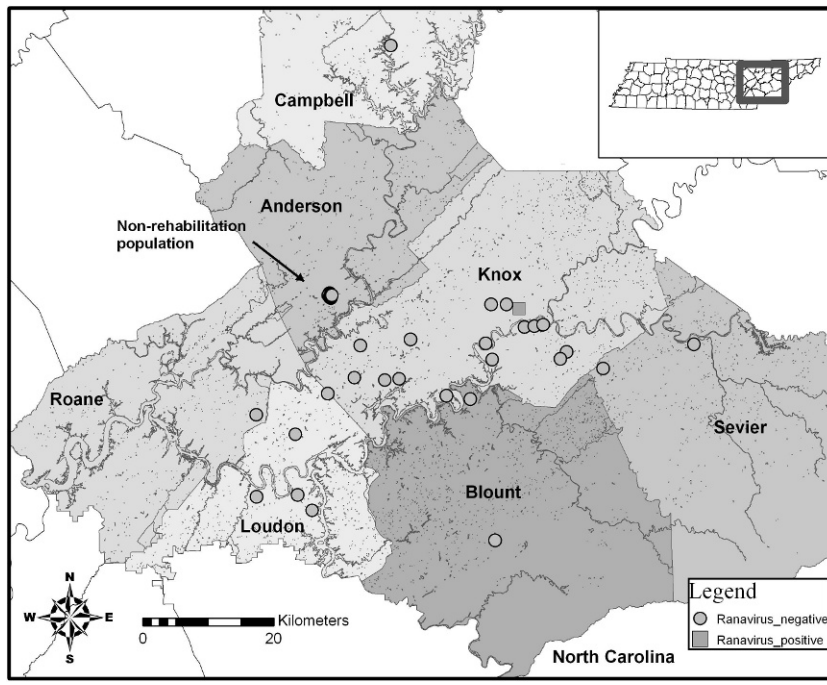

FIGURE 1. Spatial distribution of one ranavirus-positive (by polymerase chain reaction and virus isolation) and 73 ranavirus-negative eastern box turtles (*Terrapene carolina carolina*) in east Tennessee, USA. All turtles are University of Tennessee rehabilitation turtles except for the cluster of points ($n=22$) labeled nonrehabilitation (Oak Ridge). Tennessee county names are provided.

man, 2008). It is often suggested that these centers are biased toward diagnosing sick and injured animals, but this has not been definitively studied for many diseases. However, the usefulness of these centers in monitoring emerging disease lies in this bias. Diseases, such as those caused by ranavirus infection in turtles, that have variable prevalence and rapid progression to mortality may be underdiagnosed during routine monitoring of free-ranging populations. Therefore, the utility of these centers is paramount.

Prevalence of ranavirus infection in turtles presented to the three wildlife centers was low. This is not surprising, as ranaviral disease has been shown to have high mortality with quick progression to death (Johnson et al., 2007). Ranavirus disease outbreaks in amphibians typically have disease courses that run 5–50 days with up to 90% mortality (Green et al., 2002). Similar outbreaks have been poorly described for free-ranging chelonians. Furthermore, few studies have evaluated the background prevalence of ranavirus infections in chelonians outside the context of disease outbreaks, and when performed, they have shown no evidence of infection (Allender et al., 2010). If this pattern is repeated, management of disease outbreaks relies on early diagnosis.

When evaluating the natural home range buffers (8–40 ha), it is apparent that the home ranges of the ranavirus-positive and -negative turtles overlaps, thereby potentially facilitating natural transmission. Turtles show strong propensities to return to their natural home range when displaced and the home range of displaced turtles may be up to 7.5 times larger than nondisplaced turtles (Hester et al., 2008). In cases in which the natural home range is larger, more overlap occurs; there were seven turtles with overlapping home ranges with an 8-ha buffer, eight turtles

with a 100-ha home range, and 24 turtles with a displaced home range of 2.5 km. It is not uncommon for rehabilitated animals to be returned to a site some distance from their capture location due to subjective concerns of rehabilitators. This argues for release of these turtles as close to the site of capture as possible. This is further emphasized in the control population, which demonstrated a high turtle population density.

Limitations of this study included the biased sample population and lack of control population near each rehabilitation center. Much of the disease ecology is unknown in chelonians, so it may be possible that infections occurred in the wild and led to mortality that was undiagnosed or the animals recovered from infection prior to admittance to the rehabilitation center. The PCR assay only detects viral DNA that is present in the sample; samples collected may have been inappropriate to diagnose infection or the animal had cleared infection prior to sampling. We did not determine previous exposure, but this has been shown to be low, indicating that it is unlikely that we missed a great number of infections (Johnson et al., 2008). Few investigators have studied whole blood as a means of detecting ranaviruses, but one of us (M.C.A.) has had good success in detecting ranavirus infection in other cases of eastern box turtles using this sample.

Recently, ranaviral disease was placed on the World Organization for Animal Health list of reportable diseases for amphibians. Infection in chelonians, and specifically box turtles, in the United States may be a significant threat to populations (DeVoe et al., 2004; Allender et al., 2006; Johnson et al., 2008). To aid in disease management, more studies on the disease ecology are needed, specifically those that identify potential reservoirs, transmission, the effect of environmental factors on disease presentation in chelonians, and the spatial mapping of outbreaks. Wildlife centers can help monitor regional disease manifestation. Coordination with biologists and wildlife veterinarians is necessary to perform these investigations and to utilize these rehabilitation centers as early monitoring checkpoints for emerging diseases.

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