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Searching for *Atadenovirus* in Snakes in New Jersey, USA

Adenoviruses are double-stranded DNA viruses that infect mammals, birds, amphibians, and reptiles (Harrach et al. 2019). The *Atadenovirus* clade infects a diverse group of squamates (Ball et al. 2014; Prado-Irwin et al. 2018; Benge et al. 2019). *Atadenovirus* infection prevalence in wild reptile populations is estimated to range 10–30% (Ball et al. 2014); however, this rate seems to be highly dependent on species (Szirovicza et al. 2016; Benge et al. 2019). While *Atadenovirus* studies have focused on diverse genera globally, no studies of *Atadenovirus* occurrence have been conducted in the northeastern United States. Our aim was to assess *Atadenovirus* prevalence in wild snakes in New Jersey, USA, with a particular focus on the US-threatened Northern Pinesnake, *Pituophis melanoleucus melanoleucus*. This species is an ideal organism to test for the presence of *Atadenovirus* because it shares overwintering hibernacula with other snake species and it is susceptible to ophidiomycosis (Burger et al. 1988; Burger and Zappalorti et al. 2011; Lorch et al. 2016; Allender et al. 2020). Squamate *Atadenovirus* infections may be identified through many methods, including the minimally invasive cloacal swab (Szirovicza et al. 2016). We conducted cloacal swabbing with both an internal and external swab on each specimen to compare resulting DNA concentrations. We also tested whether these methods were successful by using a PCR experiment to verify the presence of host DNA. Lastly, we estimated *Atadenovirus* prevalence in wild New Jersey snakes, with a particular focus on the Northern Pine Snake.

Cloacal swabs were collected in the New Jersey Pine Barrens from 14 wild adult Northern Pine Snakes (Fig. 1). Additionally, we collected two adult Eastern Hog-nosed Snakes (*Heterodon platirhinos*), and one adult Northern Black Racer (*Coluber constrictor constrictor*). These snakes were collected during March 2022, at the end of hibernation. Collection of the samples from snakes was approved by the Rutgers University Animal Care and Use Committee (permit #E6-017 to J. Burger), the New Jersey Department of Environmental Protection (Endangered and Nongame Species Program), the New Jersey Division of Parks and Forestry, and with permission from private landowners. Specific locations of the dens are omitted to protect the locations from poachers and unauthorized access. Snakes were returned to their hibernation dens within a few hours.

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Two swabs were collected in the field from each snake, one taken by inserting the swab inside the cloaca, and a second taken by brushing the swab against the external surface of the cloaca. Following collection, all swabs were stored frozen at -4°C until laboratory analyses were conducted.

DNA extractions from swab samples were performed using the Qiagen DNEasy Blood and Tissue Kit (Qiagen, Hilden, Germany) via a modified protocol adapted to extract the maximum amount of DNA (Adamowicz et al. 2014). Swabs were incubated at 56°C while being shaken at 900 RPM in a VorTemp 56 shaking incubator (LabNet, New Jersey, US). The 60-min incubation time was divided into three segments of 20 min. At the end of each 20-minute segment, each swab was placed into a filter column and spun at 15,000 rpm in the Eppendorf centrifuge (Eppendorf, Hamburg, Germany) and the collected eluate was pipetted back into the original sample tube. This process helps the DNA become mechanically dislodged from the cotton swab and allows for the capture and retention of the maximum amount of DNA (Adamowicz et al. 2014). Swabs were transferred to and from the filter columns using sterilized forceps to limit the possibility of contamination. The manufacturer's protocol was followed for the remaining steps of the extraction, except at the point of DNA elution, where DNA was eluted with molecular-grade water rather than the recommended buffer.

To verify the presence of DNA in these extractions, we quantified DNA using a Qubit DNA fluorometer using the Double-Stranded DNA Broad-Range (DS-DNA BR) kit, which detects DNA sample concentrations from 0.0005 to 120 ng/µL with a detection range from 0.1 to 120 ng of DNA (Invitrogen, Massachusetts, US). Since we expected the bulk of DNA present to be of host origin, we ran a PCR test targeting the *Atadenovirus* mitochondrially encoded cytochrome c oxidase one (CO-1 gene) (Folmer et al. 1994). Next, we tested for the presence of *Atadenovirus* DNA in our extractions following an established nested-PCR protocol (Doneley et al. 2014; Prado-Irwin et al. 2018; Hyndman et al. 2019). Each PCR reaction included 12.875-µL distilled molecular-grade water, 2.5 µL each of either the inner or outer forward and reverse primer diluted to (0.05 µM), 2.5-µL EconoTaq buffer with Magnesium, 2.5-µL dNTP solution diluted to (0.25 µM), and 0.12-µL EconoTaq DNA Polymerase (Lucigen, Wisconsin, US). For the outer PCR

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FIG. 1. The Northern Pinesnake (*Pituophis melanoleucus melanoleucus*).

TABLE 1. Three snake species sampled for *Atadenovirus* in New Jersey, USA: Northern Pinesnake (*Pituophis melanoleucus melanoleucus* [PMM]), Eastern Hog-nosed Snake (*Heterodon platirhinos* [HP]), and Northern Black Racer (*Coluber constrictor constrictor* [CCC]). Swab samples were conducted at internal and external cloaca locations. DNA amount indicates concentration of DNA in ng/µL; "too low" indicates that the concentration of the sample was below 0.0050 ng/µL. CO-1 band indicates presence of host DNA. *Atadenovirus* was not detected in any snake.

No.	Species	Location	DNA conc. (ng/µL)	CO-1 band	*Atadenovirus* band
1	HP	external	too low	present	absent
1	HP	internal	1.29	present	absent
2	HP	external	too low	present	absent
2	HP	internal	0.858	present	absent
3	CCC	external	too low	present	absent
3	CCC	internal	1.76	present	absent
4	PMM	external	too low	absent	absent
4	PMM	internal	too low	present	absent
5	PMM	external	too low	present	absent
5	PMM	internal	too low	present	absent
6	PMM	external	too low	present	absent
6	PMM	internal	2.26	present	absent
7	PMM	external	too low	present	absent
7	PMM	internal	too low	present	absent
8	PMM	external	too low	present	absent
8	PMM	internal	too low	absent	absent
9	PMM	external	too low	absent	absent
9	PMM	internal	0.516	present	absent
10	PMM	external	too low	present	absent
10	PMM	internal	too low	present	absent
11	PMM	external	too low	present	absent
11	PMM	internal	0.346	present	absent
12	PMM	external	too low	absent	absent
12	PMM	internal	too low	present	absent
13	PMM	external	too low	present	absent
13	PMM	internal	0.83	present	absent
14	PMM	external	too low	present	absent
14	PMM	internal	too low	present	absent
15	PMM	external	too low	present	absent
15	PMM	internal	0.266	present	absent
16	PMM	external	0.218	present	absent
16	PMM	internal	1.19	absent	absent
17	PMM	external	too low	present	absent
17	PMM	internal	too low	present	absent

primers, we used PolFOuter and PolROuter primers (Wellehan et al. 2004). In between the inner and outer PCR, we performed a PCR cleanup using Agencourt AMPure XP beads following the manufacturer's protocol (Beckman Coulter, California, US). For the inner PCR primers, we used PolFOuter and PolFInner (Wellehan et al. 2004). For accuracy and to diagnose PCR problems, we included a positive and negative control. The positive control was an *Atadenovirus* extraction collected from an *Anolis* lizard which was analyzed in a previously published paper (Ascher et al. 2013). All PCR products were visualized using gel electrophoresis to verify that the positive and negative control lanes showed correctly.

Bayesian models can be used to understand the sensitivity of negative results given the estimated prevalence of a pathogen, but these models require an estimate of host population size (Gray et al. 2017). As there currently exists no population size estimate of our focal species, the Northern Pine Snake, we used a frequentist statistical approach called the Rule of Three, which relates the necessary sample size to detect disease with 95% confidence to the disease prevalence (Jovanovic and Levy, 1997). Derived from the Bernoulli distribution for random variables, the Rule of Three states that $3/N = p$, where N is the sample size and p is the estimated prevalence of disease.

We found that both internal and external swab techniques were able to obtain amplifiable DNA, but the internal swab approach was more effective and had a higher yield. When samples were run on the fluorometer, some swabs had concentrations that were too low to be read by the DS-DNA BR kit, which indicates a concentration of less than 0.005 ng/µL. For the swabs that had readable concentrations, the internal swabs had higher DNA concentrations (Table 1, Wilcoxon- Paired Test, $W = 149.5$, $p = 0.00326$). From our cytochrome oxidase PCR, we found that the internal swabs had higher rates of amplification (94%) than external swabs (76%), as shown by the presence of visible bands on the agarose gel (Table 1, Z-test for proportions, $z = -1.4527$, $p = 0.07353$). The DNA concentration results provide strong evidence that internal swabs may be a better diagnostic test in identifying *Atadenovirus* infections despite being more invasive. This is because the internal swab likely had an increased opportunity to pick up host DNA from the epithelial cells lining the cloaca and through fecal matter. While this method may be better for detecting DNA, care must be taken to avoid damaging the epithelial lining of the cloaca during the swabbing procedure.

A total of 17 snakes of three species were sampled from New Jersey dens: 14 wild adult Northern Pine Snakes; two adult Eastern Hog-nosed Snakes; and one adult Northern Black Racer. No snake samples yielded identifiable *Atadenovirus* (Table 1). Given our sample size of 14 pine snakes, we calculated that we would be able to detect *Atadenovirus* if it infected ≥21.4% pine snakes in the overall population. Hence, *Atadenovirus* may have occurred in the pine snake population at a lower prevalence but was undetected by our methods (Jovanovic and Levy 1997). A greater sample size would improve detectability.

There are several other possibilities for why our study did not detect *Atadenovirus*. Although these methods are commonly used to test squamates for *Atadenovirus*, to our knowledge no statistics exist about the sensitivity of the test, particularly at low DNA concentration levels and low viral loads. Additionally, *Atadenovirus* is a gastrointestinal virus and may have been better detected in an oral swab than a cloacal swab. However, cloacal swabs have been shown to test positive for the virus (Szirovicza et al. 2016; Benge et al. 2019). Second, the species studied may not naturally be susceptible to *Atadenovirus* infections, although closely related snakes, such as Corn Snakes (*Pantherophis guttatus*), have been shown to harbor infections (Abbas et al. 2011). Third, it is possible that there is no squamate *Atadenovirus* in New Jersey. Although there are human cases of *Adenovirus* in New Jersey, no studies have tested for the virus in other potential wild animal hosts, such as snakes, birds, and turtles (Marschang 2011; Killerby et al. 2019). Lastly, snakes were sampled after winter hibernation which could have lowered viral quantity or allowed snakes to completely clear infections if they had been infected previously. However, this is unlikely as snakes with similar diseases such as equine encephalomyelitis were

found to be viremic during and post hibernation (Bingham et al. 2012). Furthermore, human strains of Adenovirus are shown to be thermally stable at 10–85°C (Roos 2020). In conclusion, although our cloacal swab sampling approach appears sufficient for *Atadenovirus* DNA detection in wild snakes, increased sample sizes per snake species and site is warranted to detect occurrence if prevalence is low, as well as testing of other likely susceptible taxa such as birds and turtles, to fully understand the range of *Atadenovirus* and its potential impacts on the New Jersey Pinelands.

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