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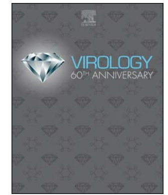

Respiratory disease in ball pythons (*Python regius*) experimentally infected with ball python nidovirus

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ABSTRACT

Circumstantial evidence has linked a new group of nidoviruses with respiratory disease in pythons, lizards, and cattle. We conducted experimental infections in ball pythons (*Python regius*) to test the hypothesis that ball python nidovirus (BPNV) infection results in respiratory disease. Three ball pythons were inoculated orally and intratracheally with cell culture isolated BPNV and two were sham inoculated. Antemortem choanal, oroesophageal, and cloacal swabs and postmortem tissues of infected snakes were positive for viral RNA, protein, and infectious virus by qRT-PCR, immunohistochemistry, western blot and virus isolation. Clinical signs included oral mucosal reddening, abundant mucus secretions, open-mouthed breathing, and anorexia. Histologic lesions included chronic-active mucinous rhinitis, stomatitis, tracheitis, esophagitis and proliferative interstitial pneumonia. Control snakes remained negative and free of clinical signs throughout the experiment. Our findings establish a causal relationship between nidovirus infection and respiratory disease in ball pythons and shed light on disease progression and transmission.

1. Importance

Over the past several years, nidovirus infection has been circumstantially linked to fatal respiratory disease in multiple python species, but a causal relationship has not been definitively established. Through experimental infections, our study fulfills Koch's postulates and confirms ball python nidovirus as a primary respiratory pathogen in this species. Our findings will provide veterinarians valuable information for the diagnosis and management of this disease and lay the groundwork for continued scientific investigation of this sometimes fatal disease. Python nidoviruses are members of a growing group of viruses that have been associated with severe respiratory disease, including bovine nidovirus and shingleback lizard nidovirus. The establishment of BPNV as a primary pathogen in pythons is an important step in understanding the pathogenic potential of this emerging group of viruses.

2. Introduction

The nidoviruses (order *Nidovirales*) are a large and diverse group of viruses that includes notable human and veterinary pathogens (De Groot et al., 2012; Graham et al., 2013; Lauber et al., 2012; Masters and Perlman, 2013; Snijder et al., 2013; Snijder and Kikkert, 2013). The discovery of a group of related nidoviruses in snakes, lizards, cattle, and nematodes has recently expanded the order (Bodewes et al., 2014; Dervas et al., 2017; Marschang and Kolesnik, 2017; O'Dea et al., 2016; Shi et al., 2016; Stenglein et al., 2014; Tokarz et al., 2015; Uccellini et al., 2014). These novel nidoviruses cluster most closely with viruses in the subfamily *Torovirinae* within the *Coronaviridae* family of the *Nidovirales* order, and form a distinct clade from viruses in the *Bafinivirus* and *Torovirus* genuses, which infect ray-finned fish and mammals, respectively. Based on phylogenetic analysis, it has been proposed that the reptile nidoviruses be classified within a distinct genus named *Barnivirus*, and that *Torovirinae* be classified as its own family due to the growing evidence of the paraphyly of *Coronaviridae*, though these viruses have not yet been formally classified (Adams et al., 2017; Batts et al., 2012; Gonzalez et al., 2003; Nga et al., 2011; Stenglein et al., 2014). Toroviruses share similar tissue tropisms of the gastrointestinal (GI) and respiratory epithelium, ultrastructural features, and genome

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organization (Batts et al., 2012; Pradesh et al., 2014; Schutze et al., 2006), and represent a group of emerging pathogens of unknown, and possibly underestimated, significance in veterinary and human medicine.

The snake-associated nidoviruses were first discovered in ball pythons (*Python regius*) and Indian rock pythons (*P. molurus*) with severe respiratory disease that had tested negative for known snake respiratory pathogens (Bodewes et al., 2014; Stenglein et al., 2014; Uccellini et al., 2014). Postmortem findings in sick pythons included stomatitis, sinusitis, pharyngitis, tracheitis, esophagitis, and proliferative pneumonia with significant mucus secretion in affected tissues; secondary bacterial infections were also noted within the respiratory tract or systemically in some snakes. In 2017, similar findings were detected in green tree pythons (*Morelia* [*M.*] *viridis*) infected by a related nidovirus (*Morelia viridis* nidovirus) (Dervas et al., 2017). Additionally, nidoviruses have been detected in antemortem oral swabs (or rarely blood) from a Burmese python (*P. bivittatus*), ball pythons, Indian rock pythons, green tree pythons, a carpet python (*M. spilota*), and boa constrictors (*Boa constrictor*) with or without documented respiratory signs (Marschang and Kolesnik, 2017). Related nidoviruses associated with respiratory disease in wild shingleback lizards and cattle have also been recently described (O'Dea et al., 2016; Tokarz et al., 2015).

Reports of nidovirus in multiple python species are highly suggestive of, but do not definitively establish, a causal relationship between viral infection and respiratory disease. This study sought to fulfill Koch's postulates through experimental infections of ball pythons with ball python nidovirus (BPNV). The goal was to conclusively establish a causative relationship between infection and respiratory disease as well as further characterize the clinical course of disease, describe useful diagnostic techniques, and to investigate possible routes of transmission.

3. Materials and methods

3.1. Generation of a diamond python cell line

A non-immortalized cell line was generated from heart tissue collected from a diamond python (*Morelia spilota*). Multiple ~ 2 mm cubes of myocardium were collected from a diamond python directly following humane barbiturate overdose euthanasia for chronic vertebral disease. Tissues were collected within 2 h of euthanasia and placed in 1.5 ml, ice-cold, sterile phosphate buffered saline (PBS) in 2 ml microcentrifuge tubes for transport to the laboratory. Tissue samples were individually transferred to a 6-well cell culture plate (Corning), washed three times with ice cold PBS, and manually minced with a sterile scalpel blade in 1.5 ml PBS with 0.25% trypsin (Gibco) and 1 mM ethylenediaminetetraacetic acid (EDTA). Samples were incubated at 37 °C with gentle agitation every 20 min (m) for a total of 60 m. Following incubation, 0.5 ml of the digested product was added per well of a 12-well cell culture plate (Corning) along with 2 ml of complete cell growth medium [Minimum Essential Medium with Earle's Balanced Salts, L-Glutamine, and Nonessential Amino Acids (MEM/EBSS; Hyclone); 10% irradiated fetal bovine serum (FBS; HyClone); 100 U penicillin; 100 µg streptomycin; 0.25 µg amphotericin B (Cellgro); and 50 µg gentamicin (Cellgro)] and placed at 30 °C in a humidified 5% CO_2 atmosphere. Wells were monitored regularly for evidence of cell adherence and replication. Partial (~ 50%) medium changes were performed weekly. When cell monolayers reached ~ 70% confluence, monolayers were washed twice with room temperature sterile PBS, 1 ml enzyme free cell dissociation buffer (Gibco) was added to each well, and the samples were incubated for 5 m at 30 °C. Cell monolayers were disrupted by gently pipetting samples up and down, and the cell/dissociation buffer mixture was transferred to a 60 mm tissue culture dish (Corning) with 7 ml of complete cell growth medium and returned to a 30 °C, humidified, 5% CO_2 atmosphere. The cells were monitored regularly for evidence of cellular replication with weekly, partial (~ 50%) medium changes. At ~ 70% confluence, monolayers were passed using 0.25% trypsin first into T25, and then into T75 tissue culture flasks (Corning). At 100% confluence, T75 flasks were trypsinized, washed in complete cell growth medium, and resuspended in 1 ml of complete cell growth medium with 20% irradiated FBS and 10% DMSO for storage in liquid nitrogen in 1.2 ml cryovials (Corning).

3.2. Isolation of BPNV

Oral swabs were collected from a ball python with upper respiratory disease that was part of a colony with a documented history of BPNV infections (Uccellini et al., 2014). Swabs were placed in 1.5 ml of viral transport medium (MEM/EBSS, 0.5% bovine serum albumin, 200 U penicillin, 200 µg streptomycin, 0.25 µg fungizone, and 10 µg ciprofloxacin; Gibco) prior to inoculation on diamond python heart (DPHt) cells. Briefly, 1 ml of the swab extracts were added to DPHt cells in T25 culture flasks. After a 3 h incubation at 30 °C, monolayers were rinsed and cell growth medium added (MEM/EBSS, 10% irradiated FBS, 200 U penicillin, 200 µg streptomycin, 0.25 µg fungizone, and 10 µg ciprofloxacin; Gibco). Cultures were maintained at 30 °C and monitored daily for cytopathic effects. At 7 days post inoculation cells were frozen at − 70 °C, thawed, and were re-inoculated onto new DPHt monolayers. The study challenge virus (deemed BPNV-148) was a passage 2 preparation.

3.3. Plaque assay

DPHt cells were incubated in complete cell medium [MEM/EBSS (HyClone), 10% irradiated FBS (HyClone), 10% Nu-Serum1 (Corning), and 2x penicillin-streptomycin solution (HyClone)] in a 6-well CELLSTAR cell culture plate (Greiner Bio-one) at 30 °C in 5% CO_2 until 90% confluence was attained. BPNV-148 stock was diluted in serum-free MEM/EBSS to generate 5 dilutions of 1×10^{-2} through 1×10^{-6}. For cell inoculation, all medium was removed and 900 µl of each dilution was placed on the cells, with serum-free MEM/EBSS added to the last well as a negative control. Cells were incubated at 30 °C in 5% CO_2 for 1 h, after which the infected medium was removed and an agarose overlay was placed [complete cell medium with 0.8% UltraPure LMP Agarose (Invitrogen)]. Assays were incubated at 30 °C in 5% CO_2 for 6 days, at which time 1 ml of 4% paraformaldehyde (EM grade; Electron Microscopy Sciences) in DPBS (Corning) was added to each well and incubated for an additional hour. The agarose overlay was removed, cells were rinsed with DPBS, and an additional 1 ml of paraformaldehyde mixture was added. Cells were placed at 4 °C overnight. The formaldehyde was removed, cells were rinsed with sterile water, and 100 µl of crystal violet (0.5% crystal violet in 25% methanol and 75% sterile water) was added and incubated for 10 min at room temperature. Crystal violet was rinsed off with sterile water, assays were dried, and plaques were counted. Plaque assays were also performed using samples collected during experimental infection studies; the same protocol was utilized.

3.4. Experimental infection

Five captive-bred ball pythons (BP A-E; 4 males and one undetermined sex) were acquired, each approximately 6 weeks old and varying in size from 77 to 106 g. All pythons were housed and treated according to the IACUC protocol (15–6063A) and Colorado State University Laboratory Animal Resources standards. Infected snakes were housed in a cubicle with separate HEPA-filtered air supply from control snakes and all snakes were housed in separate cages without direct contact. Uninfected snakes were always handled prior to infected snakes to prevent fomite transmission. Physical exams were performed and all snakes were deemed clinically healthy at the time of acquisition. Pre-infection choanal (CHS), oroesophageal (OES), and cloacal swabs (CLS) were collected and tested by qRT-PCR (see below) for BPNV. One

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