Differential disease susceptibility in experimentally reptarenavirus infected boa constrictors and ball pythons

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Running title: Reptarenaviruses cause IBD

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Abstract

Inclusion body disease (IBD) is an infectious disease originally described in captive snakes. It has traditionally been diagnosed by the presence of large eosinophilic cytoplasmic inclusions, and is associated with neurological, gastrointestinal and lymphoproliferative disorders. Previously, we identified and established a culture system for a novel lineage of arenaviruses isolated from boa constrictors diagnosed with IBD. Although ample circumstantial evidence suggested that these viruses, now known as reptarenaviruses, cause IBD, there has been no formal demonstration of disease causality since their discovery. We therefore conducted a long-term challenge experiment to test the hypothesis that reptarenaviruses cause IBD. We infected boa constrictors and ball pythons by cardiac injection of purified virus. We monitored progression of viral growth in tissues, blood, and environmental samples. Infection produced dramatically different disease outcomes in snakes of the two species. Ball pythons infected with Golden Gate virus (GoGV) and with another reptarenavirus displayed severe neurological signs within two months and viral replication was only detected in central nervous system tissues. In contrast, GoGV-infected boa constrictors remained free of clinical signs for two years despite high viral loads and the accumulation of large intracellular inclusions in multiple tissues including the brain. Inflammation was associated with infection in ball pythons but not in boa constrictors. Thus, reptarenavirus infection produces inclusions and inclusion body disease, although inclusions per se are neither necessarily associated with nor required for disease. Although the natural distribution of reptarenaviruses has yet to be described, the different outcome of infection may reflect differences in geographical origin.

Importance

New DNA sequencing technologies have made it easier than ever to identify the sequences of microorganisms in diseased tissues, i.e. to identify organisms that appear to cause disease. But to be certain that a candidate pathogen actually causes disease, it is necessary to provide additional evidence of causality. We have done this to demonstrate that reptarenaviruses cause inclusion body disease (IBD), a serious transmissible disease of snakes. We infected boa constrictors and ball pythons with purified reptarenavirus. Ball pythons fell ill within two months of infection and displayed signs of neurological disease typical of IBD. In contrast, boa constrictors remained healthy over two years despite high levels of virus throughout their bodies. This difference matches previous reports that pythons are more susceptible to IBD than boas and could reflect the possibility that boas are natural hosts of these viruses in the wild.
Introduction

Inclusion body disease (IBD) has been a vexing problem in captive snake collections for several decades (1). Classic clinical signs of IBD include neurological signs, regurgitation, and secondary bacterial infections including stomatitis and pneumonia (2). More recently, several cases of lymphoproliferative disorders have been associated with IBD in boa constrictors (2–5). Different clinical outcomes have been described for boas and pythons, with pythons reportedly experiencing a shorter, more severe, and more CNS-involved disease course (1, 2, 6, 7). Passage experiments demonstrated IBD to be transmissible, but the etiological agent remained elusive until the recent identification and isolation of arenaviruses from snakes diagnosed with IBD (1, 7–10).

Two major groups of arenaviruses (family Arenaviridae) have been identified: those that infect mammals (genus Mammarenavirus) and those that infect snakes (genus Reptarenavirus) (11–13). Arenaviruses share a number of common characteristics, including a bi-segmented ssRNA genome, with two genes on each of the small (S) and large (L) genome segments in ambisense orientation (11, 12). One possibly distinguishing feature of reptarenaviruses is that simultaneous infection by multiple viruses is common in captive snakes (14–16). Whether this is true in wild snakes is unclear, and in fact there is no published information about the natural hosts of reptarenaviruses, although IBD has been described in a number of captive snake of a number of species worldwide, and reptarenaviruses have been identified in snakes on multiple continents (2, 6, 8–10, 14, 17–19).

There is strong indirect evidence that reptarenaviruses cause IBD. First, reptarenavirus RNA detection and viral recovery is correlated with IBD diagnosis (8–10, 14). Second, cytoplasmic inclusions, the historical diagnostic hallmark of IBD, contain reptarenavirus nucleoprotein (10, 14, 20, 21). Third, several independent metagenomic next-generation sequencing studies have not identified other candidate etiologic agents (8–10, 14, 15). Nevertheless, apparently healthy snakes can be infected with reptarenaviruses and even harbor inclusion bodies (22). In fact, 5 of the first 6 apparently healthy boa constrictors that we obtained initially for this study proved to be already infected with reptarenavirus. Clearly, infection does not always, or immediately, produce disease. Therefore, the purpose of this study was to determine whether reptarenavirus infection can cause IBD, as formal demonstration of disease causality, and as a step toward identification of viral and host determinants of pathogenicity, and to study the outcome of reptarenavirus infection in snakes of multiple species.
We therefore experimentally infected boa constrictors (*Boa constrictor*) and ball pythons (*Python regius*) with reptarenaviruses. We monitored infected snakes and uninfected controls. We periodically collected blood samples and tissue biopsies to monitor virus replication, and collected environmental samples to assess possible mechanisms of transmission. Infected boa constrictors remained subclinical over two years despite high and disseminated viral loads and the accumulation of inclusion bodies. In contrast, infected ball pythons exhibited severe neurological signs within two months after infection with viral nucleic acid and protein detected only in the brain.

**Results**

To confirm absence of pre-existing virus infection in snakes to be used for experimental infections, blood, lung, and liver biopsies were collected and examined histologically and tested for reptarenavirus RNA by qRT-PCR and metagenomic NGS. Five of the first six boa constrictors we obtained initially tested positive for viral RNA (designated boas A–F). Three additional boa constrictors from a closed collection tested negative and were used for infection studies (boas G–I). We infected snakes G and H with 4x10^5 fluorescent focus-forming units (ffu) of Golden Gate Virus (GoGV), a prototypic reptarenavirus, by intracardiac injection (6). Virus had been purified from the supernatant of infected JK boa constrictor cells (8). The third snake (boa I) was mock infected. Following inoculation, snakes were monitored and blood samples and liver and lung biopsies were periodically collected. Feces, urates, shed skin, and tank swabs were also collected to assess possible routes of virus shedding (Fig. 1A).

Similar to the boa constrictors, four ball pythons were obtained and confirmed negative for pre-existing virus infection (pythons J–M). One ball python (L) was infected with 4x10^5 ffu of GoGV, and a second (M) was co-infected with 2x10^5 ffu of GoGV and 2x10^5 ffu of a reptarenavirus isolated from a boa constrictor that had exhibited stomatitis and anorexia and had been euthanized and diagnosed post-mortem with IBD (snake #37 in (14)). Our rationale for co-infecting python M was twofold: to assess the pathogenic potential of genetically diverse reptarenaviruses (the S segments of the two viruses share ~74% pairwise nucleotide identity), and to conduct a preliminary investigation of reptarenavirus multiple-infection, which is surprisingly common in captive snakes (14, 15). Feces, urates, shed skin, and tank swabs were collected.

None of the boa constrictors developed clinical signs during the two-year experiment. All snakes behaved normally and gained weight equivalently. The three boa constrictors were euthanized...
at the end of the study period, 24 months post-inoculation. Complete post-mortem examinations were performed and tissues were collected from all major organs for pathological examination and virus detection.

In contrast, infected ball pythons exhibited severe clinical signs within ~2 months of infection. Sixty-five days post-infection, python M developed an acute onset of neurological signs characteristic of IBD, including lethargy, abnormal posture, and failure to recover from dorsal recumbency (Fig. 1 inset, and Supplementary Video). This snake was immediately euthanized. Three days later (68 days post-infection) python L was observed to have focal dermatitis of unknown etiology on its right side. Further evaluation revealed that the snake had paralysis of the caudal 80% of its body and did not respond to hypodermic needle insertions in that area. It was unclear whether the dermatitis was related to infection. The snake was immediately euthanized. The control ball pythons did not display any clinical signs, and were euthanized at day 68 as well. Complete post-mortem examination of ball pythons were performed, but no ante-mortem biopsies were collected because of rapid disease onset.

We used qRT-PCR to measure viral RNA levels in tissues. Despite the absence of clinical signs in boa constrictors, high-level systemic virus replication was evident. Viral RNA was detectable in blood samples throughout infection at concentrations that ranged from $10^3$ to $10^{10}$ genome equivalents per ml of blood (Fig. 2A). Viral RNA was detected in ante-mortem liver biopsies and in all post-mortem tissues assayed: liver, lung, tonsil, spleen, kidney, colon, trachea, and brain (Fig. 2B–C). Levels of viral RNA varied, but reached concentrations exceeding 100-fold the copy number of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control mRNA. Viral RNA was also detected in feces, urates, and skin shed from boa constrictors collected throughout the two-year infection (Fig. 3). Attempts to isolate virus from these ‘environmental’ samples were unsuccessful, perhaps because samples may not have been processed or stored in a manner that preserved infectivity. These results show that boa constrictors support high reptarenavirus loads in the absence of clinical signs and shed detectable viral RNA in feces, urates, and skin.

In ball pythons, viral RNA was only detected in the central nervous system of both infected snakes, but not in other tissues tested (blood, colon, liver, lung, and kidney; Fig. 4A). Segments of genotype S2 and L2 were detected in the brain of snake L (Fig. 4B). Segments of genotype S6 and L3 were detected by qRT-PCR in the brain of snake M, which had been co-infected with GoGV (S2/L2) and snake 37 virus (genotype S6/L3/L21) (Fig. 4C). We created shotgun NGS libraries from total RNA extracted from the brains of the two infected ball pythons to confirm the absence of other organisms that could be responsible for neurological signs and did not identify other candidate
We used fluorescence microscopy with an antibody raised against GoGV NP to visualize viral protein in tissues. In necropsy tissues from infected boa constrictors, we observed large cytoplasmic NP-positive inclusions in every tissue examined: heart, intestines, liver, kidney, and brain (Figs. 5 & 6). Viral inclusions were also apparent in the liver biopsy samples from both boas taken at 16 weeks and 32 weeks post-infection, but were not evident in pre-infection tissues (Fig. 5).

In infected ball pythons, we did not detect NP-staining inclusions in any tissues except for brain (Figs. 6 & 7). Anti-NP antibody staining was present in brain cells of ball python L, but in contrast to the inclusions found in boa constrictor tissues including the brain, the staining appeared diffusely cytoplasmic (compare Figs. 6B and 6C). For the brain of python M, anti-NP staining was observed, but the fixed slices from python M were not of sufficient quality for staining by DAPI, limiting our ability to characterize infection in this specimen. Anti-NP staining was absent from all other ball python necropsy tissues, including heart, kidney, intestines, and liver. (Fig. 7).

Gross and histopathological examinations were performed on euthanized snakes. In both infected and control boa constrictors, gross lesions were mild or considered incidental. The most notable histological change in boa constrictor tissues was the presence of large eosinophilic inclusions in tissues throughout the body, and in some tissues the majority of cells were affected. Most (boa H) to virtually all (boa G) neurons in the brain and spinal cord had sharply demarcated inclusions (Fig. 8). Inclusions were most dense in the retina, neurons, bile duct epithelium, ductuli efferenti, exocrine pancreas, stomach, and kidney. Inclusion bodies were common in lymphocytes of all tissues in infected boa H but not in boa G. Inclusions were also noted in peripheral ganglia, the optic nerve, seminiferous tubules, oviductal glands, adrenal glands, hardarian glands, small intestine, respiratory epithelium, pulmonary smooth muscle, cardiomyocytes, hepatocytes, and multiple vessels. Inclusions were absent in the uninfected boa constrictor. Despite the abundant inclusions, little inflammation was observed, and that which was observed was not considered related to infection.

Pathological examinations of ball pythons revealed a markedly different picture than in boas, characterized by central nervous system inflammation and a general lack of obvious inclusions. No gross lesions were detected in python M. Regionally extensive dermatitis was found in python L, the cause and significance of which was unknown. The most significant histopathologic findings were inflammatory changes in the brain, spinal cord, and ganglia of both infected ball pythons (Fig. 9).
Infected pythons had mild to moderate lymphocytic encephalitis, lymphocytic ganglioneuritis, and lymphocytic, histiocytic, and granulocytic meningomyelitis. Neuronal necrosis and neuronophagia were also present (Fig. 9). At the site of the dermatitis observed on infected python L, multiple variably sized foci of necrosis with heterophilic infiltrates were observed. Other histologic changes included moderate lymphocytolysis in multiple lymphoid organs and minimal lymphocytic biliary dochitis (python M). In infected ball pythons, the presence of inclusion bodies was equivocal, with possible viral inclusions observed in neurons and rare bile ducts of infected python M (Fig. 10). In both pythons, multiple types of epithelial cells had eosinophilic granular material within the cytoplasm. Although these “granules” were suggestive of inclusions, the material was generally more lightly stained and indistinct when compared to typical inclusions of IBD. In control snakes (J and K), no significant gross or microscopic lesions were observed.

Discussion

Reptaretaviruses were first identified in cases of IBD, and substantial but indirect evidence suggested that infection causes disease (8–10, 14, 15). While infection of both boa constrictors and ball pythons resulted in the presence of detectable viral replication, we noted a stark contrast between the outcome in the two types of snakes. During two years of infection, boa constrictors maintained high levels of viremia ($10^8 - 10^{10}$ viral copies per mL of blood) and accumulated widespread intracytoplasmic inclusions. Despite the high viral load and numerous inclusion bodies, boas did not display overt clinical signs by the time they were euthanized, and there was a notable absence of inflammation. In contrast, infection of ball pythons produced dramatic clinical signs over the course of only ~60 days. In pythons, inclusions were extremely rare, virus was only detected in the CNS, and pronounced inflammation was observed. These findings are by and large concordant with those of two IBD transmission experiments in Burmese pythons and boa constrictors that were conducted prior to the identification of reptaretaviruses (1, 7). Additional studies will be required to untangle the factors underlying this species-specific clinical outcome. It is also likely that not all snakes (even of the same species) respond identically to infection, and additional studies using larger numbers of infected snakes could reveal variability in clinical outcomes that our study, with its relatively small numbers, missed.

It is not clear whether the infected boa constrictors would have eventually progressed to disease, and if so, over what time period. There are many examples of viruses that only produce disease after a long chronic period. For instance, HIV-1 infection typically only progresses to AIDS after years of mainly subclinical infection. It is possible that a longer chronic phase, secondary infection, stress, or
other triggers are necessary for IBD progression in boa constrictors and other less susceptible snakes. Nevertheless, reptarenavirus infection in ball pythons produced neurological signs typical of IBD, and these viruses remain the leading candidate etiologic agent for IBD in all snakes.

One possible explanation for the chronic subclinical infection in boa constrictors is that they are a reservoir host for reptarenaviruses in the wild (23). Boa constrictors (family Boidae) are native to the Americas and ball pythons (family Pythonidae) are found in Africa. It is possible that reptarenaviruses have co-evolved with and adapted to their natural reptile hosts in the Americas, as is the case for the New World lineage of mammal-infecting arenaviruses (12, 24, 25). Additional sampling of wild snakes will address this possibility.

It is possible that reptarenavirus genotype influences clinical outcome. Indeed, a large number of genetically diverse reptarenaviruses have been described, and it is possible that some reptarenaviruses would produce different disease outcomes than those observed here. For instance a reptarenavirus not studied here might cause disease in boa constrictors but not ball pythons. It would therefore be imprudent to extrapolate from these results to all reptarenaviruses. Nevertheless, prior studies have observed a strong connection between snake species and IBD clinical course, whereas no connection between reptarenavirus genotype and clinical outcome has been noted to date (1, 2, 7, 9, 14, 15). And, in our experiment, ball pythons infected with different reptarenavirus genotypes exhibited similar clinical signs: python L with GoGV and python M with GoGV and ‘snake 37 virus’ both displayed severe neurological signs. We identified a subset of the inoculated genome segment genotypes in python M’s brain (S6/L3), indicating that genotype combinations S2/L2 and S6/L3 produced similar disease, and that experimental infection could be used to further investigate intra-host, inter-reptarenavirus genotype dynamics.

One of our motivations for co-infecting python M with GoGV and ‘snake 37’ virus was to begin to investigate the phenomenon of multiple unbalanced reptarenavirus infections (14, 15). This phenomenon is surprisingly common in captive snakes and is characterized by intrahost virus populations composed of multiple distinct viral genotypes, and by an imbalance between the numbers of S and L segment genotypes in a single infection. For instance, the ‘snake 37’ virus inoculum was composed of 3 genetically distinct reptarenavirus segments: S6, L3, and L21 (GoGV is simply S2 and L2). This virus was isolated from an infected boa constrictor, and the 3 segments replicate as an ensemble in culture (14). In our survey of reptarenavirus diversity, S6 was by far the most prevalent S segment genotype, both at a population level and in individual snakes, suggesting it may be outcompeting S segments of other genotypes (14). That S6 was the only S genotype detected in the
brain of co-infected ball python M supports this suggestion, but larger studies will be necessary for a
more conclusive investigation of this intriguing phenomenon.

Despite the name IBD, the connection between inclusions and disease is clearly not
straightforward. It is now well established that reptarenavirus infection produces the inclusions
associated with IBD (8, 10, 22). However, inclusions do not necessarily indicate disease and disease
does not require inclusions. Inclusions can be found in apparently healthy snakes, and in infected ball
pythons, viral nucleoprotein was cytoplasmic but not in inclusion bodies. We speculate that inclusion
bodies may accumulate slowly, and given the rapid disease onset in ball pythons, inclusions may not
have had enough time to form. Indeed, the granular appearance of cytoplasmic anti-NP staining in
python tissues is reminiscent of the staining pattern observed in boa JK cells shortly after infection (8).
Thus, reptarenavirus infection produces inclusions and inclusion body disease, but inclusions per se are
not pathognomonic for IBD, despite assertions to that effect (16).

This study has implications for control of IBD in captive snake populations. Our data suggest
that large quantities of virus may be shed in feces, urates, and skin. Thus, infected boas could be
actively transmitting virus during a chronic and subclinical period, confounding disease control and
quarantine measures. It would be prudent to separate boa constrictors and pythons until the boa
constrictors have been confirmed by molecular methods to be free of reptarenaviruses, which have
now been unambiguously linked to disease in ball pythons.
Materials and Methods

Ethics Statement

This study including protocols for the care, handling, and infection of animals was approved by the University of California, Davis Institutional Animal Care and Use Committee (IACUC protocol #17450).

Preparation of Virus Stocks

Virus stocks for inoculation were prepared by infecting JK boa constrictor cells with low-passage stocks of Golden Gate virus (GoGV (8)) or “snake 37 virus”, the virus population isolated from snake #37 (14). Ten-cm dishes of infected JK cells were cultured as described (8). Supernatant was collected at 4, 7, 10, and 13 days post infection and stored at -80°C. Viral RNA was purified from supernatant using the Zymo Research viral RNA Kit and screened for viral RNA levels by qRT-PCR as described below. Supernatants with the highest viral RNA levels were pooled and clarified by centrifugation at 930g for 5’ at room temperature. Clarified supernatants were filtered through a 0.22 µm filter, and underlayed with a 30% sucrose cushion in a centrifuge bottle (Beckman Coulter #355618). Viruses were concentrated by ultracentrifugation at 140,000g in a Thermo Fisher F50L-8x39 rotor for 2 hours at 4°C. Supernatant was decanted and the pellet re-suspended in 1 to 2 ml PBS. Aliquots were stored at -80°C and titrated using a fluorescent focus assay as described previously (26).

Snake Husbandry and Monitoring

Three adult boa constrictors (Boa constrictor; one male control, one male infected, one female infected) and four adult ball pythons (Python regius; two female control, one female infected, one male infected) were used for this study. Control and infected snakes were housed in separate buildings, were handled independently, and each animal had its own tank and supplies. Snakes were allowed to acclimate to their housing following procurement for three weeks prior to start of the study. Whole blood was collected for overall health assessment and for arenavirus RNA qRT-PCR prior to inclusion in the study. During the acclimation and study periods, the snakes were monitored twice daily for overall health. Animals that exhibited any abnormal neurological (star gazing, head tilt, tongue flicking), gastrointestinal (regurgitation, diarrhea, constipation), or respiratory clinical signs, or that repeatedly declined food, or that exhibited steady body weight loss, were to be euthanized.

Liver and Lung Biopsies
After the acclimation period, liver and lung biopsy samples were collected under isoflurane anesthesia. Snakes were again anesthetized and surgical lung and liver biopsies were collected at 4 and 8 months post-inoculation. Biopsies were examined histopathologically and for reptarenavirus RNA by qRT-PCR and metagenomic NGS.

Snake Innoculation and Blood Sample Collection

Several weeks after the initial biopsies were collected, mock or experimental infections were administered by intracardiac injection in 200 µl PBS under general anesthesia (anesthetic protocol identical to that described for biopsies). We chose this route of infection because the natural routes of reptarenavirus transmission in the wild remain unknown and because prior studies have shown that reptarenaviruses replicate in blood cells (20). Thereafter, every 14 days for the 1st 3 months, 0.3-0.5 ml whole blood samples were collected via cardiocentesis with manual restraint, using a 25 g needle on a 1 or 3 cc syringe. A minimum of 3 blood smears were made and the remaining blood was collected in lithium-heparin tubes and stored at -80°C until testing. At 2 months, 3 months, and 18 months, an additional 0.25 ml whole blood sample was collected into a K2 EDTA tube for a complete blood count (and biochemistry panel at 18 months). After 3 months, blood was collected monthly for 9 months, then every 3 months during the second year of the study.

Euthanasia and Post-mortem Examination

Snakes were euthanized using 100 mg/kg pentobarbital administered intracardiac while under isoflurane anesthesia after either exhibition of clinical signs, or at the end of the study. A full post-mortem examination was performed.

Sections of brain, spinal cord, trachea, lung, liver, kidney, spleen, pancreas, adrenal glands, gonads, heart, tonsil and complete gastrointestinal tract were collected and placed in 10% buffered formalin, fixed and processed as 5 µm sections and stained with H&E. A second identical set of tissues were flash frozen immediately in liquid nitrogen and stored at –80 ºC.

Immunofluorescence Staining and Imaging

Paraffin mounted slides were deparaffinized with the following series of three minute washes: mixed xylenes (x2), 50% mixed xylenes to 50% ethanol, 100% ethanol (x2), 95% ethanol, 70% ethanol, 50% ethanol, and deionized water (x2). Antigen retrieval followed with a 30-minute incubation at 99 ºC in EDTA buffer (1mM EDTA with 0.05% Tween-20). Slides were then rinsed three times with deionized water, and washed in 50 mM Tris, pH 7.6; 150 mM NaCl (TBS) containing 0.025% Tween-20 for 5 minutes (x2). Permeabilization was done in PBS with 0.1% Triton-X for 5
minutes and followed with 5 minute washes in TBS with 0.05% Tween-20 (TBS-T) (x4). After washing, the slides were blocked in blocking buffer (5% Donkey Serum, 1% BSA in TBS) for 20 minutes and incubated overnight at 4 °C in anti-nucleoprotein primary antibody (8) at a 1:1000 dilution in TBS with 1% BSA, followed by washing in TBS-T for 5 minutes (x4). Donkey anti-rabbit Alexa Fluor 488 (Thermo Fisher A-21206) secondary antibody was then applied at a 1:400 solution in TBS with 1% BSA for 30 minutes at room temperature in the dark. Finally slides were washed in TBS-T (x4) and mounted using Prolong Antifade Mounting with DAPI (Thermo Fisher Scientific P36931). Imaging was performed on a Zeiss Axio Scan using a 20x lens or on a Nikon Ti microscope with an Andor Zyla 4.2 sCMOS spinning disk camera with a 100x lens. Image processing was done using the Zeiss software, Zen Microscopy and ImageJ (27).

**RNA Extraction**

RNA was extracted from solid tissue samples, feces, urate, and shed skin samples as previously described (14). Purified and DNase treated RNA samples were resuspended in 50µl of RNase/DNase free water and quantified fluorometrically. To extract RNA from blood, 250µl of whole blood was added to a 2 ml tube containing 1 ball bearing and 1 ml of Trizol reagent (Invitrogen) and homogenized using the TissueLyzer (Quiagen) for 2-3 min at 30 Hz. Homogenized samples were mixed with 200µl of chloroform, incubated at room temperature for 2 minutes, and centrifuged for 10 min at 12,000 x g at 4°C. The aqueous phase was mixed with 450µl cold isopropanol and incubated at 4°C for 1 hour. Samples were centrifuged for 10 min at 12,000g at 4°C, and the supernatant decanted. Precipitated RNA was washed with 1 ml of 75% ethanol and incubated for 10 min at 4°C. RNA was pelleted by centrifugation for 10 min at 12,000g at 4°C. Ethanol was removed and the pellet allowed to air dry before resuspending in 80µl of RNase/DNase free water. Samples were DNased using 20 units of DNaseI (NEB) and incubated at 37°C for 30 min. To DNase treated samples, 100µl of phenol/chloroform/isoamyl alcohol mixture (125:24:1 pH 4.3) was added and incubated at room temperature for 15 min, then centrifuged for 3 min at 12,000g at 4°C. The aqueous phase was transferred to a new 1.5 ml tube and RNA was precipitated using the GlycoBlue coprecipitant protocol (Ambion) with a prolonged incubation step of 30 min. Samples were DNase treated twice, followed by phenol/chloroform extraction and glycobule coprecipitation.

**Illumina Sequencing and Data Analysis**

Sequencing libraries were prepared from RNA and analyzed as previously described (14). Sequencing data have been deposited in the SRA (bioproject accession PRJNA383000).
qRT-PCR

RNA (500 ng) was added to 1µl of 250µM random hexamer oligonucleotide, and incubated at 65˚C for 5 minute. Master mix was added to final concentrations of 1X reaction buffer, 5mM dithiothreitol, 1.25mM (each) deoxynucleoside triphosphates (dNTPs), and 0.5 µl of Superscript III reverse transcriptase (Life Tech). Reactions were incubated for 5 minutes at 25 °C, then for 5 minutes at 42˚C and then for 15 minutes at 70˚C. cDNA was diluted to 100 µl (1:10) in water. Each qPCR reaction contained 5 µl diluted cDNA, 1X Hot FirePol Mix Plus (Solis Biodyne), and 0.5 µM of each primer. qPCRs were run on a Roche LightCycler 480 instrument with thermocycling conditions: 15 min at 95°C, 40 cycles of 10 sec at 95°C, 12 sec at 60°C and 12 sec at 72°C. Viral RNA levels were calculated using linearized plasmid standard curves. Primer sequences for qPCR were: S6: MDS-558 TTGATCTTCAGTCAGGACTTTACG & MDS-559 RACCTTGGTTCCACTGCTG; L3: MDS-530 ATGAGTGAGYCGACCTCCATAG & MDS-531 CRAGTGCCAATGATGTAAGAGAA; L21: MDS-538 CCTCCATTGCGCTAAACAAC & MDS-539 CAAGAGCAAGAGAGGTCAGAGAG; S2: MDS-554 CGGTGAATCCTAGTGAGGAG & MDS-555 CTACCTTGGACCCACTGGAA; L2: MDS-532 CRGRTCCACCGCCATT & MDS-533 GAGTGCTAGTGARGAAAGAGATCC; L13: MDS-785 TGTCACAATGATGACCCTCAA & MDS-786 GGGCCAGTGATGAGAGAGAC; GAPDH: MDS-921 AATATCTGCCCCATCAGCTG & MDS-923 GTTTTCCAAGAGCGTGATCC. In some instances, Sanger sequencing was used to verify qRT-PCR products.
Figure Legends

Figure 1. Timeline of experimental reptarenavirus infection of boa constrictors and ball pythons. Time of pre- and post-infection biopsies (bx) and blood samples tested are indicated. Inset images of representative infected boa constrictor and ball python at the end of their respective study periods.

Figure 2. Boa constrictors had persistently high viral loads in all tissues. Viral RNA levels were quantified by qRT-PCR (A) Blood viral RNA levels. S2/L2: viral genome segment genotypes. (B) Tissue viral RNA levels for snake G and (C) snake H. w.p.i: weeks post infection. Samples from the uninfected boa constrictor were negative.

Figure 3. Viral RNA is detectable in feces, urates, and shed skin from infected boa constrictors. Viral RNA was detected by qRT-PCR. Viral RNA was not detected in any feces, urate, or skin samples collected from ball pythons. *The positive result for this fecal sample from the control snake may have resulted from sample mislabeling; no other sample from this control animal ever tested positive.

Figure 4. Viral RNA was detectable in infected ball python brains. Viral and cellular RNA levels were quantified by qRT-PCR. (A) Reptarenavirus RNA was detected in brain but not other tissues. Viral RNA levels were normalized to levels of GAPDH mRNA in ball python L (B) and M (C) brains. Samples from uninfected snakes were negative. Controls: virus 37: virus 37 inoculum; GoGV: GoGV inoculum; J brain: uninfected python J brain. nd: not detected.

Figure 5. Reptarenavirus nucleoprotein-positive inclusions were detected in livers of infected boa constrictors throughout infection. (A) Biopsy and necropsy liver sections from infected and uninfected boa constrictors were stained with anti-NP antibody (green) and DAPI (blue). (B) Necropsy heart, kidney, and intestine sections were stained as in (A). Scale bars = 10 µM.

Figure 6. Reptarenavirus nucleoprotein detected in the brain of infected snakes. Brain sections were stained with anti-NP antibody (green) and with DAPI (blue). Sections from uninfected ball pythons J (A), infected ball python L (B), and infected boa constrictor H (C). Top, lower left, and lower right panels display increasingly zoomed images of the same sections. Scale bars = 2000, 200,
and 20 µM. Contrast speckled cytoplasmic staining in infected ball python cells (B) with inclusions in infected boa constrictor cells (C).

**Figure 7. Reptarenavirus nucleoprotein was not detected in non-CNS tissues of infected ball pythons.** Necropsy tissues from infected ball python L were stained with anti-NP antibody (green) and DAPI (blue). These images are representative of negative staining of all non-CNS tissues from all ball pythons. Scale bars = 50 µM.

**Figure 8. Inclusions were evident in infected boa constrictor brains.** Images of hematoxylin and eosin (H&E) stained brain sections from indicated boa constrictors. Infected boa constrictors (G and H) had numerous, brightly eosinophilic, cytoplasmic viral-inclusion bodies (arrow heads) within neuronal cell bodies and glial cells of the brain. Similar inclusions were found within cells of nearly every organ examined. No inflammation was associated with the inclusions. The uninfected boa constrictor (boa I) did not have inclusions. The inset shows a magnified view of the boxed regions. Scale bars = 50 µm.

**Figure 9. Inflammation in infected ball python central nervous system tissues.** Arenavirus infected pythons (L and M) had moderate lymphocytic, histiocytic, and granulocytic inflammation (asterisk) within the brain, spinal cord, and ganglia. Necrotic neurons were occasionally seen (arrows). No inflammation was detected in the uninfected python (J). HE. Scale bars = 50 µm. Central canal (C).

**Figure 10. Bile duct inclusions in ball python M.** Small eosinophilic cytoplasmic inclusions (arrows) were seen in rare bile duct epithelial cells of the infected ball python M. H&E stained tissue section; scale bar = 50 µm.

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References


allowed to acclimate
boa constrictors

weeks post inf.
-7 -4 0 5 10 18 24 34 39 43 104

blood draw

boas euthanized

ball pythons

allowed to acclimate
infection bx

weeks post inf.
-21 -18 0 10

python clinical signs & euthanasia

infection bx

bx

2nd bx

3rd bx

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weeks post infection

vRNA copies per ml blood

boa G

boa H

A

B

copies vRNA per GAPDH mRNA

biopsy necropsy

boom necropsy

10 12
10 10
10 8
10 6
10 4
10 2

biopsy necropsy

10 2
10 3
10 1
10 0
10 -1
10 -2
10 -3
10 -4
10 -5

C

Species: boa G, boa H

Liver 18 w.p.i.
Liver 39 w.p.i.
Lung 18 w.p.i.
Lung 39 w.p.i.
Tonsil
Spleen
Kidney
Lung
Colon
Trachea
Liver
Brain

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http://jvi.asm.org/ Downloaded from
Urate
Skin
Feces

LSBoa I

LSBoa H

LSBoa G

12 24 36 48 60 72 84 96

Weeks post infection

PCR
+
–
Feces
Urate
Skin

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http://jvi.asm.org/ Downloaded from
A

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B

Viral genome segment

python L brain

C

python M brain

Viral genome segment
A

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<td>39 wpi</td>
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Boa H (Infected)

Boa I (Uninfected)

B

Heart

Kidney

Intestine

Boa G (Infected)

Boa H (Infected)

Boa I (Uninfected)