Detection of nidoviruses in live pythons and boas

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Keywords
PCR, coronavirus, torovirus, snake, Boa constrictor, reptile, Morelia

Summary
Objectives: Nidoviruses have recently been described as a putative cause of severe respiratory disease in pythons in the USA and Europe. The objective of this study was to establish the use of a conventional PCR for the detection of nidoviruses in samples from live animals and to extend the list of susceptible species. **Materials and methods:** A PCR targeting a portion of ORF1a of python nidoviruses was used to detect nidoviruses in diagnostic samples from live boas and pythons. A total of 95 pythons, 84 boas and 22 snakes of unknown species were included in the study. Samples tested included oral swabs and whole blood. **Results:** Nidoviruses were detected in 27.4% of the pythons and 2.4% of the boas tested. They were most commonly detected in ball pythons (*Python* [*P.*] *regius*) and Indian rock pythons (*P. molurus*), but were also detected for the first time in other python species, including *Morelia* spp. and *Boa constrictor*. Oral swabs were most commonly tested positive. **Conclusion:** The PCR described here can be used for the detection of nidoviruses in oral swabs from live snakes. These viruses appear to be relatively common among snakes in captivity in Europe and screening for these viruses should be considered in the clinical work-up. **Clinical relevance:** Nidoviruses are believed to be an important cause of respiratory disease in pythons, but can also infect boas. Detection of these viruses in live animals is now possible and can be of interest both in diseased animals as well as in quarantine situations.

Schlüsselwörter
PCR, Coronavirus, Torovirus, Schlange, Abgottschlange, Reptilien, Morelia

Zusammenfassung
Gegenstand: Nidoviren wurden vor kurzem als mögliche Ursache schwerer respiratorischer Erkrankungen bei Pythons in den USA und Europa nachgewiesen. **Ziel** dieser Studie war, eine konventionelle PCR für den Nachweis von Nidoviren aus Proben lebender Tiere zu etablieren und die Liste der für diese Viren empfänglichen Spezies zu erweitern. **Material und Methoden:** Eingesetzt wurde eine PCR, die einen Teil des ORF1a der Python-Nidoviren nachweist, um Nidoviren in diagnostischen Proben von lebenden Boas und Pythons zu detektieren. Getestet wurden vor allem Rachenabstriche und Blut von 95 Pythons, 84 Boas und 22 Schlangen unbekannter Spezies. **Ergebnisse:** Nidoviren ließen sich bei 27,4% der Pythons und bei 2,4% der Boas nachweisen. Am häufigsten wurden sie bei Königspythons (*Python* [*P.*] *regius*) und Tigerpythons (*P. molurus*) gefunden, daneben aber auch bei anderen Pythonspezies inklusive *Morelia* spp. und bei Abgottschlangen (*Boa constrictor*). Rachenabstriche waren am häufigsten positiv. **Schlussfolgerung:** Die hier beschriebene PCR kann zum Nachweis von Nidoviren in Rachenabstrichen lebender Schlangen eingesetzt werden. Diese Viren scheinen bei in Gefangenschaft gehaltenen Schlangen in Europa relativ häufig vorzukommen und ihr Nachweis sollte bei klinischen Untersuchungen in Erwägung gezogen werden. **Klinische Relevanz:** Nidoviren gelten als bedeutende Ursache respiratorischer Erkrankungen bei Pythons, können aber auch Boas infizieren. Der jetzt mögliche Nachweis dieser Viren am lebenden Tier kann bei erkrankten Schlangen, aber auch während einer Quarantäne klinisch relevant sein.

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Introduction

The order *Nidovirales* consists of enveloped viruses with a single stranded, positive sense RNA genome. It includes the four families *Arteriviridae*, *Mesoniviridae*, *Roniviridae*, and *Coronaviridae* (4). The family *Coronaviridae* is currently divided into two subfamilies, the *Coronavirinae* and the *Torovirinae*. Coronaviruses have been found in mammals and birds, while toroviruses have been found in mammals and fish; viruses most closely related to members of the *Torovirinae* have recently been described in pythons in the USA and in Europe (1, 10, 12). The snakes in which these viruses were detected included several ball pythons (*Python regius*) (10, 12) and an Indian python (*P. molurus*) (1). All of the affected snakes had severe pneumonia, with stomatitis, tracheitis,

and esophagitis noted in individual snakes. The highest viral load was found in respiratory tissues, particularly lungs, of affected animals (10).

All of the detected snake nidoviruses were closely related to one another and pathological, histological, and electronmicroscopical findings from the cases strongly supported the hypothesis that nidoviruses are a cause of severe respiratory disease in pythons. So far, nidovirus detection has generally been described from dead, necropsied snakes using next generation sequencing methods for the detection of large portions of or complete genomes. The purpose of this study was to establish the use of a PCR for the detection of snake nidoviruses in samples from live snakes submitted to a commercial diagnostic laboratory in Europe as well as the possible detection of these viruses in additional species.

Materials and methods

Animals examined

A total of 201 snakes were included in the study. Animals and samples tested are listed in ▶Table 1. Samples had been submitted in 2014 and 2015 for testing for other viral infections, particularly for reptarenaviruses and were all believed to be from boas and pythons, even in cases in which no species was specified. Statistical comparisons of results between groups were carried out using a chi-square test.

PCR and sequence analysis

RNA was prepared from all samples using the Roche MagNA Pure 96 System with the help of MagNA Pure 96 DNA and Viral NA Small Volume Kits according to the manufacturer's instructions. PCRs for the detection of snake nidoviruses were carried out using the primers MDS-481w (GCTCSAAGACAACCCAGAAG) and MDS-482 (TTGCTGCGATGATACCTTTG) modified from Stenglein et al. (10) targeting a portion of ORF1a of described python nidoviruses. For the PCR reactions, solutions were prepared with the Real Time Ready RNA Virus Master-Kit (Roche) according to the manufacturer's instructions. Thermocycler conditions were 50 °C for 15 minutes, 95 °C for 7 minutes, nine "touch down" cycles at 95 °C for 10 seconds, at 57.5 °C for 30 seconds (–1 °C per cycle), and at 72 °C for 50 seconds, and 26 cycles at 95 °C for 10 seconds, at 49.5 °C for 30 seconds, and at 72 °C for 50 seconds followed by a final extension step at 72 °C for 7 minutes. Amplified PCR products were visualized with gel electrophoresis (Invitrogen). Reactions with a product of the expected size (ca. 260 bp) were purified using a MinElute purification kit (Qiagen) according to the manufacturer's instructions. Products were sequenced in both directions using an automatic ABI3130 sequencer (Applied Biosystems). Sequences were manually edited and primer sequences removed.

The sequences were compared to the data in GenBank (National Center for Biotechnology Information, Bethesda, MD) online (http: //www.ncbi.nih.gov) using BLASTN and BLASTX options. All sequences were compared to previously published snake nidovirus sequences (GenBank accession Nos. KJ541759.1 and KJ935003.1). Sequences were aligned using MUSCLE (2) online (http://www.ebi.ac.uk/Tools/msa/). Phylogenies were calculated using neighbor joining and maximum likelihood methods with 500 bootstraps using MEGA version 6 (11). Bayesian analysis was carried out using the HKY substitution model in MrBayes, assuming gamma distribution with 100 generations, sample frequency 10 and burn in ratio 50% to reconstruct phylogenies (3) as an application of the TOPALi v2.5 program (Biomathematics and Statistics Scotland) from nucleotide (nt) alignments.

Table 1 Samples tested for snake nidoviruses by PCR.
Tab. 1 Proben, die mittels PCR auf Schlangennidoviren untersucht wurden

Family (number tested)	Samples tested (number tested)
Pythonidae (95)	Oral or esophageal swab (87)
	Blood (62)
	Cloacal swabs (2)
Boidae (84)	Oral or esophageal swab (79)
	Blood (37)
	Cloacal swabs (2)
Unknown (22)	Oral or esophageal swab (19)
	Blood (13)
	Cloacal swabs (1)

Results

Nidoviruses were detected in a total of 30 of the 201 snakes examined (14.9%; 95% confidence interval [CI] 9.98–19.82%) (▶Table 2). This included 26 pythons (27.4% of the pythons tested, 95% CI: 18.43–36.37%), two boas (2.4% of the boas tested, 95% CI: 0–5.67%), and two snakes of unknown family. There was a highly significant difference in the percentage of positive pythons and the percentage of positive boas ($p < 0.0001$). Of the snakes in which the species was known, viruses were detected in ball pythons (*P. regius*, 16 positive), Indian rock pythons (*P. molurus*, five positive), a Burmese python (*P. bivittatus*, one positive), green tree pythons (*Morelia viridis*, two positive), a carpet python (*M. spilota*, one positive), a python of unknown species, and boa constrictors (*Boa constrictor*, two positive). The positive samples were submitted from Germany (n = 16), the United Kingdom (n = 7), Denmark (n = 1), the Czech Republic (n = 1), Austria (n = 1), Switzerland (n = 2), France (n = 1), and Belgium (n = 1).

In all cases, sequencing showed that the results were specific for nidoviruses and displayed the highest identity to previously described nidoviruses from ball pythons. PCR products from all samples were 263 bp in length. Final editing resulted in 223 bp

Table 2
Nidovirus positive snakes.

Tab. 2
Nidovirus-positive Schlangen

Species (scientific name)	Samples tested[1]	Country of origin[2]	Clinical signs, comments
Ball python (*Python regius*)	**Oral swab**, blood	D	
Ball python (*P. regius*)	**Oral swab**, blood	D	
Ball python (*P. regius*)	**Oral swab**, blood	D	
Ball python (*P. regius*)	**Oral swab**, blood	D	
Ball python (*P. regius*)	**Oral swab**, blood	D	
Ball python (*P. regius*)	**Oral swab**, blood	D	
Ball python (*P. regius*)	**Oral swab**, blood	D	
Ball python (*P. regius*)	**Oral swab**	UK	
Ball python (*P. regius*)	**Oral swab and blood** (mixed)	A	Respiratory disease
Ball python (*P. regius*)	**Oral swab**, blood	F	
Ball python (*P. regius*)	**Oral swab**, blood	D	
Ball python (*P. regius*	**Oral swab**	UK	
Ball python (*P. regius*)	**Blood**	UK	
Ball python (*P. regius*)	**Oral swab**	BE	
Ball python (*P. regius*)	**Oral swab**	CH	Mucus in oral cavity
Ball python (*P. regius*)	**Oral swab**	CH	Mucus in oral cavity
Indian rock python (*P. molurus*)	**Oral swab**, blood	D	
Indian rock python (*P. molurus*)	**Oral swab**, blood	D	
Indian rock python (*P. molurus*)	**Oral swab**, blood	D	
Indian rock python (*P. molurus*)	**Oral swab, blood**	D	
Indian rock python (*P. molurus*)	**Oral swab**, blood	D	
Burmese python (*P. bivittatus*)	**Oral swab**, blood	UK	
Green tree python (*Morelia viridis*)	**Oral swab**, blood	CZ	
Green tree python (*M. viridis*)	**Oral swab**, blood	D	
Carpet python (*M. spilota*)	**Oral swab**, blood	D	
Python (species unknown)	**Oral swab**	DK	
Boa constrictor (*B. constrictor*)	**Oral swab**	UK	
Boa constrictor (*B. constrictor*)	**Oral swab**	UK	
Unknown	**Oral swab**	UK	
Unknown	**Oral swab**, blood	D	

[1] Positive tested samples are marked in bold letters.
[2] A = Austria; BE = Belgium; CH = Switzerland; CZ = Czech Republic; D = Germany; F = France; DK = Denmark; UK = United Kingdom

long sequences from most cases, with 200 bp long sequences that could be compared from all but three positive animals. In these three animals, the quality of the sequences was sufficient to allow identification as nidoviruses with ca. 90% or greater identity to previously described nidoviruses from pythons, but was insufficient for more detailed analysis. These viruses were detected in a green tree python and two ball pythons and were excluded from further analysis. Direct comparison of all other sequences with previously described python nidoviruses showed that the detected portion of the genomes were 87–98% identical to the corresponding portion of a nidovirus detected in a ball python in the USA (10; GenBank Accession No. KJ541759.1) and 81–95% identical to a nidovirus detected in an Indian python in the Netherlands (1; GenBank accession No. KJ935003.1). The two most diverse sequences detected originated both from *Morelia* spp., one animal from Germany and one from the Czech Republic. These sequences were 95% identical to one another and 81–88% identical to the other nidoviruses detected in this study and the previously de-

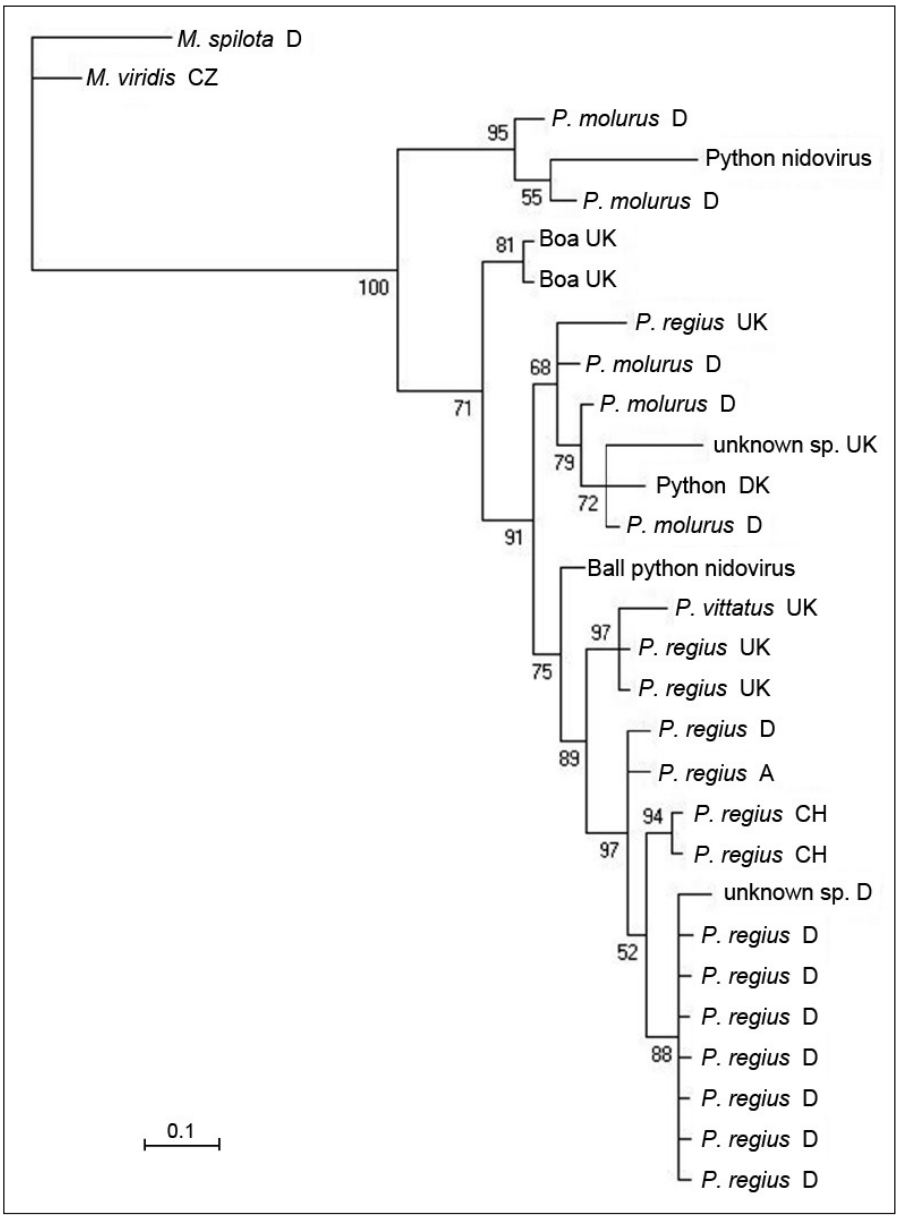

Fig. 1
Phylogenetic distance tree of partial snake nidovirus replicase gene (ORF 1a) sequences (200 bp). Unrooted Bayesian phylogeny with posterior probabilities. Snake species in which virus was detected (M. = *Morelia*, P. = *Python*) and country of sample origin are listed (A = Austria, CH = Switzerland, CZ = Czech Republic, D = Germany, DK = Denmark, UK = United Kingdom). GenBank accession numbers of snake nidoviruses used: python nidovirus: KJ935003.1, ball python nidovirus: KJ541759.1.

Abb. 1
Phylogenetischer Baum basierend auf Teilsequenzen des nidoviralen Replikase-Gens (ORF 1a) (200 bp). Bayesische Phylogenie mit A-posteriori-Wahrscheinlichkeiten. Schlangenspezies, bei denen die Viren nachgewiesen wurden (M. = *Morelia*, P. = *Python*), und das Land, aus dem die Proben stammten (A = Österreich, CH = Schweiz, CZ = Tschechische Republik, D = Deutschland, DK = Dänemark, UK = Vereinigtes Königreich) sind gelistet. GenBank Accession Numbers der eingesetzten Schlangennidoviren: python nidovirus: KJ935003.1, ball python nidovirus: KJ541759.1.

scribed python nidoviruses. The sequence analysis included a total of 19 unique sequences from 27 snakes. These sequences have been submitted to GenBank and given the accession numbers KU215836-KU215854. Phylogenetic analyses led to identical trees using multiple programs. The resulting tree is shown in ▶Fig. 1.

Discussion

Respiratory diseases are commonly found in boas and pythons in captivity (5, 8), and a number of different infectious agents have been described in association with these diseases, including ferlaviruses (6) and inclusion body disease, which is believed to be caused by reptarenaviruses (9), as well as various bacteria and fungi (7). In many cases, facultative pathogenic bacteria that can be a part of the normal bacterial flora of these animals have been detected in association with pneumonia. The description of nidoviruses as a cause of respiratory disease in pythons (10, 12) offers new insight into these diseases in snakes, requiring the establishment of new diagnostic assays in order to be able to detect these viruses in animals during quarantine or in diseased animals. Studies on nidovirus infected dead snakes demonstrated the highest viral load in the lungs (10). It therefore seemed likely that oral swabs or tracheal washes might be appropriate samples for testing live snakes. The results of this study show that PCR testing of oral swabs is a useful tool for nidovirus detection, while testing of blood samples, in contrast, is not. Tracheal wash samples might be more sensitive, although sample collection would be more invasive.

Previously nidoviruses have been mostly described in ball pythons (10, 12) and in a single Indian python (1). The findings in this study support the hypothesis that nidoviruses can mostly be found in pythons, although genetically closely related viruses were also detected in boa constrictors in two cases. This is the first report of nidoviruses in this species. The prevalence of nidoviruses in pythons was, however, significantly higher than in boas. Although sampling bias based on inclusion of samples that had been submitted for virological testing for various reasons may have influenced the results, the high number of nidovirus infections found in pythons in this study indicates that these viruses are relatively common in captive snakes throughout Europe.

This is also the first report of nidovirus detection in *Morelia* spp. It is interesting to note that the two nidoviruses from *Morelia* spp. analyzed were genetically the most diverse. Further studies are necessary to determine if there are host species specific nidovirus lineages, although the analysis of the viruses from pythons and boas does not support this hypothesis. It is possible that genetically more diverse nidoviruses may also be found in these species that were not detected by the primers used.

The finding of genetically similar nidoviruses from a wide range of European countries indicates that these viruses are widespread among captive snakes. This is not surprising considering the extensive international trade in captive reptiles. The high number of virus positive animals, particularly pythons, found in samples submitted to a diagnostic laboratory for various reasons indicates that inclusion of nidovirus screening in the diagnostic work-up might be helpful in limiting further spread of these viruses and in establishing healthier collections.

Conclusion for practice

Nidoviruses can infect various species of pythons as well as boas. They are quite common in captive pythons and can be detected in oral swabs in live animals using a PCR. Since these viruses have been associated with severe respiratory disease in affected animals, inclusion of testing for nidoviruses in snakes with respiratory disease and in quarantine situations should be considered.

Conflict of interest

The authors are employed by a commercial diagnostic laboratory offering diagnostic testing services for veterinarians.

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