Identification of snake arenaviruses in live boas and pythons in a zoo in Germany

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Keywords

Boa constrictor, Filovirus, Golden Gate Virus, inclusion body disease (IBD), *Morelia viridis*, RT-PCR

Summary

Objective: Recent studies have described the detection and characterisation of new, snake specific arenaviruses in boas and pythons with inclusion body disease (IBD). The objective of this study was to detect arenaviral RNA in live snakes and to determine if these were associated with IBD in all cases. Samples for arenavirus detection in live animals were compared. Detected viruses were compared in order to understand their genetic variability. **Materials and methods:** Esophageal swabs and whole blood was collected from a total of 28 boas and pythons. Samples were tested for arenaviral RNA by RT-PCR. Blood smears from all animals were examined for the presence of inclusion bodies. Internal tissues from animals that died or were euthanized during the study were examined for inclusions and via RT-PCR for arenaviral RNA. All PCR products were sequenced and the genomic sequences phylogenetically analysed. **Results:** Nine live animals were found to be arenavirus-positive. Two additional snakes tested positive following necropsy. Five new arenaviruses were detected and identified. The detected viruses were named "Boa Arenavirus Deutschland (Boa Av DE) numbers 1–4" and one virus detected in a python (*Morelia viridis*) was named "Python Av DE1". Results from sequence analyses revealed considerable similarities to a portion of the glycoprotein genes of recently identified boid snake arenaviruses. **Conclusions:** Both oral swabs and whole blood can be used for the detection of arenaviruses in snakes. In most cases, but not in all, the presence of arenaviral RNA correlated with the presence of inclusions in the tissues of infected animals. There was evidence that some animals may be able to clear arenavirus infection without development of IBD. This is the first detection of arenaviruses in live snakes. **Clinical relevance:** The detection of arenaviruses in live snakes is of importance for both disease detection and prevention and for use in quarantine situations. The findings in this study support the theory that arenaviruses are the cause of IBD, but indicate that in some cases it may be possible for animals to clear arenavirus infections without developing IBD.

Schlüsselwörter

Boa constrictor, Filovirus, Golden Gate Virus, inclusion body disease (IBD), Einschlusskörperchenkrankheit, *Morelia viridis*, RT-PCR

Zusammenfassung

Gegenstand und Ziel: Neue schlangenspezifische Arenaviren wurden vor kurzem bei Boas und Pythons mit der Einschlusskörperchenkrankheit (inclusion body disease, IBD) beschrieben. Ziel der Studie war, arenavirale RNA bei lebenden Schlangen nachzuweisen und festzustellen, ob Virus immer mit IBD bei den infizierten Schlangen assoziiert war. Proben für den Virusnachweis bei lebenden Tieren wurden verglichen. Ferner erfolgte ein Vergleich der nachgewiesenen Viren zur Feststellung ihrer genetischen Variabilität. **Material und Methoden:** Von 28 Boas und Pythons wurden ösophageale Tupfer sowie Vollblut mittels RT-PCR auf arenavirale RNA und Blutausstriche auf Einschlüsse untersucht. Innere Organe von Tieren, die während der Studie starben oder euthanisiert wurden, unterlagen ebenfalls einer Untersuchung auf arenavirale RNA sowie auf Einschlusskörperchen. Alle PCR-Produkte wurden sequenziert und genomische Sequenzen phylogenetisch analysiert. **Ergebnisse:** Arenaviren ließen sich bei neun lebenden Tieren sowie bei zwei Tieren nach der Sektion nachweisen. Die fünf detektierten neuen Arenaviren wurden „Boa Arenavirus Deutschland (Boa Av DE) Nummer 1–4" bzw. „Python Av DE1" genannte. Die Sequenzanalyse zeigte deutliche Ähnlichkeiten mit dem entsprechenden Abschnitt bei neu identifizierten Schlangenarenaviren. **Schlussfolgerungen:** Ösophageale Tupfer wie auch Vollblut können zum Nachweis von Arenaviren bei Schlangen eingesetzt werden. In den meisten Fällen korrelierte die Arenavirusinfektion mit dem Nachweis von Einschlüssen in den Organen. Es gab Hinweise darauf, dass in Einzelfällen arenavirusinfizierte Tiere wieder virusfrei werden können, ohne an IBD zu erkranken. Dies ist der erste Nachweis von Arenaviren bei lebenden Schlangen. **Klinische Relevanz:** Der Arenavirusnachweis bei lebenden Schlangen hat Bedeutung für die Diagnose, Prävention und Quarantäne. Die Ergebnisse dieser Studie unterstützen die Theorie, dass Arenaviren IBD verursachen, zeigen aber, dass infizierte Tiere Arenavirusinfektionen eventuell überstehen, ohne IBD zu entwickeln.

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Introduction

Arenaviruses are enveloped viruses with a genome consisting of two negative strand RNA segments: a large L and a small S segment. The S segment encodes the viral nucleocapsid protein (NP) and the glycoprotein (GP) while the L-segment encodes the RNA-dependent RNA polymerase and protein Z, the functional equivalent of the matrix (M) protein (8). A number of arenaviruses are zoonotic, causing persistent asymptomatic infections in their natural rodent hosts but severe and sometimes lethal disease in humans (7). The family *Arenaviridae* includes 25 species of viruses, at least 10 of which occasionally infect humans (13, 16). Phylogenetically, arenaviruses are arranged into Old World (OW) and New World (NW) serogroups according to their geographical distribution and genetic differences (4). In a study published in 2012, a third group of arenaviruses was described from snakes in the U.S., Golden Gate Virus (GGV) was detected in Boa constrictors (*Boa constrictor*) and California Academy of Sciences Virus (CASV) was found in annulated tree boas (*Corallus annulatus*). All animals in which these arenaviruses were detected were positive for inclusion body disease (IBD) by histological examination (15). More recently, new divergent arenaviruses were identified in boa constrictors with IBD in the Netherlands. These are closely related to the snake arenaviruses GGV and CASV (1). An additional study reported the isolation of novel arenaviruses from boid snakes with IBD in Europe. One isolate was completely characterized and represents an additional group of IBD-associated arenaviruses called University of Helsinki Virus (UHV) (12). Analysis of the genome organisation of the newly described snake arenaviruses showed that their L and NP genes were homologous to those of previously described arenaviruses, while their GP sequences were homologous to filovirus and retrovirus envelope glycoproteins (17). The finding of arenaviruses in snakes was unexpected because these viruses had been described previously only in mammals.

IBD is a severe progressive disease of boas and pythons. The name IBD comes from the presence of large cytoplasmic inclusions in a variety of cell types in IBD-positive animals (6, 14). Affected snakes may be clinically inapparent carriers or may develop severe neurological disease and die. The inclusion bodies are made of a unique protein (inclusion body disease protein) which most likely represents the arenavirus nucleoprotein and the snake arenaviruses are now believed to be the cause of IBD (12, 15).

The purpose of this study was to screen live captive boas and pythons in Germany for the detection of arenaviruses.

Materials and methods

Animals and sample collection

In January 2013, blood and esophageal swab samples were collected from each of 26 snakes in a zoological collection in Germany with a history of IBD in the collection. Samples were taken from 11 boas (*Boa constrictor*), four rainbow boas (*Epicrates cenchria*), two Indian pythons (*Python molurus*), two ball pythons (*Python regius*), four green tree pythons (*Morelia viridis*) and three garden tree boas (*Corallus hortulanus*) (▶Table 1). Sixteen snakes were kept in a quarantine section to be tested before adding them to other snakes in the show section. The other ten snakes were from the show section. In addition to the samples taken for virological testing, blood smears were also prepared from each snake and used for cytological detection of cytoplasmic inclusions typical of IBD.

Two months later, swabs were again taken from the same snakes except 11 snakes that had been euthanized (among them six boa constrictors positively tested for arenaviruses) or had died or been removed from the collection for unrelated reasons (▶Table 1). Tissues of these animals were examined histologically for the presence of inclusion bodies (▶Table 2). One green tree python (no. 23) died 8 months after the initial sampling of unrelated causes and was also examined histologically (▶Table 1).

In the second round of testing, swab and blood samples were also collected from two new snakes: one Indian python and one albino Burmese python (*Python bivittatus*) (nos. 27 and 28). In addition, swabs were taken from two rattle snakes (*Crotalus atrox*), three Solomon Islands skinks (*Corucia zebrata*) and one veiled Yemen chameleon (*Chamaeleo calyptratus*) which were kept in the same quarantine section as the snakes. All snakes appeared healthy and did not show any clinical signs at the time of sampling.

PCR and sequence analysis

Collected samples were immersed in 3 ml Dulbecco's modified Eagle's medium (DMEM, Biochrom GmbH, D-12247 Berlin, Germany) supplemented with antibiotics. Samples were then treated with ultrasound and centrifuged at 3000 x g for 15 minutes. RNA was extracted from 300 µl of the sample supernatants using the guanidinium isothiocyanate method described previously (3), and resuspended in 75 µl of RNase free water. Reverse transcription-polymerase chain reaction was carried out using the primers MDS-400 (5'-TTCATTTCTTCATGRACTTTRTCAATC-3') and MDS-402 (5'-GGSATAACAAAYTCACTTCAAATATC-3') targeting a partial sequence of the glycoprotein gene (15). Positive amplicons were gel purified (peqGOLD gel extraction kit, Peqlab Biotechnologie GmbH, D-91052 Erlangen, Germany) and submitted for sequencing by a commercial service (Eurofins MWG Operon, D-85560 Ebersberg, Germany). Sequences were edited, assembled, and compared using the STADEN Package version 2003.0 Pregap4 and Gap4 Programmes (2). The sequences were compared to data in GenBank (National Center for Biotechnology Information, Bethesda, Maryland 20894, USA) online (http://www.ncbi.nih.gov) using BLASTN and BLASTX options. Multiple alignments of nucleotide sequences were created using the ClustalW algorithm of the BioEdit Sequence Alignment Editor program (10). For phylogenetic analysis, the DNAdist and Fitch programs of the PHYLIP program package version 3.6. were used (9). Bayesian analysis was carried out using the CPRev+G and

Table 1 Boas and pythons tested for the detection of arenaviruses in whole blood and esophagus swabs by RT-PCR. Detected viruses were named Boa Av DE1–4 and Python Av DE1.

Tab. 1 Boas und Pythons, bei denen Vollblut und Ösophagustupfer mittels RT-PCR untersucht wurden. Nachgewiesene Viren wurden Boa Av DE1–4 und Python Av DE1 genannt.

No.	Species	Terrarium	1st RT-PCR test (January 2013)		IB detection in blood smears	Remarks	2nd RT-PCR test (April 2013)		Snake arenaviruses
			Blood	Swab			Blood	Swab	
Quarantine section									
1	*Boa constrictor*	3	–	+	–	Euthanized	n. d.	n. d.	Boa Av DE1
2	*Python molurus*	10	–	–	–		n. d.	–	
3	*Epicrates cenchria*	4	–	–	–		n. d.	–	
4	*Epicrates cenchria*	4	–	–	–		n. d.	–	
5	*Epicrates cenchria*	4	–	–	–		n. d.	–	
6	*Epicrates cenchria*	4	–	–	1 IB		n. d.	–	
7	*Morelia viridis*	6	–	–	–		n. d.	–	
8	*Morelia viridis*	7	–	–	–		n. d.	–	
9	*Boa constrictor*	12	–	–	–	Died due to cardiac tamponade, IBD-negative	n. d.	n. d.	
10	*Boa constrictor*	13	–	–	n. d.	Mother IBD-positive, euthanized	n. d.	n. d.	
11	*Boa constrictor*	13	+	+	–	Mother IBD-positive, euthanized	n. d.	n. d.	Boa Av DE2
12	*Boa constrictor*	13	–	+	–	Mother IBD-positive, euthanized	n. d.	n. d.	Boa Av DE2
13	*Boa constrictor*	13	+	+	–	Mother IBD-positive, euthanized	n. d.	n. d.	Boa Av DE2
14	*Boa constrictor*	13	+	+	–	Mother IBD-positive, euthanized	n. d.	n. d.	Boa Av DE2
15	*Boa constrictor*	13	–	–	–	Mother IBD-positive, euthanized	n. d.	n. d.	
16	*Boa constrictor*	13	+	+	–	Mother IBD-positive, euthanized	n. d.	n. d.	Boa Av DE2
Show section									
17	*Corallus hortulanus*	14	–	–	–	Removed from the collection for unrelated reasons	n. d.	n. d.	
18	*Corallus hortulanus*	15	–	–	–		n. d.	–	
19	*Corallus hortulanus*	16	–	–	–		n. d.	–	
20	*Python regius*	17	–	–	–		n. d.	–	
21	*Python regius*	17	–	–	–		n. d.	–	
22	*Morelia viridis*	18	–	–	–		n. d.	–	
23	*Morelia viridis*	18	–	+	–	Died 8 months later due to unrelated causes, IBD-negative, arenavirus-negative*	n. d.	–	Python Av DE1
24	*Boa constrictor*	19	–	–	+–++ IB		n. d.	+	Boa Av DE4
25	*Boa constrictor*	19	+	+	–	Died	n. d.	n. d.	Boa Av DE3
26	*Python molurus*	20	–	–	–		n. d.	–	
27	*Python molurus*	20	n. d.	n. d.	n. d.		–	–	
28	*Python bivittatus*	20	n. d.	n. d.	n. d.		–	–	

+ = positive; – = negative; n. d. = not done; IB = inclusion body; +–++ = small numbers seen. All positive PCR products were sequenced.
*Lung, heart, liver, kidney, intestine, pancreas and brain tested.

Snake no.	Species	Inclusions found in	Arenavirus detected in	Detected virus
1	*Boa constrictor*	No inclusions found in any of the tissues tested	Lung, small intestine, pancreas, kidney, brain	Boa Av DE1
10	*Boa constrictor*	No inclusions found in any of the tissues tested	Brain	Boa Av DE2
11	*Boa constrictor*	Heart blood, trachea, esophagus, stomach, small and large intestine, liver, pancreas, kidney, ureter, thyroid, brain	Heart blood, trachea, esophagus, stomach, small and large intestine, liver, pancreas, kidney, ureter, thyroid, brain	Boa Av DE2
12	*Boa constrictor*	Heart blood, trachea, salivary gland, oral mucosa, esophagus, stomach, small and large intestine, liver, pancreas, kidney, brain	Heart blood, trachea, salivary gland, oral mucosa, esophagus, stomach, small and large intestine, liver, pancreas, kidney, brain	Boa Av DE2
13	*Boa constrictor*	Trachea, lung, salivary gland, esophagus, stomach, small and large intestine, liver, pancreas, spleen, leukocytes in heart blood, kidney, thyroid, brain	Trachea, lung, salivary gland, esophagus, stomach, small and large intestine, liver, pancreas, spleen, heart blood, kidney, thyroid, brain	Boa Av DE2
14	*Boa constrictor*	Trachea, lung, salivary gland, esophagus, stomach, small and large intestine, liver, pancreas, spleen, leukocytes in heart blood, kidney, thyroid, brain	Trachea, lung, salivary gland, esophagus, stomach, small and large intestine, liver, pancreas, spleen, heart blood, kidney, thyroid, brain	Boa Av DE2
15	*Boa constrictor*	No inclusions found in any of the tissues tested	Brain	Boa Av DE2
16	*Boa constrictor*	Trachea, lung, salivary gland, esophagus, stomach, small and large intestine, liver, pancreas, spleen, leukocytes in heart blood, kidney, thyroid, brain	Trachea, lung, salivary gland, esophagus, stomach, small and large intestine, liver, pancreas, spleen, heart blood, kidney, thyroid, brain	Boa Av DE2
25	*Boa constrictor*	Trachea, lung, esophagus, stomach, small and large intestine, liver, pancreas, spleen, leukocytes in heart blood, kidney, thyroid, brain	Trachea, lung, esophagus, stomach, small and large intestine, liver, pancreas, spleen, heart blood, kidney, thyroid, brain	Boa Av DE3

Table 2
Histological and arenavirus testing in snakes that died or were euthanized after the initial round of testing.

Tab. 2
Histologische Untersuchung und Untersuchung auf Arenavirus bei Schlangen, die nach den ersten Untersuchungen starben oder euthanisiert wurden

HKY+G substitution models in MrBayes: assuming gamma distribution with 100 generations, sample frequency 10 and burn in ratio 25% to reconstruct phylogenies (11) as an application of the TOPALi v2.5 program (Biomathematics and Statistics Scotland) from deduced amino acid (aa) and nucleotide (nt) alignments to configure an optimal tree showing the posterior probability and maximum likelihood values. Bootstrapping was carried out with 100 replicates.

Results

RT-PCR showed positive signals (300 bp) from five blood samples and eight swabs originating from seven boas and one python (*Morelia viridis*) (▶Table 1, 30% of the 26 snakes tested in the first round). Of the arenavirus-positive swabs, the corresponding blood sample tested negative for arenavirus in three snakes (nos. 1, 12 and 23) (▶Table 1). Sequencing results revealed five different arenaviruses which were named Boa Arenavirus Deutschland one to four (Boa Av DE1–4) and Python Arenavirus Deutschland one (Python Av DE1).

Nucleotide (NT) identity values of Boa Av DE were 77.8–87.6% identical to GGV, 76.8–100% identical to Boa Av NL3 and 67.5–78.8% identical to CASV (▶Table 3). Boa Av DE1 was 100% identical to Boa Av NL3 (Accession no: KC508669). It was given a separate name in this study in order to simplify identification. Boa Av DE2 was detected in five boid snakes no. 11, 12, 13, 14 and 16 (▶Table 1) kept in the quarantine section of the zoo in a single terrarium. The viruses from these snakes were 100% identical to each other and 99.4% identical to Python Av DE1, but differed 12.4%, 17.1%, and 20.7% from DE1, DE3, and DE4, respectively (▶Table 3).

Multiple alignments of the Boa Av DE and three recently identified snake arenaviruses (GGV, Boa Av NL3 and CASV) showed

Table 3
Sequence identity (%) of the analyzed partial glycoprotein (GP) gene of snake arenaviruses and filoviruses. The section above the diagonal refers to the values for 194 nucleotides. The section below the diagonal shows values for 59 deduced amino acids of the detected viruses Boa Av DE1–4 and Python Av DE1 in snakes and previously described snake arenaviruses with AA sequences of the partial GP gene of filoviruses. Closest identities highlighted in bold.

Tab. 3
Sequenzidentitäten (%) der analysierten partiellen Glykoproteingene (GP-Gene) von Schlangenarenaviren und Filoviren. Der Teil oberhalb der Diagonale zeigt die Werte für 194 Nukleotide. Der Teil unterhalb der Diagonale zeigt die Werte für 59 abgeleitete Aminosäuren für die nachgewiesenen Viren Boa Av DE1–4 und Python Av DE1 und anderen Schlangenarenaviren mit AS Sequenzen des partiellen GP Gens der Filoviren. Die höchsten Identitäten sind durch Fettdruck gekennzeichnet.

GP2	BDE1	BDE2	BDE3	BDE4	PDE1	GGV	NL3	CASV
BDE1		87.6	86.0	76.8	88.1	87.1	**100**	78.8
BDE2	98.3		82.9	**79.3**	**99.4**	85.5	87.6	76.8
BDE3	98.3	**100**		77.3	83.5	**87.6**	86.0	74.2
BDE4	94.9	94.9	94.9		79.3	77.8	76.8	67.5
PDE1	98.3	**100**	**100**	**94.9**		85.5	88.1	76.2
GGV	98.3	**100**	**100**	**94.9**	**100**		72.6	87.1
NL3	**100**	98.3	98.3	**94.9**	98.3	98.3		78.8
CASV	86.4	86.4	86.4	84.7	86.4	86.4	86.4	
SUDV	30.5	30.5	30.5	30.5	30.5	30.5	30.5	28.8
LLOV	30.5	30.5	30.5	30.5	30.5	30.5	30.5	30.5
BDBV	27.1	27.1	27.1	27.1	27.1	27.1	27.1	28.8
RESTV	27.1	27.1	27.1	27.1	27.1	27.1	27.1	28.8

Abbreviations (GenBank accession numbers in brackets): GGV = Golden Gate Virus (JQ717264); Boa Av NL3 = Boa arenavirus Netherlands isolate 3 (KC508669); CASV = California Academy of Sciences Virus (JQ717262); SUDV = *Sudan ebolavirus* (ABY75325); LLOV = *Lloviu virus* (YP_004928139); BDBV = *Bundibugyo ebolavirus* (AGL73453); RESTV = *Reston ebolavirus* (ACT22802)

that the detected portion of the genomes of Boa Av DE1–4 and Python Av DE1 corresponded to positions 1025 to 1218 of the snake arenavirus GP gene. A phylogenetic tree was calculated based on NT sequences to demonstrate the distance and clustering between the five new snake Av DE viruses and the snake viruses GGV, Boa Av NL3 and CASV (▶Fig. 1). The obtained AA sequences were also compared to the corresponding sequences of Ebola viruses.[1] The deduced AA sequences showed relative similarity with the AA sequences of different Ebola viruses including: *Sudan ebolavirus* (SUDV ABY75325), *Bundibugyo ebolavirus* (BDBV AGL73453), *Reston ebolavirus* (RESTV ACT22802) and *Lloviu virus* (LLOV YP_004928139), which belong to the family *Filoviridae*.

Alignment of Boa Av DE deduced amino acid sequences showed 84.7–100% identity with the amino acid sequences from snake arenaviruses and 27.1–30.5% identity with filoviruses (▶Table 3). Phylogenetic calculations based on the deduced 59 AA sequences of the GP gene showed that Boa Av DE2 and 3 and Python Av DE1 were 100% identical to GGV and form a single monophyletic cluster as illustrated in ▶Fig. 2.

Additionally, repeated sequencing verified that there is a single nucleotide exchange at nucleotide position 159 of the GP gene sequence from Python Av DE1 which is a silent mutation. Seven snakes (nos. 10–16, ▶Table 1, ▶Table 2) were born in the zoo from previously diagnosed IBD-positive parents. Based on the animals' history and the virological findings, the decision was made to euthanize these snakes. Tissues from these animals were histologically examined for the presence of IBD-like inclusions and tested for the presence of arenaviral RNA by PCR. Five of the seven sibling boas were found to be IBD-positive by histological examination of tissues. These five animals had also tested arenavirus-positive in blood and/or swab samples. Arenavirus RNA was detected in the brains (but no other tissues) of the two IBD-negative animals from this group (▶Table 1, ▶Table 2).

The oral swab from snake no. 1 also tested arenavirus-positive. This animal was also euthanized, but no inclusion bodies were detected in any tissues. However, arenavirus was detected in several of the tissues tested by PCR (▶Table 2). An arenavirus was detected in one python (green tree python, animal no. 23) (▶Table 1) and the sequences showed 85.5% identity with GGV (▶Table 3). This animal died of unrelated causes 8 months later. No inclusions were detected in any tissues at that time and no arenaviruses were detected in any tissues, including brain (▶Table 1). The histological examinations of blood smears from all other snakes tested in the first round were negative. Inclusions were detected in the blood smear obtained from snake no. 24 (▶Table 1). A single inclusion was found in the blood smear of animal no. 6, which was arenavirus-negative (▶Table 1).

In the second round of testing, only one boa (no. 24, ▶Table 1) showed a strong RT-PCR positive signal. This snake had been housed in the same terrarium together with arenavirus positive boa no. 25 in which Boa Av DE3 was detected. Boa no. 24 had tested negative for arenaviruses in the first round of testing. Analyses of nucleotide sequences obtained from snake no. 24 (Boa Av DE4) showed an identity value of 77.3% with the NT sequences analyzed from snake no. 25 (Boa Av DE3), although Boa Av DE3

[1] Supplementary material to nucleotide sequences of Boa Av DE1–4 and Python Av DE1 (Table 4) is available for free on the journal's website (www.tieraerztliche-praxis.de).

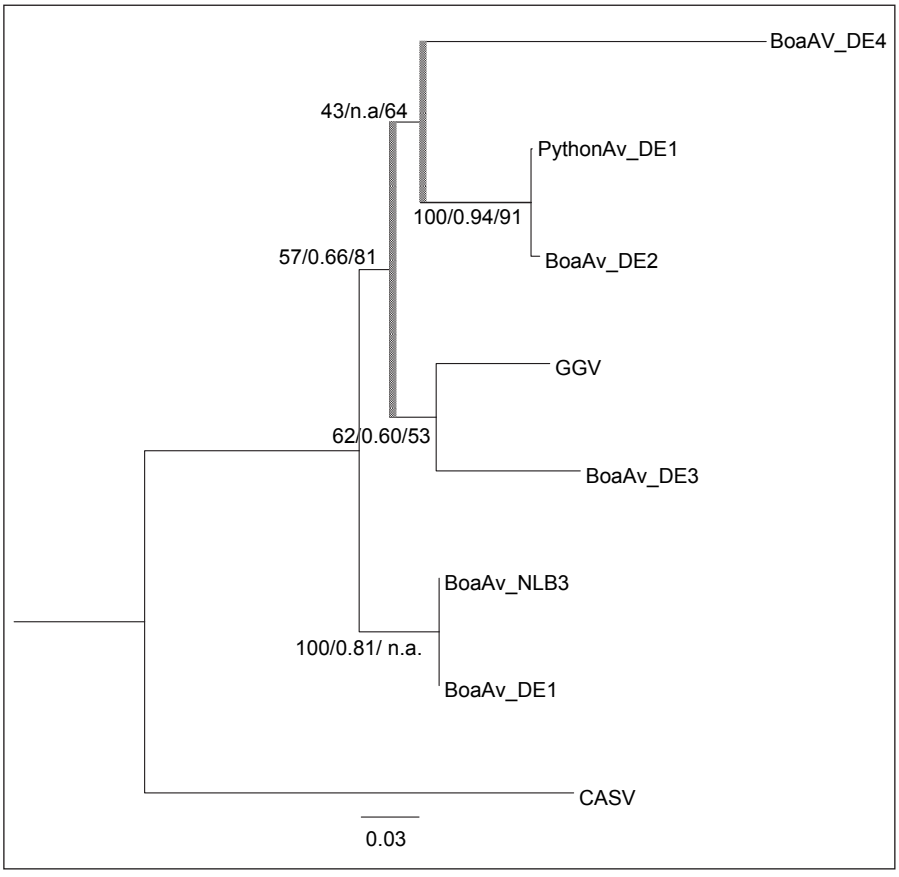

Fig. 1
Phylogenetic distance tree of snake arenaviruses. Based on a 194 nucleotide portion of the glycoprotein gene (GP2). Bootstrap, posterior probability and maximum likelihood values over 60 of 100 replicates are indicated beside the node. Branches with lower values are drawn with checkerboard lines. The maximum likelihood and Bayesian analysis resulted in the same topology tree as the FITCH analysis. GenBank accession numbers: Golden Gate Virus (GGV JQ717264), Boa arenavirus Netherlands (Boa Av NL3 KC508669) and California Academy of Sciences Virus (CASV JQ717262).

Abb. 1
Phylogenetischer Baum der Schlangenarenaviren. Basierend auf einem 194 Nukleotid langen Teil des Glykoproteingens (GP2). Neben den Knoten sind Bootstrap-, A-posteriori-Wahrscheinlichkeit- und Maximum-Likelihood-Werte über 60 aus 100 Wiederholungen angegeben. Äste mit geringeren Werten sind durch schattierte Linien gekennzeichnet. Die Maximum-Likelihood- und Bayes'schen Analysen zeigten die gleiche Topologie wie die FITCH-Analyse. GenBank Accession Numbers: Golden Gate Virus (GGV JQ717264), Boa Arenavirus Netherlands (Boa Av NL3 KC508669) und California Academy of Sciences Virus (CASV JQ717262).

showed higher identity value than DE4 to GGV and Boa Av NL3 (▶Table 3). These values indicate that these two viruses were distinct and not a transmission from one animal to the other. No arenaviruses were detected in any of the non-constrictor snakes tested and all other animals were negative as well.

Discussion

This study describes the first identification of arenaviruses in live captive snakes. These viruses appeared closely related to recently described arenaviruses in boid snakes in the United States and in the Netherlands (▶Fig. 1) and comparably related to filoviruses (▶Fig. 2). Like previously described snake arenaviruses, the detected viruses are distinct from the NWA and OWA (new and old world arenaviruses) rodent borne viruses. According to the phylogenetic topology (▶Fig. 1), the detected snake arenaviruses clustered together in three groups. Three new arenaviruses from Germany clustered as a related subgroup with the GGV, while one was identical to the previously described arenavirus found in a snake in the Netherlands (1). These findings demonstrate the variability of snake arenaviruses and also indicate that these viruses may have been transported internationally over the years, since snakes in Europe and in the USA appear to carry similar arenaviruses. A study on legal trade of reptiles has shown that a large number of these are traded through Europe and many of these animals are imported from the USA (5), making exchange of pathogens between continents likely.

A comparison of the PCR results from esophageal swabs and whole blood showed that testing of swabs resulted in more positives than testing the blood (▶Table 1). Unfortunately, the origin of most of the snakes tested in this study was not known. The majority of the snakes had been donated to the zoo from private owners. The mode of transmission of snake arenaviruses is still unknown (15). One possibility that has been discussed for IBD is that infection is transmitted by blood-feeding mites (6) or bites during fighting or aerosoles. Another possibility is that these viruses are transmitted when snakes consume infected mice or bats which carry arenaviruses or filoviruses as suggested previously (15, 17), although no similar viruses have yet been detected in these animals.

In the present study arenaviruses were found in apparently healthy snakes. None of the snakes showed any clinical signs typically associated with IBD (e. g. regurgitation, CNS signs, wasting). This has been described in IBD and arenavirus-positive snakes previously (15) and apparently clinically healthy boa contrictors with inclusion bodies in multiple tissues are regularly observed (6). The cause for this lack of clinical signs in some animals with IBD is

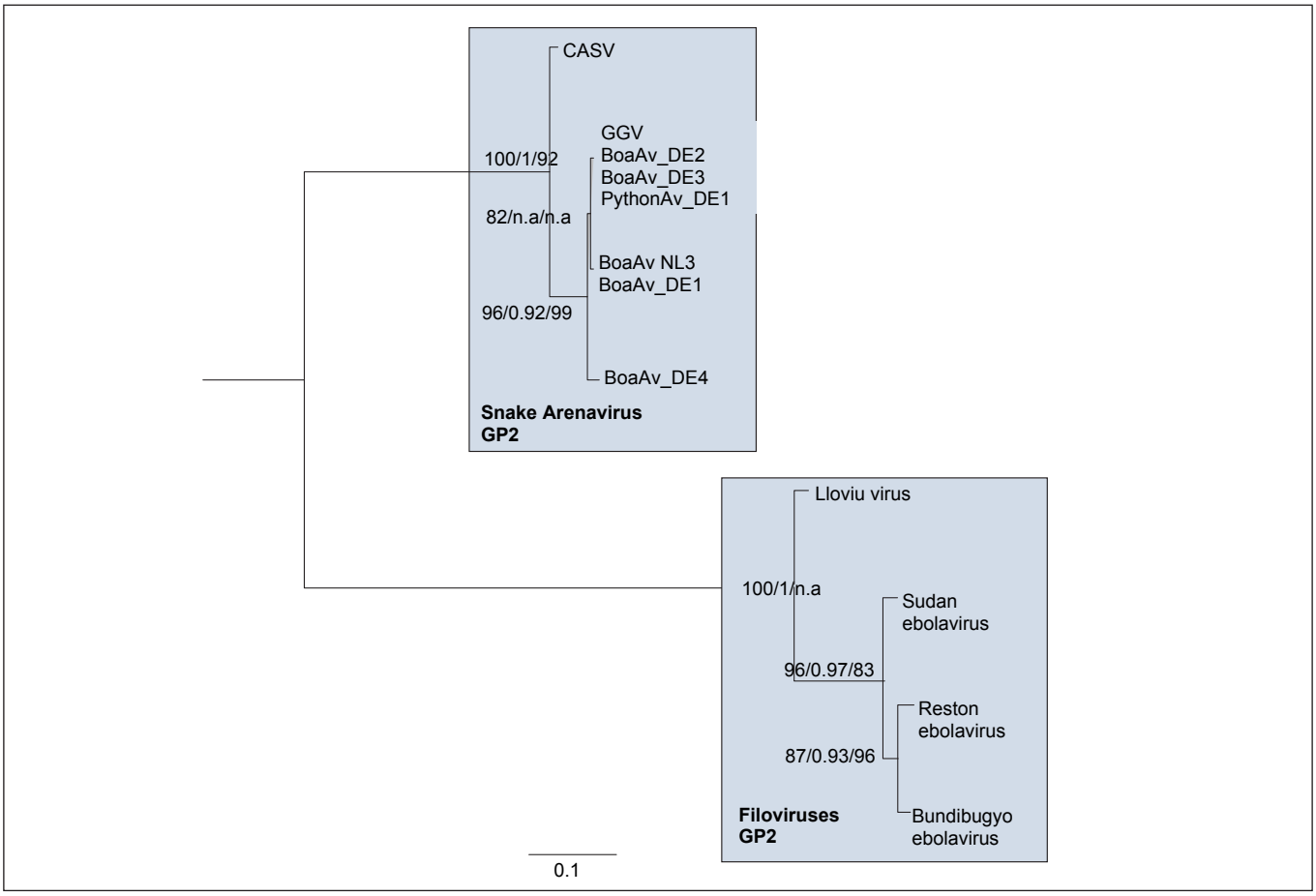

Fig. 2 Phylogenetic distance tree of snake arenaviruses and filoviruses. For the construction of the tree, 59 deduced amino acids (AA) of a portion of the glycoprotein (GP2) were analyzed using the ProtDist program followed by FITCH. Bootstrap values (for 100 replicates), posterior probability and maximum likelihood values are shown beside the branches. The Bayesian and maximum likelihood trees showed the same topology as the distance tree. AA sequences from *Sudan ebolavirus* (SUDV ABY75325), *Lloviu virus* (LLOV YP_004928139), *Bundibugyo ebolavirus* (BDBV AGL73453) and *Reston ebolavirus* (RESTV ACT22802) were chosen as outgroups. GenBank accession numbers of the AA of the GP gene of Golden Gate Virus (GGV YP_006590090), Boa arenavirus Netherlands (Boa Av NL3 AGH06039) and California Academy of Sciences Virus (CASV YP_006590086).

Abb. 2 Phylogenetischer Baum der Schlangenarenaviren und Filoviren. Für die Erstellung des Baums wurden 59 Aminosäuren (AS) eines Anteils des Glykoproteins (GP2) mithilfe des ProtDist Program gefolgt von FITCH analysiert. Bootstrap-Werte (für 100 Replikate), A-posteriori-Wahrscheinlichkeit- und Maximum-Likelihood-Werte sind neben den Ästen angegeben. Bayes'sche und Maximum-Likelihood-Bäume zeigten die gleichen Topologie wie der Distance Tree. AS-Sequenzen des *Sudan Ebolavirus* (SUDV ABY75325), *Lloviu Virus* (LLOV YP_004928139), *Bundibugyo Ebolavirus* (BDBV AGL73453) und *Reston Ebolavirus* (RESTV ACT22802) wurden als Outgroups benutzt. GenBank Accession Numbers der AS des GP-Gens des Golden Gate Virus (GGV YP_ 006590090), Boa Arenavirus Netherlands (Boa Av NL3 AGH06039) und California Academy of Sciences Virus (CASV YP_006590086).

unknown and virus strain, host factors, and time post infection could all theoretically play a role.

The cytological examination of blood smears obtained from all tested snakes in the first round of diagnostics (▶Table 1) did not show clear inclusions in any of the tested animals, although individual cytoplasmic inclusions were observed in the blood smear of one snake (boa no. 24). These were not typical of IBD but might have represented the first IBD-associated morphological changes. However, this snake was PCR-negative during the first round of testing. In the second round, the same snake (no. 24) was arenavirus-positive. It is not known, based on these results, if the initial round of testing was false negative, or if the animal became infected in the interim between the two rounds of testing, especially since this animal was housed together with an arenavirus-positive animal. It is interesting to note that the virus detected in this animal and the one detected previously in its cage mate were genetically distinct (Boa Av DE4 and 3). A single inclusion was noted in the blood of one rainbow boa (no. 6). This was not typical of IBD and no arenavirus was detected in this animal, so that the single inclusion was most likely unrelated to IBD.

The viruses found in a clutch of boa constrictors from an IBD-positive mother (nos. 10 through 16) were all identical to one another. The variation in the other viruses detected in this collection and the lack of variation in the viruses detected in these animals

Conclusion for practice

Arenaviruses are the most likely cause of inclusion body disease (IBD) in boas and pythons. Arenaviruses can be detected in live snakes using RT-PCR on esophageal swabs and whole blood. In individual cases, snakes appear to be able to clear an arenavirus infection without developing IBD. Both the direct detection of inclusions in susceptible cells as well as virus detection are therefore important for the diagnosis of IBD and for quarantine situations.

may indicate different sources of virus for different animals rather than rapid mutation in the studied portion of the genome within the collection. Further study is necessary to understand the full extent of genetic variability between these snake arenaviruses.

Following euthanasia of these seven animals from the same clutch, inclusions were found in five while no inclusions were found in the two animals that had tested arenavirus-negative while still alive (▶Table 2). These two animals were, however, arenavirus-positive in the brain but no other tissues. Since all of these animals were infected with the same arenaviruses (Boa Av DE2), it would be of interest to study whether there are specific factors that lead to a limitation of infection to the brain.

It is interesting to note that this is the first description of the detection of an arenavirus in a green tree python. This virus was genetically closely related to an arenavirus from a *Boa constrictor* (Boa Av DE2). The python showed no signs of disease and its cage mate was arenavirus-negative. The fact that this animal was both IBD and arenavirus-negative 8 months after the initial round of testing indicates that it may be possible for some animals to clear infection with arenaviruses without development of IBD, so that further study on the pathogenesis of this disease and immune responses in infected snakes is necessary.

The detection of arenaviruses in samples from clinically healthy snakes opens up possibilities for early detection of infection with these viruses. However, since an arenavirus was also detected in snakes with no signs of IBD on histological examination, further study is necessary to understand the dynamics of arenavirus infection and to determine if arenavirus infection always eventually leads to the development of IBD or if some animals may be able to clear infection and not develop disease. The development of sensitive methods for the detection of arenaviruses in live snakes will facilitate the identification of infection prior to the development of clinical disease and lower the chances for the introduction of these viruses into new collections. Correlation between arenavirus infection and the possibility of IBD-negative, arenavirus-positive animals remains to be further studied.

Conflict of interest

The authors confirm that they do not have any conflict of interest. KOH and REM work for a commercial laboratory that offers diagnostic services for veterinarians.

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