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Arenavirus Coinfections Are Common in Snakes with Boid Inclusion Body Disease

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Recently, novel arenaviruses were found in snakes with boid inclusion body disease (BIBD); these form the new genus *Reptarenavirus* within the family *Arenaviridae*. We used next-generation sequencing and *de novo* sequence assembly to investigate reptarenavirus isolates from our previous study. Four of the six isolates and all of the samples from snakes with BIBD contained at least two reptarenavirus species. The viruses sequenced comprise four novel reptarenavirus species and a representative of a new arenavirus genus.

Until very recently, arenaviruses were known as a group of mainly rodent-borne zoonotic viruses (1). The negative-sense RNA genome of arenaviruses is divided into two segments, which are designated small (S, approximately 3.5 kb) and large (L, approximately 7 to 7.5 kb); both use an ambisense coding strategy (1). The S segment encodes the glycoprotein precursor (GPC) and the nucleoprotein (NP), whereas the RNA-dependent RNA polymerase (RdRp) and the Z protein (ZP) are encoded in the L segment (1). The discovery of arenaviruses in snakes with boid inclusion body disease (BIBD) by three independent groups (2–4) has expanded the family *Arenaviridae* by a new group of viruses. In fact, the BIBD-associated arenaviruses (BIBDAVs) have been suggested to form a new arenavirus genus called *Reptarenavirus* (5). At the same time, the arenavirus study group of the International Committee on Taxonomy of Viruses (ICTV) has suggested that the genus *Arenavirus*, harboring the "classical" Old and New World arenaviruses, be renamed *Mammarenavirus* (6). According to a recent proposal, the genus *Reptarenavirus* would contain three species: alethinophid reptarenaviruses 1 (member virus: Golden Gate virus), 2 (CAS virus, CASV), and 3 (University of Helsinki virus 1 [UHV-1], boa AV NL B3 virus). While *in vitro* evidence suggests a causal relationship between arenavirus infection and BIBD (4), the *in vivo* proof is still missing. Also, the reservoir host(s) of the reptarenaviruses has not yet been confirmed; however, our recent study suggests that these viruses preferentially grow in organisms with body temperatures close to 30°C (7).

To study the diversity of reptarenaviruses, we applied next-generation sequencing (NGS) to characterize certain isolates described in our previous report (4). On the basis of phylogeny, we selected isolates originating from six *Boa constrictor* snakes and used a continuous *B. constrictor* kidney cell line, I/1Ki (4), for their propagation. While the virus isolate of snake 1 has already been almost fully sequenced (GenBank accession numbers KF297880.1 and KF297881.1), only partial L segment sequences were available for isolates from snakes 5 (KF564801.1), 8 (KF564796.1), 9 (KF564800.1), 11, and 41 (KF564797.1). We infected clean I/1Ki cultures with tissue homogenates and collected the viruses produced during the first passage by pelleting through a sucrose cushion as previously described (4). Viral RNAs were extracted from the pelleted viruses with the QIAmp Viral RNA minikit (Qiagen) according to the manufacturer's instructions, without carrier RNA. The isolated RNA was further purified and concentrated with SPRISelect beads (Agencourt). Indexed Illumina sequencing libraries were prepared with the NEBNext Ultra RNA Library Prep kit (New England BioLabs). Pooled libraries were sequenced on the Illumina MiSeq with 161-bp paired-end reads. Reads were demultiplexed, adapter sequences were removed, and sample FASTQ files were produced with the MiSeq reporter. *De novo* contiguous sequence (contig) assembly was performed with MIRA version 4.0.2 (http://mira-assembler.sourceforge.net/) and CSC (IT Center for Science Ltd., Finland) Taito supercluster. Initial contigs were used to remove the cellular RNA background with the mirabait tool, after which *de novo* assemblies were run with a subset of reads extracted with Chipster v.3.1.0 (8).

For most virus preparations, contigs of full-length or almost full-length S and L segments of arenaviruses were obtained (Table 1). The contig coverages, determined with Bowtie2 (9) in Unipro UGENE 1.14.2 (10), are presented in Table 1. The virus isolate from snake 1 served as a positive control for this study, since it represents the UHV-1 species that we have characterized in detail (4). Bowtie2 alignment of the NGS data in Unipro UGENE 1.14.2 to the UHV-1 reference L segment (KF297881.1) revealed that the sequence did not exist in the sample. Instead, two nearly complete L segment sequences were recovered from the purified UHV preparation by *de novo* sequence assembly. The database UHV sequence KF297881.1 was assembled from NGS data and by Sanger sequencing of a PCR-cloned fragment from the pGEM-T vector. At the time, we did not expect coinfection with multiple arenavi-

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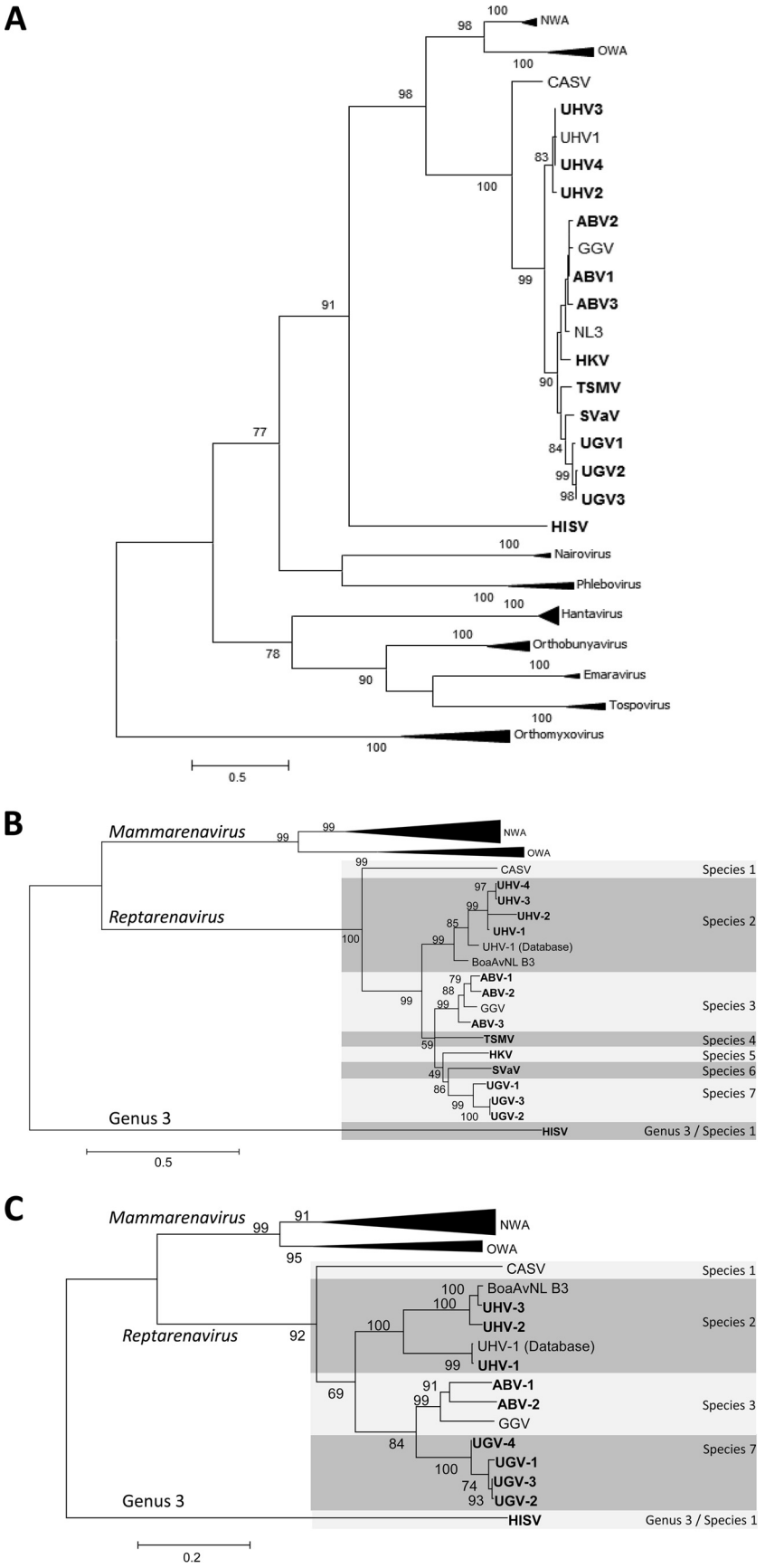
TABLE 1 Lengths, coverages, and sequence identities of the *de novo* constructed BIBDAV S and L segments[a]

Origin and genome (segment)	Length (nt)	Coverage (min-max)	No. of reads aligned	% PASC identity to other contigs (BLAST vs global alignment)	% PASC identity to database sequences (BLAST vs global alignment)	L segment prevalence in prepn (%)	GenBank accession no.
Snake 1							
UHV-1 (S)	3,393	7,071–35,430	523,677	ABV-2 (74.5 vs 66.4)	UHV-1 (99.0 vs 99.0)		KR870011
ABV-1 (S)	2,725	746–30,609	381,847	UHV-3 (71.8) vs UHV-2 (72.6)	GGV (71.7 vs 64.7)		KR870010
UHV-1 (L)	6,834	40–20,629	647,002	UHV-4 (96.0 vs 95.4)	Boa Av NL B3 (78.7 vs 79.6)	2.6	KR870020
ABV-1 (L)	6,892	688–17,159	413,735	ABV-2 (85.1 vs 85.0)	GGV (82.2 vs 82.4)	97.4	KR870021
Snake 5							
UGV-1 (S)	3,433	1,055–79,695	1096,065	UGV-3 (96.7 vs 95.7)	GGV (74.9 vs 75.6)		KR870012
UGV-1 (L)	6,787	1,310–32,480	698,732	UGV-3 (81.5 vs 81.3)	GGV (68.1 vs 70.0)		KR870022
Snake 8							
UGV-3 (S)	3,455	68–41,472	612,439	UGV-2 (99.0 vs 98.9)	GGV (73.0 vs 75.8)		KR870013
UGV-4 (S)	2,774	167–41,725	510,392	UGV-2 (86.9 vs 77.9)	GGV (66.5 vs 60.8)		KR870014
UGV-3 (L)	6,830	91–3,228	85,261	UGV-2 (99.2 vs 54.0)	GGV (67.8) vs Cupixi (54.0)	14.9	KR870023
SVaV (L)	6,506	289–2,547	78,412	UGV-1 (69.7 vs 69.6)	GGV (65.3 vs 66.2)	0.3	KR870024
ABV-3 (L)	6,641	73–2,048	50,159	ABV-1 (79.4 vs 78.3)	GGV (79.1 vs 78.1)	59.4	KR870025
TSMV (L)	6,928	11–2,439	73,643	ABV-2 (70.8 vs 72.7)	GGV (70.5 vs 72.3)	20.2	KR870026
UHV-4 (L)	6,882	127–3,210	69,701	UHV-3 (98.3 vs 98.3)	Boa Av NL B3 (79.5 vs 81.5)	0.6	KR870027
HKV (L)	6,906	3–1,655	37,354	ABV-1 (70.3 vs 72.8)	GGV (69.5 vs 73.0)	4.6	KR870028
Snake 9							
UGV-2 (S)	3,465	194–31,376	468,578	UGV-3 (99.0 vs 98.9)	GGV (73.5 vs 76.0)		KR870015
UGV-2 (L)	6,903	123–14,415	401,021	UGV-3 (99.2) vs Sn5 (81.3)	GGV (68.7 vs 71.6)		KR870029
Snake 11							
UHV-2 (S)	3,383	37–4,780	62,970	UHV-3 (93.8 vs 93.0)	Boa Av NL B3 (78.5 vs 80.0)		KR870016
HISV (S)	3,376	11–27,092	350,355	UHV-1 (15.2) vs ABV-1 (52.1)	Lassa (16.5 vs 54.0)		KR870017
UHV-2 (L)	6,894	132–2,077	63,741	UHV-4 (79.7 vs 80.0)	Boa Av NL B3 (70.4 vs 72.8)	93.4	KR870030
HISV (L)	5,913	357–4,493	115,633	TSMV (15.3) vs ABV-3-3 (50.0)	AMPV (18.8) vs BCV (48.5)	6.6	KR870031
Snake 41							
ABV-2 (S)	3,413	28–3,460	42,023	UHV-1 (74.6) vs Sn5 (76.1)	GGV (79.2 vs 79.1)		KR870018
UHV-3 (S)	3,339	10–1,055	13,563	UHV-2 (93.8 vs 93.0)	Boa Av NL B3 (80.4 vs 83.0)		KR870019
UHV-3 (L)	6,925	3–4,572	36,844	UHV-4 (98.3 vs 98.3)	Boa Av NL B3 (80.4 vs 81.9)	99.7	KR870032
ABV-2 (L)	6,938	111–2,966	49,692	ABV-1 (85.0 vs 85.0)	GGV (82.3 vs 82.8)	0.3	KR870033

[a] The viruses (abbreviations) are CAS virus (CASV), University of Helsinki viruses 1 to 4 (UHV-1 to -4), Boa Av NL B3, Aurora borealis viruses 1 to 3 (ABV-1 to -3), Golden Gate virus (GGV), Tavallinen suomalainen mies virus (TSMV), Hans Kompis virus (HKV), Suri Vanera virus (SVaV), University of Giessen viruses 1 to 4 (UGV-1 to -4), Haartman Institute snake virus (HISV), Amapari virus (AMPV), and Bear Canyon virus (BCV).

ruses and accidentally generated an "*in silico*" recombinant of the two viruses in the preparation. The sequence with accession no. KF297881.1 combines the first ~2,200 nucleotides (nt) from the 5′ end of the first L segment and ~5,000 nt from the 3′ end of the second L segment found in the preparation. In the region where the mismatch occurred, 24/26 nt are identical in both sequences. The fact that an S segment nearly identical (3367/3393 nt, >99% nucleotide sequence identity) to the UHV-1 reference S segment (KF297881.1) was recovered from the NGS data provides further evidence of the reliability of the *de novo* assembly approach chosen. Interestingly, three of the other virus preparations studied contained more than one L and S segment, indicating that several snakes were co- or superinfected with two or more reptarenaviruses. For most (3/4) samples with several reptarenaviruses, two L segment sequences were constructed; however, one of the isolates (from snake 8) contained six different L segments. Curiously, at maximum, two full-length (or nearly full-length) S segments were con-

FIG 1 Phylogenetic relationships of the newly sequenced BIBDAVs. (A) Phylogenetic tree of aligned core polymerase domains of the RdRps of segmented negative-strand viruses. The tree was reconstructed by the maximum-likelihood method in MEGA 6.06 with 1,000 bootstrap replicates. The amino acid substitution model WAG was used as suggested by MEGA as the best-fitting model. Bootstrap support values of >70 are shown at the nodes. (B, C) Maximum-likelihood trees built on the basis of RdRp amino acid sequences (B) and nucleoprotein-encoding nucleotide sequences (C). The phylogeny was reconstructed with the MEGA 5.05 software with 1,000 bootstrap replicates. The sequence data set (including representatives of Old and New World arenaviruses (OWA and NWA, respectively) and the previously reported reptarenaviruses) was compiled from the Virus Pathogen Resource database. The nucleotide sequence alignment was guided by amino acid translations with Translator X (15) and the MAFFT algorithm. The viruses (abbreviations) are CAS virus (CASV), University of Helsinki viruses 1 to 4 (UHV-1 to -4), Boa Av NL B3, Aurora borealis viruses 1 to 3 (ABV-1 to -3), Golden Gate virus (GGV), Tavallinen suomalainen mies virus (TSMV), Hans Kompis virus (HKV), Suri Vanera virus (SVaV), University of Giessen viruses 1 to 4 (UGV-1 to -4), and Haartman Institute snake virus (HISV). The newly sequenced viruses are in bold.


A
NWA
OWA
CASV
UHV3
UHV1
UHV4
UHV2
ABV2
GGV
ABV1
ABV3
NL3
HKV
TSMV
SVaV
UGV1
UGV2
UGV3
HISV
Nairovirus
Phlebovirus
Hantavirus
Orthobunyavirus
Emaravirus
Tospovirus
Orthomyxovirus
0.5
B
Mammarenavirus
Reptarenavirus
Genus 3
NWA
OWA
CASV
UHV-4
UHV-3
UHV-2
UHV-1
UHV-1 (Database)
BoaAvNL B3
ABV-1
ABV-2
GGV
ABV-3
TSMV
HKV
SVaV
UGV-1
UGV-3
UGV-2
HISV
Species 1
Species 2
Species 3
Species 4
Species 5
Species 6
Species 7
Genus 3 / Species 1
0.5
C
Mammarenavirus
Reptarenavirus
Genus 3
NWA
OWA
CASV
BoaAvNL B3
UHV-3
UHV-2
UHV-1 (Database)
UHV-1
ABV-1
ABV-2
GGV
UGV-4
UGV-1
UGV-3
UGV-2
HISV
Species 1
Species 2
Species 3
Species 7
Genus 3 / Species 1
0.2

structed per virus preparation, which may reflect stronger selection pressure or an unknown method-induced bias.

We then used pairwise sequence comparison (PASC [11], http://www.ncbi.nlm.nih.gov/sutils/pasc/viridty.cgi) to compare the sequences obtained to each other and to the arenavirus S and L segments in the databases (Table 1). According to PASC analysis and ICTV criteria, several of the viruses that we sequenced showed <76% nucleotide sequence identity to known arenaviruses and thus represent putative new alethinophid reptarenavirus species, as depicted by phylogeny (Fig. 1). For alethinophid reptarenavirus 4 (University of Giessen virus 1 [UGV-1] and UGV-2) we recovered both segments, since isolates from snakes 5 and 9 contained only a single virus each. However, for the putative representatives of alethinophid reptarenaviruses 5 to 7 (Tavallinen suomalainen mies virus [TSMV], Hans Kompis virus [HKV], and Suri Vanera virus [SVaV]), we obtained only the sequence of the L segment. Remarkably, according to the PASC analysis, one of our isolates, Haartman Institute snake virus (HISV), would be sufficiently distant from the known arenaviruses to represent a novel arenavirus genus. The S segment of HISV encodes GPC and NP by ambisense coding strategy, but despite our attempts, we were unable to obtain the full-length L segment (the ZP gene and part of the RdRp were missing). To roughly estimate the relative quantities of the viruses in each preparation, we selected primers targeting the L and S segments specific for each virus identified (the sequences of the primers used are available upon request). We performed quantitative reverse transcription (RT)-PCR with purified virus preparations and the respective primers and confirmed the presence of viruses and primer specificity by Sanger sequencing. The prevalence of L segments in the purified virus preparation is presented in Table 1.

We further tested the original tissues (stored frozen at −80°C) from the diseased constrictor snakes that had served to generate the isolates (liver tissue from snakes 5, 8, 9, and 11) by using the L segment primers for RT-PCR and confirmed the presence of these viruses in the original tissues by Sanger sequencing the products. To our surprise, the livers of snakes 5 and 9, unlike the respective isolates, were also found to have multiple reptarenaviruses (UGV-2, HKV, and ABV-3 in snake 5 and HKV and UGV-3 in snake 9) and snake 11 was found to be positive for all except ABV-2 and HISV. All of the snakes studied were from Europe. PASC analysis of the sequences revealed that the virus preparations of coinfected snakes always contained at least two different reptarenavirus species, and thus, co- or superinfection might be essential for the development of BIBD. *In vitro* studies with mammarenaviruses demonstrate that persistent arenavirus infection restricts the replication of a serologically similar but not more distant arenavirus (12). Some strains of lymphocytic choriomeningitis virus, the prototype arenavirus, induce acquired immunosuppression in their natural host (13), as also suggested for BIBD (14). We hypothesize that reptarenaviruses may establish a chronic infection in snakes. Co- or superinfection of a chronically infected snake with another reptarenavirus species might then result in amplified replication and eventually in BIBD due to immunosuppression induced by the chronically infecting virus.

The data presented here demonstrate that the recently established genus *Reptarenavirus* is likely to expand in the near future, since we identified four novel representatives of this genus from only six *B. constrictors*. Furthermore, our results suggest that co- or superinfection with reptarenaviruses is common and might be relevant for the pathogenesis of BIBD. Isolation of the individual viruses is required to confirm this hypothesis and to study the potential reassortment of reptarenaviruses.

Nucleotide sequence accession numbers.

The GenBank accession numbers of the assembled S and L segments are provided in Table 1.

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