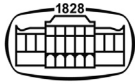

AKADÉMIAI KIADÓ

Genetic characterisation of a novel reptarenavirus detected in a dead pet red-tailed boa (*Boa constrictor*)

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RESEARCH ARTICLE

ABSTRACT

Boid inclusion body disease (BIBD) is a severe and transmissible disease of snakes worldwide. Reptarenaviruses have been identified as the aetiological agents of BIBD. We determined the almost complete genome sequence of an arenavirus detected in a female red-tailed boa that had succumbed in a private collection in Hungary. We used a combination of next generation sequencing and Sanger sequencing methods. Based on the analysis of the obtained sequence data, the virus, tentatively named Coldvalley virus, seemed to belong to the *Reptarenavirus* genus of the *Arenaviridae* family. This classification was confirmed by the genome structure (bisegmented single-stranded RNA) characteristic of the genera *Mammarenavirus* and *Reptarenavirus*. The pairwise comparison of the nucleotide and amino acid sequences, as well as the topology of the maximum likelihood phylogenetic trees, suggested that the newly-characterised Coldvalley virus can be classified into the species *Rotterdam reptarenavirus*.

KEYWORDS

arenavirus, *Boa constrictor*, boid inclusion body disease, reptarenavirus, next generation sequencing

INTRODUCTION

Boid inclusion body disease (BIBD) is a transmissible, often fatal disease commonly encountered among captive snake collections worldwide. The disease mainly affects the members of the families Boidae and Pythonidae (Argenta et al., 2020). BIBD was first recognised in the 1970s (Schumacher et al., 1994). The name of the disease originated from the large eosinophilic inclusions described in the cytoplasm of almost all cell types of the infected animals. Until description of the causative agents as reptarenaviruses, the diagnosis of BIBD had been based on the detection of inclusions by light microscopy in blood smears and tissue samples (Wozniak et al., 2000; Stenglein et al., 2012; Hetzel et al., 2013; Stenglein et al., 2017). Infected animals manifesting clinical illness show poor body condition and variable nervous system signs, including opisthotonus (stargazing), head tremors, disorientation, regurgitation and loss of co-ordination. Infection of blood cells may lead to immunosuppression which might be accompanied by secondary infections (e.g. bacterial, fungal, and protozoal) and neoplastic diseases (Carlisle-Nowak et al., 1998).

Reptarenaviruses (family *Arenaviridae*) are medium-sized, enveloped viruses with linear, single-stranded, ambisense RNA genome. Their bisegmented genome consists of a large (L) and a small (S) genome segment. The 7.5-kb-long L segment encodes the RNA-dependent RNA polymerase (RdRp; also called L protein) and the zinc-binding matrix protein (Z). The S segment is 3.5 kb in length and contains the genes of the glycoprotein precursor (GPC) and the nucleoprotein (NP) (Radoshitzky et al., 2019). The number of studies describing the

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genetic diversity of reptarenaviruses from different reptile collections is relatively low (Bodewes et al., 2013; Turchetti et al., 2013; Stenglein et al., 2015; Abba et al., 2016; Keller et al., 2017; Stenglein et al., 2017; Argenta et al., 2020). Yet, these low number of studies described a marked genetic diversity among arenaviruses infecting snakes. The genetic variability of reptarenaviruses is maintained through several mechanisms. The RdRp of reptarenaviruses lacks proofreading ability, therefore the accumulation of point mutations during replication is a major evolutionary mechanism that leads to divergence and creates novel genetic lineages over time. Additionally, reassortment and recombination between the genomic segments of reptarenaviruses result in new constellation of genes as reported previously (Hepojoki et al., 2015; Stenglein et al., 2015).

The main goal of our study was to characterise the genome and determine the phylogeny and classification of a reptarenavirus detected by PCR in a dead boa constrictor. To this end, we applied virus-specific RT-PCRs and viral metagenomics.

MATERIALS AND METHODS

Tissue samples (liver, stomach, intestine, kidney, ovary, heart, trachea, tongue, oesophagus) were collected from an adult, female, captive red-tailed boa (*Boa constrictor*) that had succumbed at a private owner in Hungary in 2012. Information about the body condition and health status of the snake prior to death was not available. For a targeted examination to check the presence of reptarenavirus in the specimen, RNA was extracted with the TRI Reagent (Molecular Research Center) and was reverse transcribed (RT) using random hexamer primers and AMV Reverse Transcriptase (Promega). Amplification of a fragment of the viral *GPC* gene of reptarenaviruses was attempted by a consensus PCR with degenerate primers (Stenglein et al., 2012). In the 25-µl volume, the reaction mixture contained 3 µl reverse-transcribed DNA, 400 µM dNTP mixture, 1x DreamTaq buffer and 2.5 U DreamTaq DNA polymerase (Thermo Fisher Scientific) and 500 nM MDS-435 (5′-TAYA-CAACCAMMGCYCTGTT-3′) and 500 nM MDS-400 (5′-TTCATTTCTTCATGRACTTTRTCAATC-3′) primers. In the sequence of the primers, we used the nucleotide ambiguity codes recommended by the International Union of Pure and Applied Chemistry. The cycling protocol consisted of a denaturation step at 95 °C 3 min, 45 cycles of the steps at 95 °C 30 s, 47 °C 30 s, and 72 °C 1 min, followed by a last elongation step at 72 °C 7 min. The PCR products were purified from agarose gel using Gel/PCR DNA Fragments Kit (Geneaid). The products were directly sequenced applying BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100 Avant Genetic Analyser (Applied Biosystem).

Based on its estimated highest virus content, the liver sample was selected and prepared for next generation sequencing (NGS) with Ion Torrent PGM™ using a protocol described previously (Bányai et al., 2014). Sanger sequencing was carried out in order to confirm the NGS data and to generate sequence information missing from the viral metagenomics approach.

The NGS reads were assembled with the CLC Bio software (http://www.clcbio.com), and the assembled contigs and sequences, obtained by Sanger method, were aligned and edited with the Geneious Prime® v.2020.2.4 (Kearse et al., 2012), TranslatorX, and AliView software (Abascal et al., 2010; Larsson, 2014). BLAST was used for sequence identification (Altschul et al., 1990). Phylogenetic analysis and pairwise identity values were obtained with the MEGA X software (Kumar et al., 2018). Recombination analysis was made with the RDP4 software (Martin et al., 2015).

Virus isolation from homogenised tissue samples was also attempted. VH 2 (Russell's viper heart, ATCC CCL-140) cells were plated for isolation of the arenavirus from the liver, stomach, intestine, kidney, heart and trachea. The cells were cultured in Dulbecco's Modified Eagle Medium (Lonza Bioscience) supplemented with 5 V/V% fetal bovine serum (Thermo Fisher Scientific), 1 V/V% Penicillin-Streptomycin-Amphotericin B Mixture (Lonza Bioscience) and 1 V/V% Non-Essential Amino Acid Solution (Lonza Bioscience) in a 24-well multidish (Thermo Fisher Scientific), and were maintained at 28 °C with 5% CO_2. Three blind passages were carried out, the cells were examined daily using light microscope and the cultures were treated three-times with freezing and thawing cycles before nucleic acid purification and RT-PCR using the above-described method.

RESULTS

The reverse transcription PCR (RT-PCR) targeting the *GPC* gene of arenaviruses gave positive results and the sequencing confirmed the presence of an arenavirus in all the 9 examined organs of the red-tailed boa. BLAST analysis of the first sequences suggested that a virus most similar to the University of Helsinki virus (UHV) was identified. Attempts for the isolation in cell culture remained unsuccessful, arenavirus RNA could not detected even after the third blind passage. A large fragment of the viral genome sequence could be determined by the viral metagenomics approach, directly from the liver sample. After combining this with the supplementary information, obtained from the Sanger sequencing, two contigs were assembled and, based on bioinformatics analyses, one L and one S segment was identified. The combined nucleotide sequence was 8,755 bases in length and covered the whole L and the partial S genome segments (Table 1). The obtained partial genome sequence was deposited in the GenBank database under accession numbers MZ818775 and MZ818776, respectively.

The genomic organisation of the newly detected reptarenavirus, that we propose to name Coldvalley arenavirus, was similar to that of members of the genus *Reptarenavirus*. The L segment encoded the RdRp in the virus genome-complementary sequence and the Z in the virus genome-

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Table 1. General features of the novel reptarenavirus (Coldvalley arenavirus) genome

Genome segment	L		S	
Length of the segment (nt)	6,860		1,895*	
Reference	UHV-3 (KR870032)		UHV-3 (KR870019)	
Coverage to reference	100%		56.8%	
Length of the 5′ end (nt)	87		ND	
Length of the OFR (nt)	348	6,207	ND	1,755
Encoded protein	**Z**	**RdRp**	**GPC**	**NP**
Protein size (aa)	115	2,068	NA	584
Length of the IGR (nt)	172		111*	
Length of the 3′ end (nt)	47		29	

*partial sequence; ND: not determined; ORF: open reading frame; IGR: noncoding intergenic region; Z: zinc-binding matrix protein; RdRp: RNA-dependent RNA polymerase; GPC: glycoprotein precursor; NP: nucleoprotein.

sense sequence, while the S segment encoded the NP in the virus genome-complementary sequence. No additional sequence from the GPC could be obtained. The phylogenetic analyses and pairwise identity calculations revealed that the sequence of the Coldvalley arenavirus was most similar to, and grouped together on the phylogenetic trees with, the sequences of multiple strains named University of Helsinki vrus 3 (UVH-3).

DISCUSSION

Isolation and propagation of reptarenaviruses using mammalian (Vero, Vero E6, A549, BHK-21, HEK293FT), reptilian (VH 2, IgH 2, JK, I/1Ki, V/5Lu, V/1Liv, VII/2Liv), and arthropod (tick cell line, RAE/CTVM1, BME/CTVM2) cell lines have been reported with various degrees of success (Stenglein et al., 2012; Hetzel et al., 2013; Abba et al., 2016; Korzyukov et al., 2016; Keller et al., 2017; Korzyukov et al., 2020). Unfortunately, the virus isolation attempts in our study remained unsuccessful. It is conceivable that the organ samples processed in our study contained insufficient quantity of infective virions for the inoculation, or the virions could not bind to, or enter the cells. Other potentially susceptible cell lines, such as for example the JK and I/1Ki of boa origin were not available in our laboratory (Hepojoki et al., 2015).

According to the current taxonomic demarcation criteria, the classification of reptarenaviruses is based on pairwise sequence comparisons of coding complete genomes and on some biological properties (e.g. host range, transmission patterns and pathobiology). Nucleotide (nt) identities higher than 40% and 35% for the L and S segments, respectively, appoint members into the same genus. These viruses also have to form a monophyletic clade in the maximum-likelihood trees generated based on the nt sequences of *RdRp* and *NP*. Two arenaviruses should be classified in a common species if their nt sequence identity values are higher than 80% and 76% for the S and L segments, respectively, and the amino acid (aa) sequence similarity values are higher than 88% in case of the NP. There are currently five accepted species of the genus *Reptarenavirus*: *California reptarenavirus, Golden reptarenavirus, Giessen reptarenavirus, Ordinary reptarenavirus* and *Rotterdam reptarenavirus* (Radoshitzky et al., 2019).

The pairwise identity calculations showed 98.8–99.3% nt identity in case of the complete L segments, 98.8–99.3% nt and 99.3–99.4% aa identity for RdRp, 98.6–99.7% nt and 97.9–100% aa identity for Z, and 98.8% nt and 98.9% aa identity for the NP compared to the available UHV-3 strains. The three genes tested were found to be identical in length, with high nt identity values (RdRp 6145/6207, NP 1752/1736, Z 340/345 identical nt/full nt). Few nt changes resulted in changes in the aa sequence (RdRp 2050/2068, NP 578/584, Z 113/115 identical aa/full aa). However, different genomic regions of the novel sequence shared similarly high identity values with that of other reptarenaviruses, e.g. with the RdRp and Z of UHV-4 (KX527590, KR870027) and L20 strains, as well as an UHV-1 strain (KR870020) (up to 99.2% nt and 99.5% aa identity), and with the NP of ROUTV (KC508669) and UHV-2 (KR8700016) strains (up to 98.5% nt and 98.9% aa identity). Although the complete sequence of the S segment of the Coldvalley arenavirus could not be determined, the identity values and phylogeny of the available sequences appointed this virus to be a member of the genus *Reptarenavirus*.

Besides the above-mentioned sequences, the RdRp of UHV-1 (KF297881) and ROUTV (KC508670) strains, type sequences of the *Rotterdam* species (*Reptarenavirus* genus), showed relatively high nt and aa identities with the Coldvalley arenavirus sequences (82.1–87.2% nt and 84.9–87.8% aa identity) and with each other (86.7% nt and 90.7% aa), and also clustered together on the phylogenetic trees. Regarding the NP, the ROUTV (KC508669) strain grouped together with the Coldvalley arenavirus (98.5% nt and 98.9% aa identity), while the UHV-1 (KF297880) represented a slightly more distant cluster (77.6% nt and 85.0% aa identity). This finding suggested potential recombination events among variable reptarenaviruses that have been described in other studies (Stenglein et al., 2015). We also performed a recombination analysis (data not shown) according to which no recombination affected the UHV-3, but rather did the UHV-1 strains. Co-infection is a common event among reptarenaviruses and reassortment of the S and L segments leads to unbalanced segment ratio in the progeny virions, where the number of the detected L segments usually exceed the number of the S segments (Hepojoki et al., 2015). This phenomenon may be related to the function of the S segment that codes for the viral glycoprotein, responsible for the cell entry, as well as NP, encoded also by this segment that plays a role in viral replication (Keller et al., 2017). This segment may be strongly affected by selection constraint and the most effective S segments may spread among the progeny virions during co-infection.

Despite the availability of numerous complete reptarenavirus genome segment sequences in the GenBank, in most cases the individual S and L segments cannot be

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Fig. 1. Maximum-likelihood phylogenetic trees based on the nt sequence of the RNA-dependent RNA polymerase (RdRp) (A), and the nucleoprotein (NP) (B) genes of reptarenaviruses. The tree was constructed with MEGAX software with 1,000 bootstrap replicates using the best-fit model of nucleotide evolution with MEGAX (GTR+G+I and K2+G+I for the RdRp and NP, respectively). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap analyses is shown next to branch nodes (when ≥70%). The virus sequences are marked with the GenBank accession number and the abbreviated name of the isolate. Symbols demonstrate the classified reptarenavirus species: ●– *Giessen reptarenavirus*, ■– *Ordinary reptarenavirus,* ◆– *California reptarenavirus*, ▼– *Golden reptarenavirus*, ▲– *Rotterdam reptarenavirus*. Abbreviations: ArSV-1 – Arabuta snake virus 1, ArBV-1 – Aramboia boa virus 1, ABV - Aurora borealis virus, BSV-1 – Bis spoeter virus, CASV – California Academy of Science Virus, FStV-1 - Frankfurter Strasse virus 1, GauV-1 – Gaucho virus 1, GOGV – Golden Gate virus, HKV-1 – Hans Kompis virus 1, HJV-1 – Hipoen jatkoon virus 1, KaBV-1 – Kaltenbach virus 1, KePV-1 – Keijut pohjoismaissa virus 1, KUKV-1 – Kiva uusi kaarme virus, KMHV-1 – Kuka mitae haeh virus 1, MNTV-1 – Mistä näitä tulee virus 1, PVaV-1 – Peilihimmeli vakooja virus 1, PJV-1 – Peto jauhoski virus 1, PAV-1 – Porto Alegre virus 1, SauV-1 – Saudades virus 1, SVaV-1 – Suri Vanera virus – SVaV, TSMV-1 – Tavallinen suomalainen mies virus, UGV – University of Giessen virus, UHV – University of Helsinki virus

matched to each other. This fact and the occurrence of reassortment complicate the simple classification of reptarenavirus strains.

At present in the GenBank, the L and S sequences are available only from one UHV-3 isolate (GenBank acc. no. KR870032 and KR870019, respectively) collected from a red-tailed boa in Germany in 2012 (Hepojoki et al., 2015). This German strain and the Coldvalley arenavirus from Hungary showed close genetic relationship, and were detected in the same time interval from the same host species, implying common origin of these viruses. There are a number of additional, closely related strains (such as additional UHV-3, UHV-1, UHV-4, L20) found in red-tailed boas. Unfortunately, complete genome sequences are scarce, but the phylogenetic analyses and common host species suggest that UHV-3, together with the Coldvalley arenavirus, belong to the species *Rotterdam reptarenavirus.* According to our knowledge this is the first described reptarenavirus infection and nearly complete genome sequence in Hungary.

The popularity of boas in exotic animal collections and the intense trade of reptiles may contribute to the intensive spread of these viruses, while the potential for mutation, recombination and reassortment may promote successful

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infection of variable hosts. Indeed, multiple reptarenavirus infection in the same animal is known (Hepojoki et al., 2015; Stenglein et al., 2015; Keller et al., 2017). The possible long incubation period (weeks or months for boas) and unapparent infections require the implementation of rigorous quarantine. The genomic characterisation of individual viral strains may lead to the development of effective tools against the infection as no vaccine against BIBD is currently available (Hetzel et al., 2020).

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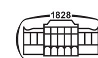

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