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Partial characterization of a new adenovirus lineage discovered in testudinoid turtles

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ABSTRACT

In the USA and in Hungary, almost simultaneously, adenoviruses of a putative novel lineage were detected by PCR and sequencing in turtles belonging to four different species (including two subspecies) of the superfamily Testudinoidea. In the USA, partial sequence of the adenoviral DNA-dependent DNA polymerase was obtained from samples of a captive pancake tortoise (*Malacochersus tornieri*), four eastern box turtles (*Terrapene carolina carolina*) and two red-eared sliders (*Trachemys scripta elegans*). In Hungary, several individuals of the latter subspecies as well as some yellow-bellied sliders (*T. scripta scripta*) were found to harbor identical, or closely related, putative new adenoviruses. From numerous attempts to amplify any other genomic fragment by PCR, only a nested method was successful, in which a 476-bp fragment of the hexon gene could be obtained from several samples. In phylogeny reconstructions, based on either DNA polymerase or hexon partial sequences, the putative new adenoviruses formed a clade distinct from the five accepted genera of the family *Adenoviridae*. Three viral sub-clades corresponding to the three host genera (*Malacochersus*, *Terrapene*, *Trachemys*) were observed. Attempts to isolate the new adenoviruses on turtle heart (TH-1) cells were unsuccessful. Targeted PCR screening of live and dead specimens revealed a prevalence of approximately 25% in small shelter colonies of red-eared and yellow-bellied sliders in Hungary. The potential pathology of these viruses needs further investigation; clinically healthy sliders were found to shed the viral DNA in detectable amounts. Based on the phylogenetic distance, the new adenovirus lineage seems to merit the rank of a novel genus.
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1. Introduction

Adenoviruses (AdVs) are common infectious agents in a large number of vertebrate animals worldwide. The family *Adenoviridae* is presently divided into five genera (Harrach et al., 2011). *Mastadenovirus* and *Aviadenovirus* were established long ago and currently contain exclusively mammalian or avian AdVs, respectively. The most recently approved genus, *Ichtadenovirus*, was established for the only known piscine AdV isolated from the white sturgeon (Benkő et al., 2002; Benkő and Doszpoly, 2011). Two additional genera consist of AdVs of mixed host origin. The first representatives of the genus *Atadenovirus* (Both, 2011) were found in ruminants and birds, and an atadenovirus was also demonstrated in a marsupial host (Thomson et al., 2002). The name of the genus reflects the strikingly high A + T content observed in the genome of the first characterized atadenoviruses (Benkő and Harrach, 1998). Because of their large phylogenetic distance from the avian and mammalian AdVs, this genus had been hypothesized to have coevolved with some lower vertebrates, most probably with reptiles (Harrach, 2000). This hypothesis was supported by the first genome-wide analysis of a reptilian AdV (snake adenovirus 1), revealing the presence of numerous genus–specific genes and ORFs (Davison et al., 2003). Interestingly however, the nucleotide (nt) composition of the SnAdV-1 genome (50.21% G + C) was not biased (Farkas et al., 2008). Development of a nested PCR with consensus primers, targeting the DNA-dependent DNA polymerase gene, enabled the recognition of numerous novel atadenoviruses in many additional squamate hosts (snakes and lizards) (Wellehan et al., 2004; Papp et al., 2009; Hyndman and Shilton, 2011; Abbas et al., 2011). Based on the sequence of shorter or longer genome parts, the G + C content of every atadenovirus of squamate origin examined to date was found to be non-biased. This notion prompted a hypothesis on the correlation between A + T richness and assumed occasional host switches of adenoviruses (Benkő and Harrach, 2003). The fifth genus, *Siadenovirus*, initially contained the only frog AdV isolate (FrAdV-1) together with turkey adenovirus 3, an exceptional avian AdV known for its broad host spectrum and strong pathogenicity (Davison et al., 2000; Beach

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et al., 2009). While it had been hypothesized that siadenoviruses had co-evolved with amphibians (Benkő and Harrach, 2003; Davison et al., 2003), this was seriously challenged by the high A + T content of every known siadenovirus and by the discovery of further new siadenoviruses in birds of different species (Zsivanovits et al., 2006; Katoh et al., 2009; Wellehan et al., 2009; Kovács et al., 2010; Park et al., 2012), as well as in Sulawesi tortoises (Rivera et al., 2009). Birds are known to be infected by aviadenoviruses, atadenoviruses, and siadenoviruses. The detection of a siadenovirus in Sulawesi tortoises indicated that besides birds and ruminants, some reptiles might also harbor AdVs from diverse genera.

From 2007 on, hitherto unseen AdVs, representing clearly a separate lineage that most probably merits the status of a new genus, were detected by PCR and sequencing in a number of turtles belonging to several species of the superfamily Testudinoidea (Turtle Taxonomy Working Group, 2011). In the present work, attempts to characterize this new AdV lineage and the results of a pilot study aiming at the assessment of the prevalence in some small pond slider populations in Hungary are described.

2. Materials and methods

2.1. Origin and processing of the samples

The first specimens found, almost simultaneously in the USA and Hungary, to contain the DNA of a new virus, were from a captive red-eared (*Trachemys scripta elegans*) and a dead yellow-bellied (*Trachemys scripta scripta*) slider, respectively. Subsequently, further closely related AdV sequences were detected in four eastern box turtles (*Terrapene carolina carolina*) and in a pancake tortoise (*Malacochersus tornieri*) in the USA.

For preliminary assessment of the prevalence of the newly recognized viruses in small colonies of pond sliders in Budapest, samples from dead or live individuals were collected from four provisional turtle shelters. The sliders, kept in these shelters, had been released unlawfully to natural and public waters then recaptured and moved to closed ponds without a practice of quarantining. In addition, three European pond turtles (*Emys orbicularis*) and one Hermann's tortoise (*Testudo hermanni*) have also been sampled.

From dead animals, individual or mixed homogenates of the internal organs (lung, liver, spleen, kidney, and intestine) were used, and the extraction of the DNA was carried out with a method described in detail earlier (Jánoska et al., 2011). From live individuals, nasal, oral and cloacal swabs were collected and processed with the use of the E.Z.N.A. Stool DNA Kit (OMEGA Bio-Tek) according to the manufacturer's instructions.

2.2. PCR primers and conditions

For the initial screening, a nested PCR method with consensus primers targeting the DNA-dependent DNA polymerase gene of AdVs was used (Wellehan et al., 2004). The utility and efficiency of this PCR in detecting members of apparently every genus in *Adenoviridae* have been tested and well documented before (Zsivanovits et al., 2006; Papp et al., 2009; Rivera et al., 2009; Wellehan et al., 2009; Kovács et al., 2010; Jánoska et al., 2011; Kaján et al., 2011; Vidovszky and Boldogh, 2011; Zadravec et al., 2011). The sequence of the primers as well as the reaction conditions have been published previously (Wellehan et al., 2004). The amplification of longer fragments from the DNA polymerase gene with only the outer primers, or as a semi-nested PCR was also attempted on every positive sample.

To obtain additional adenoviral genome fragments (from the genes of IVa2, penton base, or pVII) from the positive samples, numerous family- or genus-specific, consensus PCR primers were tested, of which all but one failed. The only PCR that resulted in specific products from a portion of the previously positive samples was surprisingly a nested method designed to amplify a 476-bp fragment from the gene of the major capsid protein, the hexon of siadenoviruses. These somewhat degenerate primers were designed on the basis of highly conserved amino acid (aa) motives selected by aligning the hexon sequence of four siadenoviruses known at that time from turkey, frog, raptorial birds and a great tit (Pitcovski et al., 1998; Davison et al., 2000; Kovács et al., 2010; Kovács and Benkő, 2011). The name and sequence of the primers are: sihexfoout (5′–TTY AAY CAY CAY MRN AAY MGN GG–3′), sihexreout (5′–CAT YTG NAY NAR VHA CCA RTC–3′), sihexfoin (5′–GAR TGG TGG TTY MGN AAR GAY CC–3′), and sihexrein (5′–GGN AVN ARN AND CKR TCR TTN CC–3′). These PCRs were carried out in 50 μl of final volume. The reaction mixture consisted of 37.25 μl distilled water, 5 μl of 10 × buffer (DreamTaq, Fermentas), 0.25 μl thermo-stable DNA polymerase enzyme (DreamTaq, Fermentas), 1 μl (50 μM) of each (forward and reverse) primer, 2 μl of $MgCl_2$ (25 mM), 1.5 μl of dNTP solution of 10 mM concentration, and 2 μl target DNA. The PCR program involved an initial denaturing at 94 °C for 5 min, followed by 45 cycles of denaturing at 94 °C for 30 s, annealing at 46 °C for 60 s, and elongation at 72 °C for 60 s. The final elongation was performed at 72 °C for 3 min. In the second round of this nested PCR, 2 μl of reaction mixture from the first round was used as target with an identical cycling program.

Based on the partial sequences obtained from the DNA polymerase and hexon genes of the putative testudine AdVs, specific primers for a nested PCR to amplify the approximately 11-kb-long central genome part were designed and tested on a number of samples with the use of different DNA polymerase enzymes including KOD XL (EMD Millipore, Merck), FailSafe (Epicentre), and Phusion (Finnzymes).

2.3. Sequencing and sequence handling

Sequencing of every amplicon was first attempted on both strands directly with the respective PCR primer. The reactions were performed with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The electrophoresis was done by commercial services on ABI PRISM 3100 Genetic Analyzers. If the trace signals indicated mixed target (i.e. the presence of more than one virus in the sample was supposed), the PCR products were cloned by using the CloneJET PCR Cloning Kit (Thermo Scientific Fermentas) according to the manufacturer's instructions, and subsequently the cloned fragments were sequenced with the matching pJET1.2. Forward and Reverse Sequencing Primers.

The programs for handling, identification, processing, contig joinings and editing of the nucleotide sequences have been described in detail elsewhere (Doszpoly et al., 2011; Hemmi et al., 2011).

2.4. Phylogenetic analyses

Alignments of the deduced aa sequences were prepared using the MultAlin Interface (Corpet, 1988) with default parameters (Blossum62) and occasional manual editing. Phylogenetic calculations were performed using the TOPALi v2.5 program package (Milne et al., 2009) as described in detail earlier (Doszpoly et al., 2011; Hemmi et al., 2011). First the model selection program for the DNA polymerase and the hexon aa sequences was run, which indicated the RTRev+G substitution model as the most suitable for both the MrBayes and the PhyML calculations. The Bayesian inference of phylogeny was carried out by the MrBayes method (Ronquist and Huelsenbeck, 2003) with the following parameters:

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