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Adenovirus and mycoplasma infection in an ornate box turtle (*Terrapene ornata ornata*) in Hungary

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Abstract

A female, adult ornate box turtle (*Terrapene ornata ornata*) with fatty liver was submitted for virologic examination in Hungary. Signs of an adenovirus infection including degeneration of the liver cells, enlarged nuclei and intranuclear inclusion bodies were detected by light microscopic examination. The presence of an adenovirus was later confirmed by obtaining partial sequence data from the adenoviral DNA-dependent DNA-polymerase. Phylogenetic analyses revealed that this novel chelonian adenovirus was distinct from previously described reptilian adenoviruses, not belonging to any of the recognized genera of the family *Adenoviridae*. As a part of the routine diagnostic procedure for chelonians the detection of herpes-, rana- and iridoviruses together with *Mycoplasma* spp. was attempted. Amplicons were generated by a general mycoplasma polymerase chain reaction (PCR) targeting the 16S/23S ribosomal RNA (rRNA) intergenic spacer region, as well as, a specific *M. agassizii*
PCR targeting the 16S rRNA gene. Based on the analyses of partial sequences of the 16S rRNA gene, the *Mycoplasma* sp. of the ornate box turtle seemed to be identical with the recently described eastern box turtle (*Terrapene carolina carolina*) *Mycoplasma* sp. This is the first report of a novel chelonian adenovirus and a mycoplasma infection in an ornate box turtle (*Terrapene ornata ornata*) in Europe.

Keywords: Ornate box turtle; Terrapene ornata ornata; Adenovirus; Mycoplasma

**Introduction**

Adenoviruses (AdVs) are double stranded non-enveloped viruses with a diameter of 80-110 nm. They are present in a wild range of vertebrate species, including mammals, birds, reptiles, amphibians and fish. They belong to the family *Adenoviridae* which presently contains four accepted genera (*Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*) and a fifth proposed genus (*Ichtadenovirus*) containing the white sturgeon AdV 1, the only known fish AdV so far (Benkő et al., 2005; Kovács et al., 2003).

Adenovirus-like viruses have already been detected in various reptilian hosts, including snakes and lizards, as well as in Nile crocodiles (*Crocodilus niloticus*) (Fry et al., 1994; Ramis et al., 2000; Kim et al., 2002; Wellehan and Johnson, 2005; Kübber-Heiss et al., 2006), a leopard tortoise (*Geochelone pardalis*) (Wilkinson, 2004) and Sulawesi tortoise (*Indotestudo forstenii*) (Rivera et al., 2008). Based on available sequence data, most of the snake and lizard AdVs examined have been shown to belong to the genus *Atadenovirus* (Wellehan et al., 2004, Farkas et al., 2008; Papp et al., 2009). From chelonians molecular data is currently available only from the Sulawesi tortoise AdV (Rivera et al., 2008). Interestingly, the recently detected Sulawesi tortoise AdV appears to be a member of the genus *Siadenovirus* (Rivera et al., 2008).
Mycoplasmas can infect different reptile species, but they are most often detected from chelonians. Some of them are members of the normal flora living in association with the host, others may cause diseases leading even to the death of the animal (Brown et al., 2002). *M. testudinis* is a non-pathogenic agent of the excretory tract in Greek tortoises (*Testudo graeca*), *M. agassizii* (Brown et al., 1999) or *M. testudineum* (Brown et al., 2004) is causing the upper respiratory tract disease in wild and captive tortoises in the USA and Europe. In the eastern box turtles (*Terrapene carolina carolina*) the detected *Mycoplasma* sp. was associated with clinical signs of upper respiratory tract disease-like syndrome (Feldman et al., 2006). Due to its high incidence and clinical importance mycoplasma detection is a part of the routine diagnostic procedures for chelonians in many laboratories.

This is the first description of a novel AdV and a box turtle *Mycoplasma* sp. infection in an ornate box turtle (*Terrapene ornata ornata*) in Europe characterized by fatty liver degeneration and inclusion body hepatitis.

**Material and methods**

**Case history**

On February 12, 2008 the carcass of a female ornate box turtle (*Terrapene ornata ornata*) was delivered to Szent István University, Faculty of Veterinary Science, Department of Pathology and Forensic Veterinary Medicine for postmortem evaluation. The turtle might have been dead for several days before finding it. Prior to its death the turtle was hibernated at 6-12°C beginning in November and monitored weekly. Before hibernation the turtle was fed daily with insects (*Acheta domesticus*, *Gryllus bimaculatus*, *Gryllus assimilis*; *Zophobas morio* larvae) purchased in a pet shop. Drinking water was constantly available in a shallow dish.
Gross examination and microbiological investigations

A routine pathological dissection was carried out with macroscopic examination. Various organs were examined and samples fixed in 4% buffered formalin for later histological investigations. These tissues were then embedded in paraffin, cut in ultra-thin sections and stained with hematoxinil-eosin. Bacterial culture was carried out from the liver of the turtle on blood agar and Drigalski agar at 24°C for 48 hours. Samples of the liver, lung and intestines were collected for virus isolation and polymerase chain reaction (PCR). Small pieces of the organs were homogenized in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, USA), diluted, passed through a 0.2 µm filter and used for inoculation of turtle heart (ATCC CCL-50) cell monolayer supplemented with DMEM and 2% fetal bovine serum. The cultures were incubated at 28°C in 5% v/v CO₂ and observed daily for cytopathic effects.

DNA extraction and PCR

Small tissue specimens of the liver, lung and intestine were used for DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. A nested PCR targeting a portion of the adenoviral DNA-dependent DNA-polymerase gene (Wellehan et al., 2004) was carried out. The sample was also screened for chelonian herpesviruses (VanDevanter et al., 1996), ranaviruses (Marschang et al., 1999) and invertebrate iridoviruses (Jakob et al., 2002, Weinmann et al., 2007) by PCR according to the protocols described by the authors. For detection of mycoplasma infection a general mycoplasma PCR (Lauerman et al., 1995), amplifying a fragment from the end of the 16S ribosomal RNA (rRNA) gene through the intergenic spacer region (ISR) to the beginning of the 23S rRNA gene, was used. After obtaining and sequencing the amplicon of the general PCR, a specific M. agassizii PCR (Brown et al., 1999) was carried out targeting the 16S rRNA gene to gain more sequence data.
Sequencing and sequence analyses

PCR products were directly sequenced by using the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) according to the manufacturer instructions. The sequencing reaction was analyzed on an ABI PRISM® 3100 Genetic Analyzer. New sequences were compared with sequences deposited into GenBank (National Center for Biotechnology Information, Bethesda, USA) using BLASTX and BLASTN (Atschul et al., 1997). Pairwise and multiple alignments of nucleotide and amino acid sequences were made with the EMBOSS Pairwise Alignment Algorithms (Labarga et al., 2007) and the Multalin computer program (Corpet, 1988), respectively. The aligned sequences were edited with the GeneDoc program (Nicholas and Nicholas, 1997).

Phylogenetic analyses were carried out with several programs. Bayesian analysis was performed using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with gamma-distributed rate variation, a proportion of invariant sites and Rtrev aminoacid substitution model. Maximum likelihood analysis was carried out by using the PhyML online web server (Guindon et al., 2005) with Rtrev aminoacid substitution model. Distance matrix analysis was performed with programs included in the PHYLIP (Phylogeny Inference Package, version 3.68) program package (Felsenstein, 1989). The trees were visualized using the TREEVIEW 1.6.6 program (Page, 1996).

Results and discussion

Adenoviral infections are very common in different reptile species, but have only been described in chelonians in two cases – in leopard tortoise (Wilkinson, 2004) and in Sulawesi tortoises (Rivera et al., 2008) - so far. There is only limited information published about the AdV of the leopard tortoise which was isolated together with a herpesvirus. The tortoise
exhibited biliverdinuria, wasting and episodes of hemorrhages were observed. The Sulawesi tortoise AdV caused severe systemic disease with very high mortality rate, 92 of 105 animals died due to the infection. Multifocal hepatic necrosis with amphophilic to basophilic intranuclear inclusions and diffuse hepatic lipidosis were observed in the Sulawesi tortoises. Other pathologic changes included myeloid necrosis in the bone marrow and severe necrotizing enterocolitis.

Pathologic findings observed during macroscopic and microscopic examination of the box turtle were typical of an adenovirus infection. Upon opening its body cavity the liver appeared clay yellow in color. A small amount of transparent fluid was observed in the stomach, the mucosa of which was intact and wrinkled. Transparent phlegm was seen in the intestines and its mucosa had a slightly reddish color. The microscopic examination of the liver revealed degeneration of liver cells, a pronounced vacuolization of the cytoplasm and pyknosis of nuclei (Fig. 1). Large vacuoles were observed in the cytoplasm of some liver cells, in some cases leading to marginization of the nuclei, whereas only small, fine vacuoles were present in other cells. A light-bordered bright purplish blue, intensely staining inclusion body was also seen in some nuclei. These nuclei were substantially enlarged, with chromatin marginalization. Lymphoid cell infiltration was seen along the blood-vessels of the liver. The submucosa of the small intestine also showed lymphoid cell infiltration.

Although attempts at virus isolation were unsuccessful, the sample was PCR positive for adenoviruses supporting our observations described above. Chelonian herpesviruses, as well as ranaviruses and invertebrate iridoviruses were not detected by PCR. Nucleotide sequencing confirmed our PCR results. The sequence of the 275 bp long fragment of the partial DNA-polymerase (Accession number: EU828750) gene indicated a novel AdV, with a balanced GC content (49.45%). Comparison of the Box turtle AdV sequence with the corresponding region of other AdVs showed that it was unique and distinct from previously known adenoviral
sequences. Amino acid sequence alignment of the DNA-polymerase gene showed that motifs found in all known AdVs were less conserved in the Box turtle AdV (see Supplementary material). Phylogenetic analyses also gave an interesting result (Fig. 2; see Supplementary material). While the Sulawesi tortoise AdV seems to be related to siadenoviruses, the Box turtle AdV did not cluster to any known genera. Although there were differences between the branching topologies obtained by the different programs, all calculations confirmed the distinctness of the Box turtle AdV. The analyses were based on a relatively short nested PCR product (91 amino acid), therefore final conclusions cannot be drawn; further investigations are needed to reveal its genome organization, further genes (hexon, protease) should be included into phylogenetic calculations to establish the exact taxonomic position of this exceptional virus. Characterization of other turtle and tortoise AdVs would also give more information about the evolution and origin of chelonian AdVs.

Mass mortality in reptiles due to an adenoviral infection was demonstrated previously only in a few cases (Kim et al., 2002; Rivera et al., 2008), usually single animals died, therefore the pathogenicity of reptilian adenoviruses and their ability to cause primary disease remains uncertain. Factors leading to immune suppression have been postulated to play a role in the development of adenoviral disease, including stress (transportation, overcrowding), inadequate housing, hibernation, co-infection with other pathogens (viruses, bacteria and/or parasites), etc. In our instance the death of the turtle was also a single case. Additional research is necessary to determine the prevalence of AdVs in chelonians and its clinical importance.

Bacterial culture of the liver resulted in no growth; however, mycoplasma DNA was detected by PCR. Sequences of the ISR (Accession number: FJ159565) and from the 16S rRNA (Accession number: FJ159564) were obtained from the detected Mycoplasma sp. The analysis of the amplified partial 16S rRNA sequence showed it to be identical with the formerly
identified *Mycoplasma* sp. of the eastern box turtle (Feldman et al., 2006). There was only 1
nucleotide difference between the overlapping sequences of the 16S rRNA gene. Pairwise
alignment of the amplified fragment containing the less conserved ISR sequence and the
corresponding region of the other two *Mycoplasma* spp. using testudinian hosts, *M. agassizii*
(AY780801) and *M. testudineum* (AY973570) demonstrated 87.9% (5.1% gaps) and 75.2%
(11.3% gaps) sequence identity, respectively. Unfortunately the homologous sequence of the
eastern box turtle *Mycoplasma* sp. was not available. Comparison of the chelonian
mycoplasma ISR sequences also confirmed that the box turtle *Mycoplasma* sp. is a distinct
species, since it differs from the previously characterized *Mycoplasma* spp. described in
chelonians.

Although we could detect the presence of the *Mycoplasma* sp. by PCR in our case, there were
no pathological lesions consistent with disease caused by mycoplasma. Successful isolation of
the detected infectious agents and experimental transmission studies should be carried out in
order to clarify the role the Box turtle AdV and the *Mycoplasma* sp. plays in the death of the
turtle.

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*Atadenovirus*. Virus Res. 132, 132-139.


**Figure captions:**

Fig. 1. Inclusion body hepatitis in an ornate box turtle. Histological section of the liver (magnification: 300x, staining: hematoxilin-eosin); 1. inflammatory cell infiltration, 2. cross-section of a blood-vessel, 3. physiological pigmentation, 4. intranuclear inclusion body in the hepatocytes.

Fig. 2. Unrooted phylogenetic tree based on maximum likelihood analysis of partial adenoviral DNA-dependent DNA-polymerase amino acid sequences showing the clustering of adenoviruses: Bayesian posterior probability values for branchings are in bold. Bootstrap values for the maximum likelihood tree are given for 100 data sets. Bayesian posterior probability values and bootstrap values are shown only for the main branches. Abbreviations of the adenovirus types: B, bovine; C, canine; D, duck; F, fowl; Fr, frog; M, murine; O, ovine; P, porcine; S, simian; T, turkey; TS, tree shrew. GenBank accession numbers are as follows:
Agamid AY576678; B1 NC_006324; B4 AF036092; C1 U55001; Chameleoniid AY576679; D1 AC_000004; Eublepharid AY576677; F1 AC_000014; F9 AC_000013; Fr1 AF224336; Gekkonid AY576681; H2 J01917; H4 AY487947; H40 L19443; Helodermatid AY576680; M1 AC_000012; O7 U40839; P3 AB026117; P5 AF289262; Parakeet EU056825; Meyer AY644731; S21 AC_000010; Scincid AY576682; Snake DQ106414; Sulawesi EU056826; T3 AF074946; TS NC_004453.
Figure 2.
Alignment of partial adenoviral DNA-dependent DNA-polymerase amino acid sequences generated by the Multalin computer program. Lines separate genera and the Box turtle adenovirus is in bold font.
Unrooted phylogenetic tree of partial adenoviral DNA-dependent DNA-polymerase amino acid sequences showing the clustering of adenoviruses. The tree was constructed with the programs included in the PHYLIP (Phylogeny Inference Package, version 3.572c) program package. For distance matrix analysis, the aligned sequences were processed first with PROTDIST (Jones-Taylor-Thornton substitution model) and then with the FITCH (Fitch-Margoliash; global rearrangements) program. For bootstrap analysis, the SEQBOOT program was run before PROTDIST and FITCH. The most probable tree was calculated with the
CONSENSE program. The length of the branches indicates the phylogenetic distance between the different viruses. Bootstrap values are given for 100 data sets and shown only for the main branches.