Szent István University
Postgraduate School of Veterinary Science

Genetic investigation of goose, turkey and other bird adenoviruses

Brief Version of the PhD Thesis

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1. Introduction

According to the official virus taxonomy, adenoviruses originating from birds are now classified into three different genera within the family *Adenoviridae*. Each of the two genera, *Si-* and *Atadenovirus*, contains one adenovirus isolated from birds. The genome of both of these viruses has been fully sequenced and published. From the members of the conventional *Aviadenovirus* genus that comprises the majority of avian adenovirus isolates, the full genomic sequence was known only from two fowl adenovirus types. From adenoviruses of turkey or water fowl, short, partial sequences of the hexon gene have been determined and preliminary phylogenetic calculations with these have indicated that adenoviruses of bird species belonging to the anseriformes or galliformes represent distinct genetic lineages, respectively. On the contrary, adenoviruses isolated from turkey are very similar to those from chicken.

With the use of recent, sensitive diagnostic methods based on the detection of the nucleic acid of microorganisms frequent occurrence of adenoviruses in Hungarian goose flocks has been revealed but the possible etiological role of these adenoviruses in clinical diseases is yet to be determined.

Molecular cloning and sequencing of a Hungarian turkey adenovirus strain has been started in our laboratory. The first aim of our work was to finish the complete genome sequencing of this strain. Furthermore we planned to sequence the complete genome of a Hungarian goose adenovirus strain, too. With different computer programs, we planned to identify genes that are common in every adenovirus, as well as to characterize eventual new open reading frames (ORFs).

Besides the genome sequencing, typing of additional turkey, goose, fowl and wild bird adenoviruses was attempted with PCR amplification and sequencing of a part of the hexon gene. The feasibility of other genes (DNA polymerase) in typing was also planned to be tested.

Successful completion of the planned investigations held out a promise to produce new results significant from both, theoretical and practical points of view. We expected that the theoretical results would help improve the taxonomic classification and interpretation of the assumed evolutionary pathway of bird adenoviruses. Amelioration of the diagnostic methods and exploration of eventual relationships between certain diseases and avian adenovirus types may supply important data for the poultry industry.

2. Materials and methods

2.1. Sequencing of the turkey and goose adenovirus 1 strains

In Hungary, a virus strain (designated as D90/2) was isolated by Vilmos Palya, DVM from the trachea of 10-week-old turkeys showing respiratory signs. It was propagated on chicken embryo liver cells. The Hungarian goose adenovirus strain P29 was also isolated by Vilmos Palya, DVM. For this purpose, goose embryo liver cells were used. We propose these two strains to be the prototype strains of turkey adenovirus 1 (TAdV-1) and goose adenovirus 1 (GoAdV-1). TAdV-1 was sequenced by random cloning, whereas GoAdV-1 by a shotgun sequencing approach.

2.2. Investigation of further bird adenoviruses by PCR

For comparative purposes the prototype strains of the original TAdV-1 and -2 types, originating from Belfast, were investigated. Furthermore, partial sequence characterisation of 10 strains, belonging to different fowl adenovirus types (FAdV-2–8b, -10, -11) from a prototype strain collection was carried out. Isolates or samples originating from adenovirus-infected animals were also tested. From these samples, the amplification of the partial DNA-dependent DNA polymerase gene or the gene of the major capsid protein, the partial hexon gene was attempted.

2.3. Bioinformatics

Sequences were handled and merged using the Staden Package. Virtual restriction endonuclease analysis of the TAdV-1 and GoAdV-1 genome was achieved using the program pDRAW32. The full genome sequences were annotated with the help of the Artemis program. The derived amino acid sequences were searched for the presence of putative domains by InterProScan Sequence Search. Phylogenetic calculations were performed using the Phylip package.

3. Results

3.1. Virtual restriction endonuclease fragment analysis of the genomes, and the investigation of TAdV-1 and -2 strains of Belfast

The pattern from virtual restriction endonuclease analyses of the Hungarian turkey adenovirus (D90/2) and goose adenovirus (P29) strains differed remarkably from those published for the Irish TAdV-1 and -2 or GoAdV-1, -2 and -3, respectively. Furthermore, the sequence of the PCR product from the hexon gene showed that the supposed progeny of the original TAdV-1 from the strain collection of Belfast is identical to FAdV-8a.

3.2. Features of the genome of turkey and goose adenovirus 1

The complete genome sequences of TAdV-1 and GoAdV-1 have been deposited in GenBank Nucleotide database with accession numbers GU936707 and JF510462, respectively.

The full genome of the TAdV-1 was found to consist of 45,413 bp. This is the longest adenovirus genome sequenced to date, and its G+C percentage (67.55%) is the highest among all fully sequenced adenovirus genomes. The inverted terminal repeat (ITR) in the TAdV-1 genome was found to be 95-bp long. The full genome of the GoAdV-1 is 43,376 bp with a G+C percentage of 44.6%, and ITRs of 39 bp. The genome organisation of the two strains is presented on Fig. 1.

In both genomes, a set of 16 central genes were found in the same order and direction as in every adenovirus genome annotated to date. This conserved genome part is followed directly with the U exon and the fiber genes as in the three sequenced FAdV genomes. In TAdV-1, one complete and two truncated fiber genes were found, whereas in GoAdV-1 two complete ones. At the left part of the TAdV-1 genome, homologues of ORF0, ORF1, ORF1A, ORF1B, ORF2, ORF12, ORF13, ORF14 and ORF24 are present. At the right side of the genome, homologues of the lipase, ORF8, ORF9, ORF11, ORF17, ORF20, ORF20A, ORF22 and ORF26 were found. Only one new ORF (ORF50) is hypothesised in the right end, its derived product shows no significant similarity to any proteins in the NCBI GenBank. At the left part of the GoAdV-1 genome, homologues of ORF1, ORF2, ORF12 and ORF24, and two new ORFs (ORF51, ORF60) were annotated. In the right end, besides the homologues of ORF20, ORF20A and ORF22, two copies of the lipase gene (supposedly duplicated) and eight new ORFs (ORF52-ORF59) were found.

TAdV-1:

GoAdV-1:

Fig. 1: Genome organisation of turkey adenovirus 1 (TAdV-1) and goose adenovirus 1 (GoAdV-1). The six lighter grey bars represent the six reading frames; exons are merged by thin lines. Genes highlighted in green are conserved in every adenovirus sequenced to date, red ones are common only to every aviadenovirus, and the homologues of yellow ones are present in certain aviadenoviruses only. White ORFs show no significant similarity to any protein sequences. Fiber-2-L and fiber-2-R in the genome of TAdV-1 represent the two resulting mutilated fiber genes originating from the break of the second fiber gene. DBP – DNA-binding protein; ism – repeat region; ITR – inverted terminal repeat; polimeráz – DNA polymerase; proteáz – protease; pTP – terminal protein precursor; U-exon – U exon

The coloured figure is available in the digital version of the thesis, which can be downloaded from the homepage of the Postgraduate School of Veterinary Science: http://phd.univet.hu/lapok/A-index-ert.htm

3.3. Phylogenetic analyses

Calculated on hexon sequences, the five accepted genera of adenoviruses are clearly distinct from each other on the phylogenetic tree, supported by high bootstrap values. TAdV-1 and GoAdV-1 clustered with other members of the genus Aviadenovirus, but their phylogenetic distance from the accepted species of the genus seemed to be adequate for the establishment of two new species (Fig. 2).

The partial nucleotide sequences of the DNA-dependent DNA polymerase gene from ten prototype strains of fowl adenoviruses were submitted to the GenBank and assigned to Accession Numbers HM853995 to HM854004. By phylogenetic analysis the types belonging to the same species appeared as monophyletic clusters. Moreover the types of species FAdV-D and -E had identical amino acid sequence in this region, respectively. The five species of FAdVs are distinct on the tree, supported by high bootstrap values.

The aviadenoviruses of bird species belonging to the order Anseriformes or Galliformes represent distinct genetic lineages, respectively. The PCR screening and sequencing of chicken samples revealed the result, that the FAdV types most common in Hungary belong to the species FAdV-D and -E, or sometimes FAdV-A. In wild bird samples besides aviadenoviruses at- and siadenoviruses were also detected.

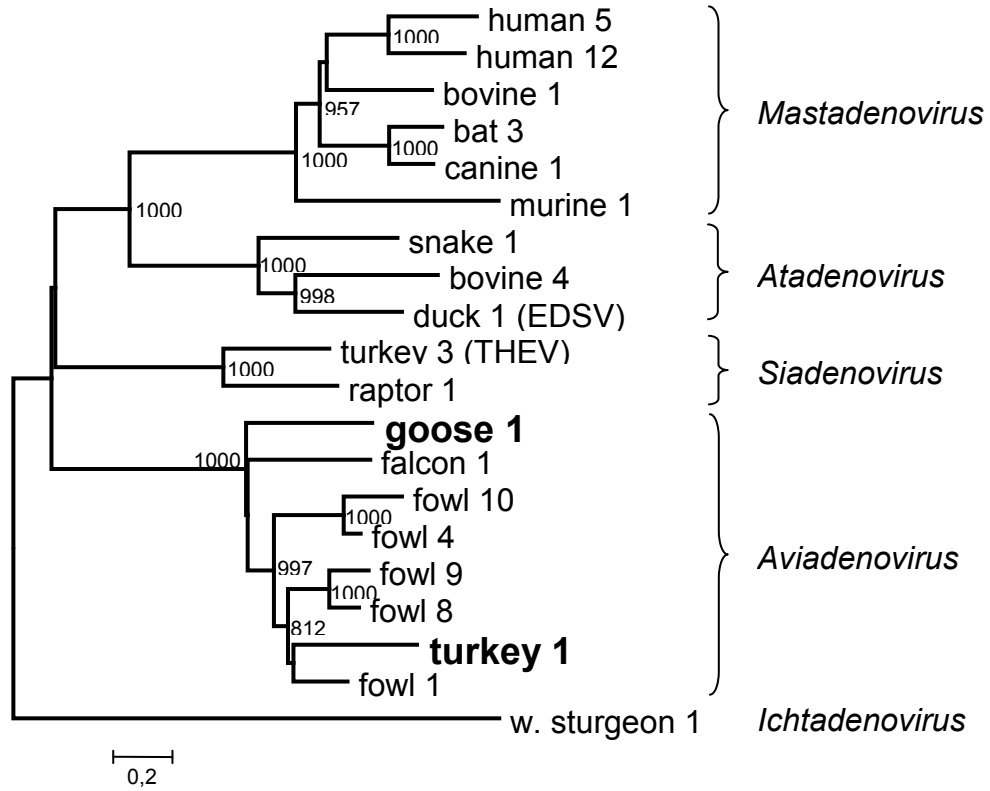

Fig. 2: Phylogenetic tree based on complete hexon amino acid sequences from adenoviruses. The tree was rooted for visualisation using the white sturgeon adenovirus 1. Adenovirus types are represented by the host animal and the type number of the virus. The turkey- and goose adenovirus 1 sequenced in the present work are in bold with larger fonts. Genus names are in italic. Bootstrap values are given for 1000 datasets if over 750. EDSV – egg drop syndrome virus; THEV – turkey hemorrhagic enteritis virus

4. Discussion

4.1. Turkey and goose adenovirus 1

The first complete genome sequences from non-chicken aviadenoviruses were determined. These results are compared to the work of other laboratories.

Most likely the original TAdV-1 and GoAdV-1 isolates have perished and no sequence is available from them. Thus, after discussing with fellow researchers on the field, we propose that the Hungarian strains D90/2 and P29 be considered as prototype strain of TAdV-1 and GoAdV-1, respectively. We propose to use these type numbers even though the virtual restriction endonuclease pattern of the genomes differed from that published for the original TAdV-1 and GoAdV-1 strains. Since these original strains are unavailable by now, it seems to be logical to fill in the gaps, instead of continuing the numbering. No sequence is available from the old strains, and that is not expected to change, as the presently available specimen of the Irish TAdV-1 is identical with the FAdV-8a type (strain 58).

Based on our results (genome organization, homologue genes, splice sites, protease cleavage signals, host species), it can be concluded, that both viruses, sequenced hereby, belong to the genus *Aviadenovirus*, and this is supported by the results of phylogenetic analyses, too. Yet both viruses are distinct from the already accepted aviadenovirus species. Accordingly, an official proposal will be made for the International Committee on Taxonomy of Viruses, claiming that Turkey adenovirus B is the species proposed for the type TAdV-1. The GoAdV-1 type will be proposed to be assigned into the *Goose adenovirus* species.

4.2. Phylogenetics of the fowl adenovirus reference strains; a polymerase gene based PCR for their diagnostics

The phylogenetic analysis based on the DNA-dependent DNA polymerase grouped the fowl adenoviruses in five major clades, which corresponded to the five accepted fowl adenovirus species. This nested PCR is the first method capable of recognising every adenovirus that might infect chickens, i.e. the members of all the three genera (*Avi-*, *At-* and *Siadenovirus*). The consensus primers were not designed specifically based on fowl adenoviruses, turkey hemorrhagic enteritis virus (THEV, TAdV-3) or the egg drop syndrome virus (EDS, duck adenovirus 1), thus the target range of this PCR seems not to be limited to the already known viruses. The recognition of a couple of novel adenoviruses in wild or exotic birds has already been published using it, and it is likely that newer fowl adenoviruses will also be found in the future. The amplified part of the DNA polymerase gene is so much conserved that it allows a preliminary, genus and species classification only. For the

intraspecies differentiation, the sequence of additional genome parts, preferably of the hexon, are needed.

4.3. PCR-screening of other bird samples; phylogenetics of new adenoviruses

In accordance with earlier findings, most of the fowl samples investigated contained adenoviruses belonging to species FAdV-D and -E. Types belonging to these species cause the most common adenoviral disease of chickens, the inclusion body hepatitis. Additionally FAdV-1 was also diagnosed from other chicken samples. In one case it could be connected to pathological findings, as some samples from Poland originated from a flock with gizzard erosion. Members of species FAdV-B and FAdV-C were detected once only, respectively. No members of FAdV-B species were detected in Hungary up to now, so this was the first Hungarian isolation and typing.

In a goose flock with hepatitis and hydropericardium, a novel goose adenovirus was detected. Although no amino acid mismatching could be detected in the polymerase sequence among the investigated four goose strains, their hexon sequences showed greater variation. The two strains, investigated on this region, too, differed remarkably. There was only 60% identity between the nucleotide sequence of this stretch from the completely sequenced P29 strain and the goose adenovirus strain causing hydropericardium. Based on this it can be hypothesised, that the four investigated goose strains belong to one species, but to at least two types.

Some bird adenoviruses were classified into the genus *Siadenovirus*, but as the number of bird adenoviruses within the genus rises constantly, it is not surprising. Interestingly even more bird adenoviruses were classified into the genus *Atadenovirus*. In one sample, two different adenoviruses could be detected. Based on the results of phylogenetic calculations, the two adenoviruses represent two, distinct virus species. All but one bird atadenovirus samples formed a monophyletic clad, which harbour bird atadenoviruses exclusively. The hosts of these viruses belong to the order Passeriformes in almost every case. The only exception within the clad is the duck adenovirus 1 (EDSV). Atadenovirus was found in six samples of house sparrow. Five from these six adenoviruses might represent one species, because they do not differ significantly in the polymerase sequence. It is assumed, that atadenoviruses have coevolved with passeriform birds for a long time, and their presence in these birds might be the consequence of a rather ancient host switch.

5. New scientific results

1. The complete genome sequences of non-chicken aviadenoviruses were determined for the first time. By the analysis of a turkey and a goose adenovirus it was concluded, that they belong to the genus *Aviadenovirus*.

2. The partial nucleotide sequence of the DNA-dependent DNA polymerase gene was determined from ten reference strains of fowl adenoviruses (FAdVs). The sequences were acquired by using a very reliable, sensitive, nested PCR. By providing these data, the veterinary diagnostic value of the PCR method was significantly increased.

3. A fowl adenovirus isolate was molecularly typed as member of the species *Fowl adenovirus B*, demonstrated for the first time in Hungary.

4. PCR-screening of samples originating from various fowl and wild birds yielded known fowl adenovirus types and new adenoviruses.

5. It was determined that most of the fowl adenovirus strains from Hungary or the neighbouring countries belong to species FAdV-D and -E, more rarely to FAdV-A.

6. It was revealed that the newly detected wild bird adenoviruses belong usually to the genus *Aviadenovirus*, but in passeriform birds, at- and siadenoviruses are also common.

6. Scientific publications

6.1. In peer-reviewed journals

Kaján Gy.L., Kecskeméti S.: **Egy tyúk-adenovírus B fajba tartozó típus első magyarországi izolálása**, Magy. Állatorv. Lapja, 2011., [in press]

Kaján Gy.L., Sameti, S., Benkő M.: **Partial sequence of the DNA-dependent DNA polymerase gene of fowl adenoviruses: A reference panel for a general diagnostic PCR in poultry**, Acta Vet. Hung., 59. 279-285, 2011.

Kaján Gy.L., Stefancsik R., Ursu K., Palya V., Benkő M.: **The first complete genome sequence of a non-chicken aviadenovirus, proposed to be turkey adenovirus 1**, Virus Res., 153. 226-233, 2010.

Ivanics É., Palya V., Markos B., Dán Á., Ursu K., Harrach B., Kaján Gy., Glávits R.: **Hepatitis and hydropericardium syndrome associated with adenovirus infection in goslings**, Acta Vet. Hung., 58. 47-58, 2010.

6.2. Book chapter

Harrach B., Kaján Gy.L.: **Aviadenovirus**, In: *The Springer Index of Viruses.* Eds.: Tidona, C.A., Darai, G., Berlin: Springer, 2011., [in press]

6.3. Congress proceedings/abstracts

Kaján Gy.L., Davison, A.J., Harrach B., Palya V., Benkő M.: **Beyond mammalian adenoviruses: genome and phylogeny of goose adenovirus**, 8th Int. Adenovirus Meeting, Zürich, 133, 2006.

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Kaján Gy.L., Davison, A.J., Harrach B., Palya V., Papp T., Stefancsik R., Benkő M.: **Sequencing and comparative analysis of a goose and a turkey adenovirus**, Acta Microbiol. Immunol. Hung., Abstracts of the 15th Int. Congr. Hung. Soc. Microbiol., 54. (Supplement) 53, 2007.

Kaján Gy.L., Davison, A.J., Palya V., Harrach B., Benkő M.: **Full genome sequence analysis of a goose and a turkey aviadenovirus**, 9th Int Adenovirus Meeting, Dobogókő, 92, 2009.

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Vet. Virol., 20 years of ESVV: Integrating classical and molecular virology, Budapest, 110, 2009.

Kaján Gy.L., Davison, A.J., Palya V., Harrach B., Papp T., Stefancsik R., Benkő M.: **Comparative genome analysis of aviadenoviruses isolated from goose and turkey**, XIV. Int. Congr. Vir., Istanbul, 217, 2008.

6.4. Other publications in peer-reviewed journals

Mayer B., Kis Zs., Kaján Gy., Frenyó L.V., Hammarström, L., Kacskovics I.: **The neonatal Fc receptor (FcRn) is expressed in the bovine lung**, Vet. Immunol. Immunopathol., 98. 85-89, 2004.

Papp T., Fledelius, B., Schmidt, V., Kaján Gy.L., Marschang, R.E.: **PCR-sequence characterization of new adenoviruses found in reptiles and the first successful isolation of a lizard adenovirus**, Vet. Microbiol., 134. 233-240, 2009.