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Diversity and genetic features of avian adenoviruses

Summary of PhD thesis

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Introduction

The family *Adenoviridae* is divided into five genera (*Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus*). Birds are among the few hosts that can be infected by rather divergent adenoviruses (AdV) classified into three different genera, namely *Aviadenovirus*, *Siadenovirus* and *Atadenovirus*.

Chicken, turkey, goose and duck were the first bird species in which AdVs were found as these economically important species were the most intensively studied. A large number of the so-called fowl adenoviruses (FAdVs), which were found in chicken were described already several decades ago. Twelve FAdV serotypes (FAdV-1 to -8a, -8b to -11) are grouped into 5 species, named *Fowl aviadenovirus A* to *E*. Duck adenovirus 1 (DAdV-1) is a member of the genus *Atadenovirus* and is characterized by severe drop in egg production and shell formation disorders in the host; this virus can infect chickens as well. Turkey adenovirus 3 (TAdV-3) has been found in turkey, chicken and pheasant causing different clinical signs in each species. At the start of this study, only 3 AdVs were known from turkey, out of which TAdV-1 and -2 belong to the genus *Aviadenovirus*, while TAdV-3, responsible for haemorrhagic enteritis of turkey is a member of the genus *Siadenovirus*. Full genome sequences in GenBank were only available for TAdV-1 and -3.

Pigeons infected with AdVs were first reported in Belgium in 1984, but by now AdV infection has been observed all over the world. Evidences for the presence of AdVs in pigeons were compiled by electron microscopy, histology and serology. Up to now the only distinct pigeon adenovirus (PiAdV) strain that could be isolated on chicken embryo liver cells was reported in Germany in 1998. PCR methods applied in routine FAdV diagnostics were also used for the molecular detection of PiAdVs; however, these methods were not fully reliable due to the significant sequence differences between FAdVs and PiAdVs. Little was known about the prevalence and diversity of PiAdVs in the Hungarian pigeon lofts.

As to wild and exotic birds, AdVs have been found in representatives of the orders Falconiformes (falcon adenovirus 1) and Psittaciformes (psittacine adenovirus 1 and 2, budgerigar adenovirus 1, Meyer's parrot adenovirus 1). Great tit adenovirus 1 and raptor adenovirus 1 detected in two owl and a hawk species were described and characterized by our research group. Except for the great tit (*Parus major*), all the above listed host species are captive animals either for hobby purposes or under the aegis of nature conservation in order to breed them artificially. The international literature on AdVs of free-living birds was limited at the time of the start of this study and no information was available about the prevalence and diversity of AdVs in the Hungarian avifauna. AdVs are known to show stringent host and cell specificity, which makes their isolation challenging as there are no cell

lines available from a wide range of avian species. Molecular techniques can be applied for in depth studies of these non-isolated AdVs.

Objectives

The aim was to determine the genomic organization of two novel turkey aviadenoviruses (TAdV-4 and -5) in order to get a better overview of the diversity and evolution of aviadenoviruses.

Another goal was to study the prevalence and diversity of AdVs present in Hungarian pigeon lofts and characterize the most common types. The PCR methods recommended for the diagnosis of avian and pigeon AdVs were tested and compared with a sensitive, nested PCR system which had already been found to be broadly applicable for the detection of various AdVs.

An objective was to study the evolution and relation of fibre genes of aviadenoviruses involving the fibre gene sequences of the newly described PiAdV-2a, TAdV-4 and TAdV-5.

In order to get a better insight of the diversity of AdVs in a broad range of bird species, an extensive AdV screening was performed among free-living birds and bird species that could be found in the natural Hungarian avifauna and among captive exotic birds from Hungarian zoos and breeding lofts. Investigation of co-evolution of the host species and their AdVs was studied by the help of phylogenetic methods.

Materials and methods

Origin of the samples

The TAdV-4 and -5 strains designated TNI1 and 1277BT respectively, was isolated in the United Kingdom from turkey breeds. The Hungarian TAdV-5 strain (D1648) from turkeys showing respiratory signs was isolated on chicken embryo fibroblast cells by Dr. Vilmos Palya.

In total, 97 racing and fancy pigeon samples originating from 27 lofts were subjected for screening for the presence of AdVs. Different organ (liver, lungs, kidneys and intestine) samples were obtained from dead or euthanized pigeons showing clinical signs suggesting young pigeon disease syndrome (YPDS). Dropping samples were also collected from clinically healthy specimen originated from lofts in which earlier the presence of AdVs had been suspected.

In this study, 124 specimens from 21 parrot species, 115 other exotic bird specimens from 27 species and 434 individual samples originating from 83 wild, free-living bird species from the Central-European avifauna were screened for the presence of AdVs. Most of the parrot and exotic bird samples (cloacal swabs, dropping and organ samples) originated from the Budapest Zoo and two private flocks. Cloacal swabs from free-living Passeriform birds, white storks (*Ciconia ciconia*), gulls (Laridae), and common kestrels (*Falco tinnunculus*) were collected during bird ringing activities from Hungary and Ukraine. From Croatia, an extracted DNA collection from cloacal swabs containing mostly gull and cormorant (*Phalacrocorax sp.*) samples was obtained. Carcasses of wild birds died in accidents were collected from the territory of Hungarian national parks (Aggtelek and Hortobágy) by the help of rangers and veterinarians. Cloacal swabs from rescued birds were kindly provided by Budapest Zoo and the bird hospital of Hortobágy.

Polymerase chain reactions

Cell supernatants, cloacal swabs, organ and dropping samples were subjected to DNA isolation. The diagnostics of AdVs was performed by a highly sensitive consensus nested PCR which is specific for the conserved DNA polymerase gene of AdVs. According to our earlier experiences, this so-called pan-AdV PCR, published by Wellehan et al. (2004) is suitable for the detection of all the so far described AdVs, regardless the genus they belong to. From the novel virus types, further sequences were attempted to be amplified by genus specific PCR methods. Viruses (PiAdV-2a, TAdV-5), chosen for full genome analysis were sequenced by primer walking method with individually designed, specific PCR primers.

DNA sequencing and bioinformatics

The isolated strains of TAdV-4 and TAdV-5 (1277BT) were subjected to next generation Illumina sequencing by the Austrian partner. Sequencing of the genome of the Hungarian TAdV-5 strain and the non-isolated PiAdV-2a was performed by primer-walking method and Sanger sequencing. The sequences were edited and assembled in CLC Genomics Workbench v.4.0 program or in the Staden program package. Prediction of genes and open reading frames was performed by the help of Artemis and JavaScript DNA translator 1.1 programs. Splice patterns were identified manually based on conserved splice signals. Full genome maps were visualized by CLC Main Workbench 7.6.

The deduced amino acid (aa) sequences were aligned by the help of Clustal Omega and Muscle programs. In order to find a model that fits our dataset the best, model selection was performed by Topali 2.5 program package or online via the ProtTest server prior to phylogenetic calculations. For phylogenetic calculations, two different methods were applied depending on the length of the available sequences. If only short sequences (<200 aa) were available, distance matrix analysis was performed. Bayesian algorithm could be used only when long enough sequences were obtained after alignments and editing. These analyses were carried out by the Topali 2.5 program package. For editing and visualization of phylogenetic trees, Mega6 program was applied.

Results

Turkey adenoviruses

The whole genome sequence of TAdV-4 was found to be 42,940 base pair (bp) long, while the genome of TAdV-5 strain 1277BT consists of 43,686 bp. The TAdV-4 genome showed 48.5% G+C (guanin+citozin) content, while this ratio proved to be slightly higher (51.6%) in TAdV-5. The sequence of a 27,931 bp long continuous fragment of the Hungarian TAdV-5 strain (D1648) genome could be determined by primer-walking method. Between the homologous 27,931 bp long genome fragments of the two TAdV-5 isolates, 41 nucleotide (nt) (0.15%) differences could be identified. TAdV-4 genome encodes a single fibre gene, while in TAdV-5 two fibre genes could be found.

According to the phylogenetic calculations based on the full polymerase and hexon genes and the partial fibre gene sequence, the closest relative of TAdV-4 is FAdV-5, while based on the hexon L1 region it constitutes a monophyletic group with TAdV-2 (which cannot be shown on other phylogenetic trees as this is the single region which is known from TAdV-2). Based on all phylogenetic calculations, the closest relative of TAdV-5 is FAdV-1.

Pigeon adenoviruses

From the 97 screened pigeon samples, 48 (49.5%) were found to be positive for AdVs. From 33 pigeons, four types of organ samples (liver, kidneys, lungs and intestines) were screened separately and among these birds 22 (66.7%) proved to be infected with AdVs. Contrarily, in the 47 pigeons screened exclusively based on liver samples, much lower positivity (36.2%) was observed. Droppings of clinically healthy individuals, originated from lofts where previously AdV infections were observed, showed a remarkable, 52.9% AdV positivity.

The analysis of the positive samples showed that more than half of the positive pigeons were infected with aviadenoviruses, while in 13 cases siadenoviruses were identified. Based on the partial sequence of the adenoviral polymerase gene, 3 types of aviadenoviruses could be differentiated. Among these, the most common type, PiAdV-2 could be detected in 33 cases. By the further comparison of PiAdV-2 nt sequences, two subtypes PiAdV-2a and -2b could be distinguished. Another new aviadenovirus type, PiAdV-3 could only be found in a single sample, as well as the earlier published PiAdV-1. From siadenoviruses two types were identified, the more abundant type (PiAdV-4) could be found in 12 cases, while PiAdV-5 is a rather rare type represented by a single detection. PiAdV-4 and -5 show a remarkable difference (34.4%) in their aa sequence, even in the short 90 aa long partial polymerase gene region. Based on the phylogenetic calculations, PiAdV-1, -2

and -3 constitute a firm monophyletic group within the genus *Aviadenovirus*, while PiAdV-4 and -5 do not show a close relationship within the genus *Siadenovirus*.

A 31,314 bp long continuous genome region from PiAdV-2a was sequenced by primer-walking method. Two fibre genes could be identified in the genome of PiAdV-2a. Out of the two genes, the first one (fibre-1) is vestigial, only the tail and a short part of the shaft domain could be recognized, but the head domain which is the most important region from a biological point of view is missing.

Fibre genes of avian adenoviruses

According to the phylogenetic calculations, fibre genes of the AdVs carrying only one fibre gene in their genome constitute a monophyletic group with the second fibre (fibre-2) gene of the AdVs possessing two fibre genes, however within this group they are still separated from each other. The fibre-1 gene of AdVs carrying two fibre genes constitutes a distinct monophyletic group; the vestigial fibre-1 gene of PiAdVs belongs to this cluster as well.

Adenoviruses of parrots and other exotic birds

According to the results of the pan-AdV PCR, 10 out of the 124 (8.1%) parrot samples proved to be positive for AdVs. Samples from a cockatiel, 3 budgerigars, a rosella, a scarlet-chested parrot, and a red-fronted parakeet contained PsAdV-2, while new types of siadenoviruses were detected in two additional specimens.

From other 28 exotic bird species, 16 out of the 115 screened samples proved to contain AdVs. Siadenoviruses were diagnosed in 11 cases, while avi- and atadenoviruses were detected only once. Among siadenoviruses, 4 novel types could be distinguished and the avi- and atadenoviruses proved to be new types, too.

Adenoviruses of free-living birds

For the presence of AdVs, 434 specimens of wild birds originating from 83 species were screened. Positive PCR results were obtained in 102 cases, so 23.5% of the samples proved to contain AdVs. Aviadenoviruses were detected in 59 specimens and by the detailed sequence analysis 28 novel types could be distinguished. Siadenoviruses showed a great diversity, too; 13 novel types have been described. Atadenoviruses were found only in 7 specimens; however, 6 AdVs out of them proved to be novel atadenovirus types. The 7th case was the detection of DAdV-1 in a white stork sample.

Discussion

Turkey adenoviruses

In collaboration with Austrian scientists, the whole genome of TAdV-4 and TAdV-5 were sequenced on Illumina platform and they were annotated and analyzed. A Hungarian strain of TAdV-5 has been partially sequenced by primer-walking method.

Both in TAdV-4 and TAdV-5, balanced G+C contents were observed, suggesting long co-evolution between the AdVs and their current host species, which is actually true for all the so far described aviadenoviruses. From AdVs that can infect birds, most probably the aviadenoviruses are the ones that co-evolved with birds, while the si- and atadenoviruses possessing lower genomic G+C content got into bird species via several host switches later in time. The Hungarian and the British TAdV-5 strains were isolated in different locations and times but based on the sequence of their 27,931 bp long homologous genome region it has been confirmed that the two strains are almost identical and they share a common ancestor.

Genome organization of TAdV-4 was found to be similar to that of FAdV-5, while TAdV-5 has identical genome organization to FAdV-1 which is in accordance with the results of the phylogenetic calculations.

Based on their genetic features, different host species and phylogenetic positions, the establishment of two novel aviadenovirus species for TAdV-4 and TAdV-5 was proposed towards the International Committee of Virus Taxonomy. Our proposal was accepted; *Turkey aviadenovirus C* species has been established for TAdV-4 and *Turkey aviadenovirus D* for TAdV-5.

Pigeon adenoviruses

By the AdV screening of Hungarian pigeon lofts, unexpectedly high positivity (almost 50%) was observed and novel avi- and siadenovirus types were identified. A novel aviadenovirus, PiAdV-2 proved to be the most abundant type. It was confirmed that the earlier published PiAdV-1 and the novel PiAdV-3 aviadenoviruses are rather rare types in the Hungarian pigeon flocks. PiAdV-1, -2 and -3 constitute a monophyletic group within the genus *Aviadenovirus* suggesting their stringent co-evolution with their host species. This is the first report of the presence of siadenoviruses in pigeon. PiAdV-4 was found to be more abundant, while PiAdV-5 was detected only in a single case. These two viruses show a remarkable evolutionary distance on the phylogenetic tree. The lack of signs of co-evolution could be an indication of distinct, independent host switches from an unknown source to pigeon.

Earlier published PCR methods were tested and they were found to be unreliable for the detection of PiAdVs other than PiAdV-1; instead, pan-AdV PCR developed by Wellehan el al. (2004) should be applied for diagnostic purposes. It was showed that the type of the organ sample has quite a major impact on the outcome of the diagnosis. Kidneys and lungs seem to be much more reliable sample types than liver, however these can show false negative results, too, thus the most precise method is the screening of diverse organ samples when AdV infection is suspected. Circoviruses and *Escherichia coli* bacteria were suspected to be the main causative agents of YPDS. AdVs were almost concluded not to play any role in YPDS. Contrarily, AdVs can be detected very often in pigeons suffering from this disease according to the results of the current study. Remarkable number (52.9%) of virus shedding pigeons were found in lofts where YPDS had been observed in the past.

The G+C content of PiAdV-2a is well balanced (48.85%) suggesting a stringent co-evolution between the virus and the current host species. Two fibre genes were found to be encoded in the PiAdV-2a genome, however most probably only fibre-2 is still functional, while fibre-1 (similarly to PiAdV-1 fibre-1 gene) lost its ability to bind host cell receptors as it does not contain the most important, C-terminal head domain. Phylogenetic position of PiAdV-2a was determined based on polymerase, hexon and fibre gene sequences, congruently suggesting the common origin of PiAdV-1 and -2a.

Fibre genes of avian adenoviruses

In the genome of TAdV-4, a single, while in the case of TAdV-5 and PiAdV-2a two fibre genes were found encoded, however, the first fibre of PiAdV-2a is vestigial. Phylogenetic analysis of fibre genes showed that the presence of two fibre genes within the same genome is not the result of simple gene duplication, but most probably these genes possess different origin; one of the fibre genes could had been obtained by an ancient aviadenovirus from a different AdV genus. Probably the ancient aviadenoviruses possessed 2 fibre genes out of which the recent aviadenoviruses carrying a single fibre gene lost one of the fibre genes later during the evolution. This lost gene could be the fibre-1 which is in accordance with the observation in PiAdVs where the fibre-1 gene still can be found in the genome, but it is severely deficient and most probably not functional.

Diversity of avian adenoviruses

The majority of the AdVs known to infect birds belong to the *Aviadenovirus* genus, however, in the past decade numerous types of siadenoviruses were detected in birds, too. The *Atadenovirus* genus includes the least known avian AdV types. In our AdV screening, performed in total on 673 wild and exotic bird specimen belonging to 131 species, 19% of the

samples were found to be positive and thus the number of the known AdV types substantially increased. In total, 33 new aviadenovirus types (including the new PiAdV and TAdV types), 21 novel siadenovirus types (including the new PiAdV types) and 8 new atadenovirus types have been described. On a phylogenetic tree, based on an 87 aa long region of the DNA-dependent DNA polymerase gene of AdVs, the three genera, avi-, si-, and atadenoviruses are clearly separated from each other.

Aviadenoviruses were detected in 60 individuals of wild and exotic birds. Genus *Aviadenovirus* comprises exclusively AdV types derived from bird hosts. According to our theory, this is the AdV lineage that co-evolved with avian hosts, and the co-speciation of host and virus could be clearly observed on the phylogenetic trees. The host specificity of aviadenoviruses is very stringent; certain types of viruses can only be found in a single host species and usually they are not able to cross the host barrier. In gull species (family Laridae) an extraordinary AdV diversity was observed; in 3 species a total of 10 different AdV types were found. We confirmed that a single bird species can be infected with multiple AdV types, furthermore even a single specimen could carry more types of AdVs regardless which AdV genus they belong.

In this study, siadenoviruses were detected in 36 cases from wild and exotic bird species. By the sequence analysis of these viruses 19 new types were identified (plus 2 types, PiAdV-4 and -5 detected in pigeons). With these results, the number of the published siadenovirus types increased from 10 to 31. Based on earlier studies, amphibians were supposed to be the original hosts of siadenoviruses, however this theory was recessed because no more members than frog adenovirus 1 has been described from amphibian hosts. The *Siadenovirus* genus shows a remarkable phylogenetic distance from all the other AdV genera, however within the genus the clustering of the different AdV types according to their host species is not as clear as in the case of aviadenoviruses. A novel siadenovirus from Gouldian finches was described in parallel by our group and a research group in the United States. The G+C content of the Gouldian finch adenovirus 1 is very low 34.7%, which is rather common among the members of genus *Siadenovirus* and suggests a recent host switch. Siadenoviruses are usually more pathogenic and their ability to cross the host barrier is higher than that observed in aviadenoviruses. In our study, we further confirmed this theory by the detection of TAdV-3 in host species other than poultry; it was found in a white stork. We detected PsAdV-2 in Europe for the first time, furthermore it was found in 5 different parrot species as a further confirmation of its exceptional ability to cross the host barrier. In spite that most of the siadenoviruses are reported from avian hosts, we do not suppose this animal group to be the original host of siadenoviruses. Our results suggest that siadenoviruses do not possess as stringent co-evolution with their host species as aviadenoviruses. It is very likely that siadenoviruses got into avian hosts at a later time point

in evolution, via multiple, independent host-switches. Their high pathogenicity and low genomic G+C content supports this theory.

Atadenoviruses have been detected in wide range of animals, such as ruminants, squamate reptiles and birds. These atadenoviruses show a rather clear divergence on the phylogenetic tree, constituting three monophyletic branches. According to the genomic G+C contents and the phylogenetic relations, the presumptive origin of this virus genus are the squamate reptiles and some members of them got into avian hosts later during evolution. Atadenoviruses have been described only in a limited number of groups of birds, so according to our hypothesis, the host switches could have happened several times from reptiles to avian species, but not to all groups of birds, only to ostriches, parrots, passeriform birds and the members of the Galloanserae superorder.

The broad AdV screening revealed that birds represented by almost 10,000 species worldwide are unfailing sources of novel AdV types. Although, the precise interpretation of the obtained results is a challenge because the sequenced DNA fragments are too short, it is already clear that the diversity of avian AdVs is grandiose and even these short DNA sequences helped the exploration of the phylogeny of avian AdVs. The confirmed ability of si- and atadenoviruses for host switches draws our attention that such possible events cannot be excluded in the future; domesticated, economically important species can be infected with novel AdVs derived from wild birds and this can even result in severe disease outbreaks.

New scientific results

1. In collaboration with Austrian scientists, the complete genome sequence of turkey adenovirus 4 and 5 were determined and annotated, also their phylogenetic position was identified.

2. Extremely high (almost 50%) AdV positivity was found among Hungarian racing and fancy pigeons.

3. Two novel pigeon aviadenovirus (PiAdV-2 and -3) and two novel siadenovirus types (PiAdV-4 and -5) have been described and it was demonstrated that two of these are much more prevalent than the earlier published and characterized pigeon adenovirus 1. This is the first report of siadenoviruses in pigeon.

4. The genome of pigeon adenovirus 2a variant was partially sequenced and annotated.

5. A phylogenetic study of the aviadenoviral fibre genes suggested that probably the ancient aviadenoviruses possessed 2 fibre genes out of which the recent aviadenoviruses carrying a single fibre gene lost the fibre-1 gene. We assume the loss of this fibre-1 gene is an ongoing event in PiAdVs.

6. The pathogen psittacine adenovirus 2 was detected for the first time in Europe. It has been found in five parrot species in which the presence of psittacine adenovirus 2 has not been reported yet. This serves as another confirmation for the unique ability of psittacine adenovirus 2 to cross the host barrier.

7. The number of known avian adenovirus types has been multiplied. In total, 62 novel adenovirus types have been described, out of which 33 belong to genus *Aviadenovirus*, 21 to genus *Siadenovirus* and 8 to genus *Atadenovirus*. High (19%) AdV positivity was found in wild and exotic birds.

Scientific publications

Scientific publications in peer-reviewed journals

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