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DETECTION AND PARTIAL GENETIC CHARACTERISATION OF NOVEL AVI- AND SIADENOVIRUSES IN RACING AND FANCY PIGEONS (*COLUMBA LIVIA DOMESTICA*)

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Up to now, only a single adenovirus (AdV) isolate seemingly specific for pigeons, hence named pigeon AdV-1 (PiAdV-1), has been characterised at DNA sequence level. In the present work, the prevalence and diversity of AdVs occurring in domestic pigeon were examined by a survey performed on randomly collected samples using a very efficient, consensus nested PCR targeting the viral DNA polymerase gene. The newly detected viruses were characterised by sequencing and phylogeny analysis. Amplification of additional genome fragments was attempted by the use of several other PCR methods aiming at the hexon gene. During a 4-year survey, samples from dead or live, healthy pigeons originating from 27 lofts were examined in Hungary. Almost 50% of the samples (48 out of 97) proved to be positive for AdV. Sequence analysis revealed the presence of four hitherto unknown pigeon AdV types. PiAdV-1 was also identified in one sample. Two novel viruses named PiAdV-2 and -3 were found to belong to the genus *Aviadenovirus*, and two other novel types (PiAdV-4 and -5) to the genus *Siadenovirus*. This is the first report on the occurrence of siadenoviruses in birds belonging to the order Columbiformes. Approximately two-thirds of the PiAdV-2 genome was sequenced and analysed.

Key words: Pigeon, adenovirus, aviadenovirus, siadenovirus, phylogeny, PCR

Family *Adenoviridae* is divided into five genera (*Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus*). The presence of adenoviruses (AdVs) has been described in representatives of a large number of vertebrate species including primates as well as fish, amphibians and reptiles (Harrach et al., 2011). Birds are known to be common hosts for AdVs and this fact was mirrored by the large number of the so-called fowl AdVs (FAdVs) described from chicken already several decades ago (Smyth and McNulty, 2008). Moreover, birds are among the few hosts that can be infected by rather divergent

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AdVs classified into three different genera, namely *Aviadenovirus*, *Siadenovirus* and *Atadenovirus*.

Aviadenoviruses have been detected almost exclusively in birds and this genus is thought to have cospeciated with the class of Aves during evolution (Harrach and Kaján, 2011). Twelve FAdV serotypes (FAdV-1 to 8a, 8b to 11) are grouped into five species, named *Fowl aviadenovirus A* to *E* (informally abbreviated as *FAdV-A–E*) (Harrach and Kaján, 2011; Kaján et al., 2011). Additional aviadenovirus serotypes have been described from goose, duck and turkey (Kaján et al., 2012; Marek et al., 2014*a* and *b*). As to wild birds, aviadenoviruses have been found in representatives of the orders Falconiformes (Schrenzel et al., 2005), Psittaciformes (Raue et al., 2005*a*; Wellehan et al., 2005) and Charadriiformes (Bodewes et al., 2013). From Columbiformes, a single AdV named pigeon adenovirus 1 (PiAdV-1), has been characterised by DNA sequence (Hess et al., 1998; Marek et al., 2014*b*).

The presence of atadenoviruses and siadenoviruses in birds has been hypothesised to be the result of interclass host switches (Benkő and Harrach, 2003). Atadenoviruses presumably have coevolved with squamate reptiles (order Squamata) (Pénczes et al., 2014). The first members of the genus *Siadenovirus* were discovered in poultry showing different signs of serious disease (Smyth and McNulty, 2008), as well as in a frog (Davison et al., 2000). While the number of siadenoviruses detected in a wide range of birds is increasing (Zsivanovits et al., 2006; Kovács et al., 2010; Park et al., 2012; Lee et al., 2014), there are only two approved members of the genus *Atadenovirus* known to be capable of infecting birds. Pigeons have not been found to harbour AdVs from any of these two genera until now.

Adenoviral infection in pigeons was first reported from Belgium in 1984 (Coussement et al., 1984), but by now it has been observed all over the world. Evidences for the presence of AdV in pigeons were compiled by electron microscopy, histology and serology (de Herdt et al., 1995; Vereecken et al., 1998). De Herdt et al. (1995) described two different disease manifestations that had been suspected to be caused by specific pigeon AdVs. According to their classification, type I or classical adenovirus affects predominantly fledglings during their first racing season. The clinical signs include anorexia, enteritis, vomiting and diarrhoea. Post-mortem examination reveals inclusion body hepatitis. This disease often occurs in association with *Escherichia coli* infection. The so-called type II adenovirus or necrotising hepatitis is not age dependent and appears sporadically. The clinical signs affect the respiratory tract and the most frequent pathological finding is hepatic necrosis. A third condition, in which the potential role of AdVs has been examined, is the so-called Young Pigeon Disease Syndrome (YPDS). This multifactorial disease complex affects the age group of 4–12 weeks. Atypical clinical signs include anorexia, depression, ruffled feathers, vomiting and a fluid-filled crop. Generally, less than 20% of the young birds are affected but morbidity and mortal-

ity might sometimes reach 50%. Raue et al. (2005b) suggested that the primary aetiological agent of YPDS is circovirus by causing depletion of the lymphoid tissues and immunosuppression. Secondary bacterial infections were also found to contribute to the pathogenesis of YPDS, but the presence of AdVs could not be confirmed by PCR. It has to be added that the primers used for these examinations were not universal but rather specific for FAdVs and PiAdV-1 (Meulemans et al., 2001; Raue et al., 2002). In other surveys, AdVs have been detected in a few cases only (Freick et al., 2008; Stenzel et al., 2012).

Initial attempts to isolate AdVs from pigeons have been made by using primary chicken cells, consequently almost every known FAdV type has been obtained (McFerran et al., 1976; Vereecken et al., 1998). The first distinct pigeon AdV strain was reported by Hess et al. (1998). On chicken embryo liver cells, they isolated a virus that was found to be a serologically novel type. This was further supported by the sequence of the PCR-amplified hexon gene fragment. The virus was named as PiAdV-1 and its whole genome sequence has been published recently (Marek et al., 2014b).

Our aim was to examine the prevalence and diversity of AdVs present in Hungarian pigeon lofts. We performed a PCR screening by using a very sensitive nested method with highly degenerate primers that recognise the most conserved portion of the adenoviral DNA-dependent DNA polymerase (*pol*) gene (Wellehan et al., 2004). Phylogenetic analysis of the sequence of amplicons permits preliminary genus classification of the given AdV. Then additional PCRs appropriate for the detection of AdVs from the respective genera can be applied. We also intended to test the PCR methods recommended for the diagnosis of avian and pigeon AdVs previously. The viruses in the positive samples were further studied in order to determine the nucleotide (nt) sequence of longer gene fragments or preferably complete genes. Approximately two thirds of the genome of the most frequently detected, novel PiAdV type could be sequenced.

Materials and methods

Origin of samples

Almost 100 birds, including 78 racing and 19 fancy pigeons from 27 different lofts, were examined for the presence of AdVs from various regions of Hungary. Every loft experienced some clinical disease for shorter or longer periods. Samples from internal organs (lung, liver, kidney, intestine) or liver samples exclusively were collected and examined from 33 and 47 birds, respectively. The pigeons were either found dead or humanely euthanised because of severe disease. The clinical signs included anorexia, vomiting, diarrhoea, weight loss and ruffled feathers. Besides these 80 carcasses, 17 cloacal swabs from live, healthy specimens from three lofts were also tested.

Table 1
Sequences of the tested PCR primers and results of the reactions on the different PiAdV types

| Reference | Primer sequences | Size of amplicon | Avidenovirus | | | | Siadenovirus | | | | | |
|------------------------|--|------------------|--------------|----------|----------|---------|--------------|---------|--|--|--|--|
| | | | PiAdV-1 | PiAdV-2a | PiAdV-2b | PiAdV-3 | PiAdV-4 | PiAdV-5 | | | | |
| Wellehan et al., 2004 | polFouter: 5'-TNMGNGGNGMNTGYTAYCC-3' | ~500 and 272 bp | | | | | | | | | | |
| | polRouter: 5'-GTDGCRANSHNCCRTABARNGMRTT-3' | | + | + | + | + | + | + | | | | |
| | polFinner: 5'-GTNTWYGYATHTGYGGHATGTAYGC-3' | | | | | | | | | | | |
| | polRinner: 5'-CCANCCBDRTRTGNARNGTRA-3' | | | | | | | | | | | |
| Raue et al., 2005b | Hex-s: 5'-CAGGCCCAAYTYCATCGG-3' | 181 bp | + | | | | | | | | | |
| | Hex-as: 5'-GTGATGACGSGACATCAT-5' | | | | | | | | | | | |
| Raue et al., 2005a | HexL1-s: 5'-ATGGG NKCNAC YATYTCGAYAT-3' | ~590 bp | + | + | | | | | | | | |
| | HexL1-as: 5'-AAR TRTCNCKRAAN SCGATGTA-3' | | | | | | | | | | | |
| Mase et al., 2009 | HexF1: 5'-GAYRGYHGGR TNBTGGA YATGGG-3' | ~800 bp | | + | | | | | | | | |
| | HexR1: 5'-TACTTATCNACRGCT GRTTCCA -3' | | | | | | | | | | | |
| Meulemans et al., 2001 | HexonA: 5'-CAARTTCAGRCAGACGGT-3' | 897 and 580 bp | | | | | | | | | | |
| | HexonB: 5'-TAGTGTGCMCGSGACATCAT-3' | | + | | | | | | | | | |
| | HexonC: 5'-SKCSACYTAYTTCGACAT-3' | | | | | | | | | | | |
| | HexonD: 5'-TTRTCWCKRAADCCGATGTA-3' | | | | | | | | | | | |
| Dospoly et al., 2013 | sihexfoot: 5'-TTYAAYCAYMRNAA YMGNGG -3' | 710 and 430 bp | | | | | | | | | | |
| | sihexreout: 5'-CATYTGNA YNA RHACCARTC-3' | | | | | | | | | | | |
| | sihexfoin: 5'-GARTGGTGGT YMGNA ARGAYCC-3' | | NT | NT | NT | NT | NT | NT | | | | |
| | sihexrein: 5'-GGNAVNARNANDCK RTCTTNCC -3' | | | | | | | | | | | |

+: positive; -: negative; NT: not tested; *: modified positions are marked with bold letters

DNA extraction from the tissues was carried out as described recently (Doszpoly et al., 2014). For the excrements, the E.Z.N.A.[®] Stool DNA Kit was used following the manufacturer's instructions (Omega BioTek).

PCR and sequencing

A consensus nested PCR targeting the *pol* gene was performed first (Wellehan et al., 2004). The positive samples were further tested by different PCRs depending on the virus type deduced from the partial *pol* sequences. Most of the additional (simple or nested) PCRs have been described for the amplification of fragments from the gene of the hexon, the major capsid protein (Meulemans et al., 2001; Raue et al., 2005a and b; Freick et al., 2008; Mase et al., 2009; Stenzel et al., 2012; Doszpoly et al., 2013). As shown in Table 1, we altered slightly the sequence of some primers in order to increase their sensitivity. Specific primers complementary to the already determined sequences of the newly detected AdVs were also designed for the use in primer walking sequencing strategy.

The PCRs were carried out in a TPersonal Thermocycler (Biometra, Göttingen, Germany) with REDTaq[®] ReadyMix[™] PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Different cycling programs, published along with the above-mentioned primers, were used. To check the results, 10- μ l aliquots of the reaction mixtures were electrophoresed in 1% agarose gels. The amplicons were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Sequencing of the products was performed with the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) from both directions with the appropriate PCR primers.

Primary sequence analysis and phylogenetic reconstructions

For sequence editing and aligning, the Staden 7.1 Package and BioEdit 7.2.5 program were applied. For sequence identification, different Blast algorithms were used at the website of the NCBI. The deduced amino acid (aa) sequences were aligned by the ClustalW program with default parameters. Gaps and primer sequences were trimmed off in the GeneDoc 2.7. application. The Phylip program package was used to perform distance matrix analyses by applying JTT model, fitch (with global rearrangements) (<http://mobyli.pasteur.fr>, Pasteur Institute, Paris). Phylogeny reconstructions were visualised with Mega6.

Genome sequencing and annotation

Genome sequencing of the most frequently detected pigeon AdV type has been initiated by PCR amplification of some additional conserved gene fragments. The conserved target sequences were selected from alignments of different aviadenoviruses. Every DNA fragment was sequenced first directly to confirm the homogeneity of the respective PCR product. Longer genome fragments

for sequencing were molecularly cloned after confirmations. For annotation and schematic presentation of the genome, the CLC program was used. The newly determined sequences were submitted to GenBank and assigned to accession numbers KX555529–KX555532, KX555534 and KX673408.

Results

Prevalence and diversity of adenoviruses in pigeon samples

Out of the 97 samples, 48 (49.5%) were found positive for the presence of adenoviral DNA. The positivity rate showed slight fluctuation according to the type of samples (Table 2). From the 33 birds, from which multiple organs had been screened separately, 22 (66.7%) proved to be positive, while from the 47 liver samples 17 (36.2%) only. A surprisingly high proportion, 9 (52.9%) out of the 17 cloacal samples of healthy birds were also positive.

Table 2

Results of the AdV screening PCR targeting the viral DNA polymerase gene

| | Pool of four organs | Liver | Lung | Kidney | Intestine | Liver | Faeces/ cloacal swab | Total |
|----------------|---------------------------|-------|------|--------|-----------|-----------|----------------------------|-----------|
| Tested | 33 | 33 | 33 | 33 | 33 | 47 | 17 | 97 |
| Positive | 22 | 10 | 17 | 14 | 14 | 17 | 9 | 48 |
| PiAdV-1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 |
| PiAdV-2a | 7 | 2 | 5 | 3 | 4 | 11 | 4 | 22 |
| PiAdV-2b | 2 | 0 | 1 | 0 | 2 | 6 | 0 | 8 |
| PiAdV-2a+2b | 3 | 2 | 3 | 2 | 2 | 0 | 0 | 3 |
| PiAdV-3 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| PiAdV-4 | 7 | 3 | 6 | 7 | 4 | 0 | 5 | 12 |
| PiAdV-5 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| Positivity (%) | 66.7 | 30.3 | 51.5 | 42.4 | 42.4 | 36.2 | 52.9 | 49.5 |

Every PCR product proved to be of adenoviral origin. Six different *pol* sequences were obtained. The phylogeny inference showed that four types of sequences were from aviadenoviruses and two from siadenoviruses. Among aviadenoviruses, the sequence of PiAdV-1 (Marek et al., 2014b) was identified in a single case. Another aviadenovirus, representing clearly a novel type was detected in 22 birds. Compared to this sequence, an almost identical *pol* sequence was found in 8 samples. Three additional samples seemed to contain both sequence variants. A fourth type of aviadenovirus *pol* sequence was obtained also just from a single sample. Siadenoviral *pol* sequences were identified in 13 cases;

12 contained identical sequences and only one a second type of siadenovirus. The presence of DNA of FAdVs or any other known AdVs of poultry origin was not detected at all.

Table 2 presents the distribution of the positive samples according to the type of sample and AdV detected. For the most frequently found putative novel aviadenovirus, we propose the name pigeon adenovirus 2 (PiAdV-2). The two variants of the PiAdV-2 *pol* sequences differed from each other in 6 out of the 272 nt only, yet had identical aa sequences. We named the more abundant variant PiAdV-2a, while the less frequent one PiAdV-2b. The divergence between the aa sequence of the corresponding *pol* fragments of PiAdV-1 and -2 was 23.3%. The third type of aviadenoviral *pol* sequence, proposed to be named PiAdV-3, was detected in a single case only. It showed 26.7 and 27.8% aa sequence divergence compared to PiAdV-1 and -2, respectively.

The frequently detected siadenoviral *pol* sequences showed no nt divergence on the 272-bp-long fragment. We propose the name PiAdV-4 for this putative virus. The *pol* sequence of the other putative siadenovirus, named PiAdV-5, differed from PiAdV-4 in 97 nt on the 272-bp-long *pol* sequence (35.6%), and was found in one specimen only.

As many of the 27 lofts were represented only by a single sample, we are not able to draw a firm conclusion about the distribution of the detected viruses on loft level (Table 3). However, loft 3 was represented by 8 samples out of which none showed AdV positivity, but we still think this sample number is not enough to conclude that this loft was free of AdV infections. We did not observe multiple infections within one specimen; however, we did see a mixed infection with the two variants of PiAdV-2 (a and b) in 3 specimens. Furthermore, we confirmed the presence of both PiAdV-2 and -4 within the same loft in 4 cases (lofts 1, 4, 10 and 21).

Phylogenetic place of pigeon adenoviruses

The phylogenetic tree, based on the *pol* aa sequences, showed PiAdV-2 and -3 constituting a monophyletic group with PiAdV-1 within the genus *Aviadenovirus*, whereas PiAdV-4 and -5 belonging to genus *Siadenovirus* (Fig. 1). Interestingly, these latter PiAdVs are not monophyletic though both of them appeared clearly in the clade of avian siadenoviruses.

Further characterisation of certain novel AdVs

Amplification of the hexon gene fragment from the newly found PiAdVs was successful only with samples containing PiAdV-2a or -4 (Table 1). Since different primer sets gave positive reaction, comparison of the hexon gene fragments was not possible. Nonetheless, the partial hexon sequences further supported the distinctness and genus affiliation of the respective viruses.

Table 3

Distribution of AdV-positive samples among pigeon lofts based on the results of PCR targeting the viral DNA polymerase gene

| Loft | Total samples | AdV positive | PiAdV-1 | PiAdV-2 | PiAdV-3 | PiAdV-4 | PiAdV-5 |
|-------|---------------|--------------|---------|---------|---------|---------|---------|
| 1 | 45 | 22 | | 21 | | 1 | |
| 2 | 8 | 4 | | | | 4 | |
| 3 | 8 | 0 | | | | | |
| 4 | 4 | 3 | | 1 | | 2 | |
| 5 | 1 | 0 | | | | | |
| 6 | 1 | 0 | | | | | |
| 7 | 1 | 1 | | 1 | | | |
| 8 | 1 | 0 | | | | | |
| 9 | 1 | 1 | | 1 | | | |
| 10 | 3 | 2 | | 1 | | 1 | |
| 11 | 1 | 0 | | | | | |
| 12 | 1 | 1 | | | | 1 | |
| 13 | 1 | 0 | | | | | |
| 14 | 1 | 0 | | | | | |
| 15 | 1 | 1 | | | 1 | | |
| 16 | 1 | 1 | | 1 | | | |
| 17 | 4 | 1 | | 1 | | | |
| 18 | 1 | 1 | | 1 | | | |
| 19 | 1 | 0 | | | | | |
| 20 | 1 | 1 | | 1 | | | |
| 21 | 3 | 3 | | 1 | | 2 | |
| 22 | 2 | 1 | | | | 1 | |
| 23 | 2 | 2 | | 2 | | | |
| 24 | 1 | 0 | | | | | |
| 25 | 1 | 1 | | | | | 1 |
| 26 | 1 | 1 | | 1 | | | |
| 27 | 1 | 1 | 1 | | | | |
| Total | 97 | 48 | 1 | 33 | 1 | 12 | 1 |

Partial genome sequence of pigeon adenovirus 2a

Approximately two thirds of the genome of the most frequently demonstrated novel virus, PiAdV-2a was sequenced. During annotation of the 31,314-bp-long contig, 26 complete and 3 partial genes were identified. Among the family-common genes, in the central part of the genome, a frequent trait of aviadenoviruses, i.e. the presence of two fibre genes of different sizes was identified. The schematic partial genome map is presented in Fig. 2. The genome organisation largely corresponded to that of PiAdV-1 (Marek et al., 2014b). Interestingly, however, every gene of PiAdV-2a seemed to encode a smaller protein than its counterpart in PiAdV-1. Among the few exceptions were ORF13 and the pVIII gene that

had identical size in the two viruses, respectively. Furthermore, the two fibre genes of PiAdV-2a were significantly shorter than those in PiAdV-1. The shorter fibre1 of PiAdV-2a consists of 141 aa compared to 246 aa in PiAdV-1. This is a more than 40% difference. Fibre2 of PiAdV-2a was found to contain 552 aa as opposed to 749 in PiAdV-1. The three major structural domains, including the tail, shaft and knob regions, could be recognised in the deduced aa sequence of fibre2 only, while in fibre1 only the tail and the shaft domain can be found.

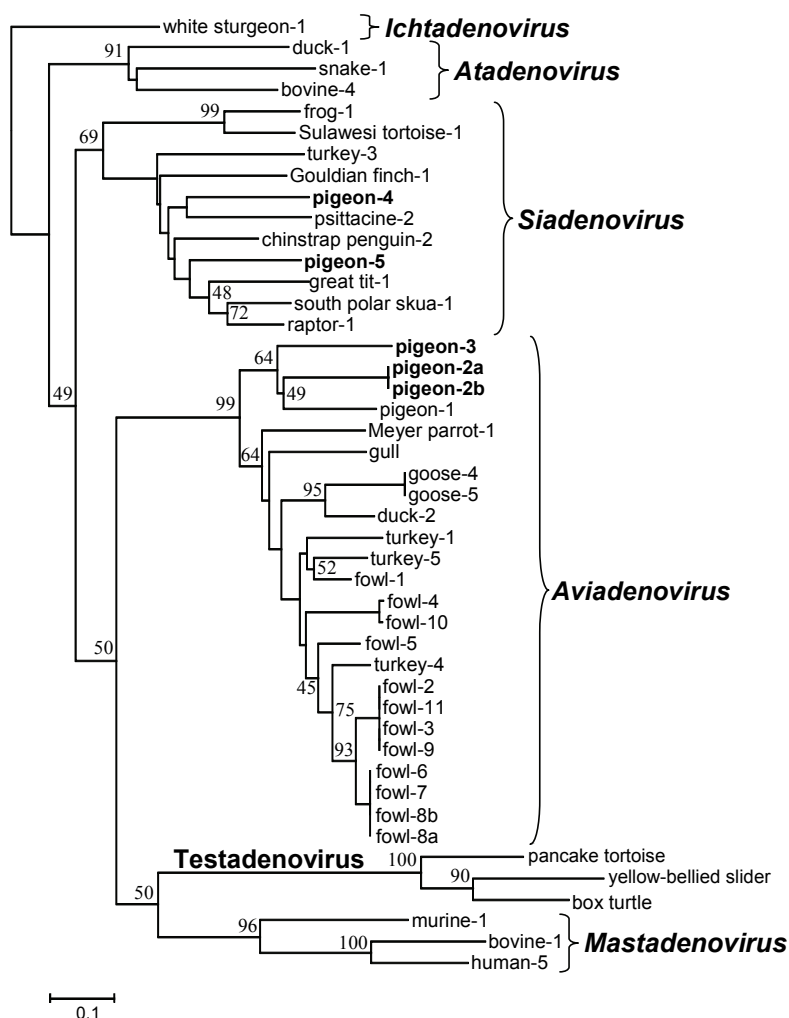


Fig. 1. Phylogenetic tree based on distance matrix analysis of the deduced amino acid sequences of the 272-bp-long DNA-dependent DNA polymerase gene fragments. Unrooted calculation. For visualisation, the white sturgeon adenovirus 1 was selected as outgroup. Bootstrap values are based on 100 samplings. The viruses are marked by the host name and type number omitting the abbreviation AdV. The bar shows 0.1 nucleotide changes per site

Splicing donor and acceptor sites were predicted in the genes of the terminal protein precursor (pTP), 33K and DNA-binding protein (DBP), as well as in ORF12, 13, 14 and 14A. Homologues of these ORFs are present in most aviadenovirus genomes studied to date. ORF1A and ORF20, flanking the studied genome portion, were sequenced incompletely. The short ORF corresponding to the so-called U exon was identified between the genes of pVIII and fibre1 (Fig. 2). The other exons belonging to the gene of the hypothetical 'U-exon protein' have not been identified in aviadenoviruses yet (Podgorski et al., 2016). No novel ORFs, specific for PiAdV-2a, were found in the examined genome fragment.

Discussion

Here, we describe the results of the first survey intended to assess the prevalence and diversity of AdVs in domestic pigeons in Hungary. The overall prevalence, found by PCR screening of almost 100 samples, was extremely high (49.5%) especially if compared to the results of similar studies reported earlier from Germany and Poland, where only sporadic occurrence of PiAdVs was revealed (Raue et al., 2005b; Freick et al., 2008; Stenzel et al., 2012). The main reason behind the significant discrepancy is most likely the diverse performance of the PCR systems applied in the different screenings. We used a nested PCR with consensus primers that recognise the *pol* gene (Wellehan et al., 2004). The superiority of this PCR in detecting AdVs belonging to different genera has been documented in numerous cases in the past decade. It was originally designed for the recognition of novel atadenoviruses in lizards, but its sensitivity and robustness allowed the discovery of many new aviadenoviruses (Wellehan et al., 2005; Bodewes et al., 2013), siadenoviruses (Kato et al., 2009; Wellehan et al., 2009; Kovács et al., 2010; Park et al., 2012; Ballmann and Vidovszky, 2013; Joseph et al., 2014; Lee et al., 2014), atadenoviruses (Szirovicza et al., 2016) and mastadenoviruses (Vidovszky et al., 2015) in a wide range of hosts. Even the members of a hitherto unknown genus (with the proposed name Testadenovirus) could be detected (Doszpoly et al., 2013).

We revealed the existence of four novel AdV types in pigeons. The two aviadenoviruses, PiAdV-2 and -3 share close common ancestry with PiAdV-1. The partial genome analysis of PiAdV-2a revealed high similarity to PiAdV-1. These corroborate the genuine pigeon origin of all the three viruses. Nonetheless, the phylogenetic distances separating them merit the establishment of two novel species. PiAdV-1 is classified into the species *Pigeon aviadenovirus A* (Marek et al., 2014b). We propose that PiAdV-2 and -3 be members of *Pigeon aviadenovirus B* and *C*, respectively. We also demonstrated the occurrence of PiAdV-1 in Hungary.

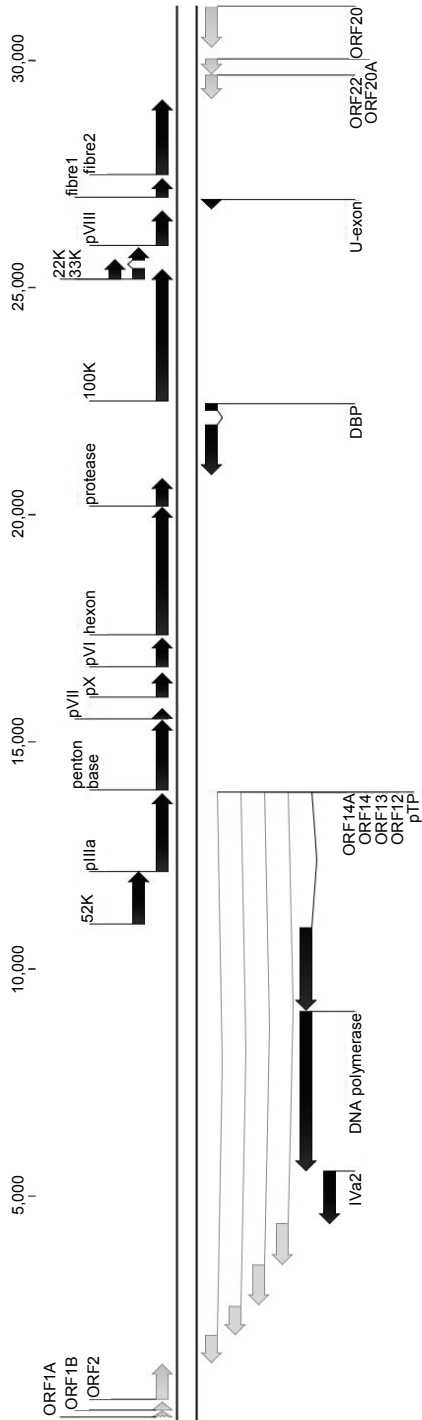


Fig. 2. Schematic presentation of the sequenced part of the genome of PiAdV-2a. Black arrows show genes conserved in every genus and grey arrows depict genes specific for the genus *Aviadenovirus*

The evolutionary origin of siadenoviruses is yet unclear (Kovács and Benkő, 2011). Their presence in different birds is supposed to be the result of multiple host switch events. This theory is supported by the elevated pathogenicity and unusually wide host range of siadenoviruses (Benkő and Harrach, 2003; Wellehan et al., 2009; Ballmann and Vidovszky, 2013). The pathogenicity of the novel pigeon siadenoviruses described in this study has not been investigated yet. The large phylogenetic distance between PiAdV-4 and -5 implies that one or perhaps both of these viruses might have spread from some other host to pigeon.

In our experiments, the performance of the hexon-based PCRs was found to be poor. It seems that the level of variability of this gene does not support the design of primer sets universally applicable throughout the family *Adenoviridae*. The hexon can be a better target for genus- or type-specific PCRs. Even the nested primer set, originally designed on the basis of the most conserved aa stretches of the known siadenovirus hexons (Kovács, unpublished results) failed to recognise PiAdV-5. This was surprising, as this PCR had been successfully used for the demonstration of AdVs belonging to a different genus (Dospoly et al., 2013).

The relationship between the newly found PiAdVs and YPDS or other disease conditions remains to be elucidated. In several recent papers, the crucial role of pigeon circovirus (PiCV) in YPDS was emphasised (Raue et al., 2005b; Stenzel et al., 2012). The prevalence of PiCV in healthy racing pigeons in Hungary was reported to be as high as 62% (Cságola et al., 2012). Our material originated predominantly from diseased and dead birds; therefore, it would be of special interest to find out the frequency of PiCV in these samples. We plan to perform such a PCR survey in the near future.

It is worth mentioning that we found AdV-shedding birds among the healthy individuals in a considerable proportion (52.9%). AdVs usually establish persistent infection, but virus shedding is not continuous, and infected birds may easily produce negative PCR results. Different stress factors, such as crowding, transportation, racing and most likely PiCV infection may trigger reactivation of AdVs, thus facilitating their PCR detection. Most of our samples were derived from racing pigeons. These birds are kept for hobby purposes and they participate in competitions on a regular basis. This means that they are regularly transported in crowded trucks or vans collecting pigeons from different lofts, and this stressful journey is followed by a challenging trip to the home loft on their own wings. Besides stress this is a regular way to exchange viral or bacterial agents between lofts.

Little is known about the tissue tropism of PiAdVs. As it can be deduced from the data in Table 2, the individual PiAdVs might replicate with different efficiency in the organs. Although the number of positive cases for each PiAdV did not allow statistical analysis yet, it is clear that sampling has a major impact on the outcome of diagnosis. Hepatitis or hepatic necrosis is commonly associated

with AdVs in poultry, therefore the liver is often the only organ collected for PCR. It seemed that lung and kidney samples might be more appropriate especially in case of siadenovirus involvement.

In conclusion, our results show that at least five distinct PiAdVs circulate in the Hungarian domestic pigeon population. The complex aetiology of YPDS might deserve a closer look by using the most universal and robust *pol* PCR when searching for PiAdVs as possible secondary pathogens.

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