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The distribution of pigeon adenoviruses in Northern Chinese pigeon and turtledove flocks provides further evidence of viral crosstransmission

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OBJECTIVE
To understand the prevalence, genetic diversity, and potential pathogenicity of adenoviruses present in pigeon and turtledove populations.

METHODS
Nested PCR and Sanger sequencing methods were used to identify the genotype and percentage of various adenoviruses in the feces of pigeon (*Columba*) and turtledove (*Streptopelia*) populations. In Beijing, China, a total of 194 fresh feces samples from meat-use pigeons (*C livia domestic*a), homing pigeons (*C livia domestica*), wild pigeons (*C livia domestica*), and turtledoves (*S decaocto* and *S chinensis*) were collected using noninvasive sampling collection techniques. Their partial DNA-dependent DNA polymerase gene sequences were obtained using nested PCR and double-ended Sanger sequencing, and their genotypes were then ascertained based on sequence alignment.

RESULTS
A total of 6 genotypes of adenovirus were detected in pigeon and turtledove flocks, including pigeon adenovirus (PiAdV)-1, PiAdV-2A, PiAdV-3, PiAdV-4, PiAdV-5, and a novel adenovirus genotype (PiAdV-6). Among them, PiAdV-1 was found widespread in flocks of pigeons exhibiting extensive presentations of hepatic necrosis. Highly conserved PiAdV-4 and PiAdV-5 were found to be nonpathogenic and extensively distributed in all pigeon and turtledove groups.

CONCLUSIONS
These findings imply the presence of diverse PiAdVs in pigeon and turtledove flocks, and the wild pigeons and wild turtledove birds are potentially serving as natural sources of these viruses.

CLINICAL RELEVANCE
This study provides supportive evidence of the pathogenicity of different genotypes of adenovirus in pigeon flocks and also implies that stopping the transmission of the virus brought by wild pigeons and turtledoves may be important for the prevention of diseases associated with PiAdVs.

Keywords: pigeon adenoviruses, pigeon and turtledove flocks, PiAdV-6, extensive hepatic necrosis, viral crosstransmission

Adenoviruses are nonenveloped double-stranded DNA viruses with a linear genome that ranges from about 25 to 45 kb.[1,2] The family Adenoviridae occurs within the order Rowavirales, and there are 87 International Committee on Taxonomy of Viruses–accepted adenovirus species. Adenoviruses have been divided into 6 genera: *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, *Mastadenovirus*, *Ichtadenovirus*, and *Testadenovirus* (https://talk.ictvonline.org/taxonomy/).[3,4] Reportedly, adenoviruses can infect a large number of vertebrate species. It was believed that *Atadenovirus*, *Mastadenovirus*, and *Siadenovirus* genera could infect chickens and wild birds.[5,6] Birds are considered common hosts for adenoviruses,[7] and this fact is mirrored by a large number of fowl adenoviruses detected in chickens several decades ago.[8] As to wild birds, *Aviadenoviruses* have been found in representatives of the orders Falconiformes, Psittaciformes, and Charadriiformes.[9–12]

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Adenoviral infection in pigeons was first described in Belgium in 1984 and has since been observed worldwide.[13] Two adenovirus-associated disease courses in specific pigeons have been described.[14] Adenovirosis type I, also known as classical adenovirosis, primarily affects young pigeons, which exhibit weight loss, vomiting, and diarrhea for about a week.[14] Furthermore, it was considered to be a stress-induced infectious disease, especially exhaustion in pigeon races.[15–18] The adenovirosis type II is also known as necrotizing hepatitis, which affects pigeons of all ages and is characterized by sudden death and extensive hepatic necrosis.[14] The pigeon mortality rate is very high, with approximately 30%, but it can reach 100% in pigeon lofts with necrotizing hepatitis infections.[16] Although the clinical characteristics of these 2 adenovirus-associated diseases have been described in detail and virions were observed in tissues by electron microscopy, the causative agents of them have never been clearly identified. A series of pigeon adenoviruses (PiAdVs), including PiAdV-1, PiAdV-2A, PiAdV-2B, PiAdV-3, PiAdV-4, and PiAdV-5, has been identified in epidemiological investigations or in sick pigeons,[19–22] but they still did not establish a definite association with these 2 different adenovirus-associated disease courses.[22,23] The serotype or genotype of adenoviruses responsible for the 2 adenovirus-associated disease courses remains unknown. In addition, adenovirus diversity has also received relatively little attention. Perhaps as a result of the defect in the testing method that failed to detect novel PiAdV, researchers in one investigation discovered only 1 carrier of AvAdV-4 in 235 pigeons in southern China and no other genotypes.[24] There have not been any current studies into the diversity of PiAdV-3, PiAdV-4, and PiAdV-5 despite the fact that they were initially discovered and named in Hungary in 2016.[21] Recently identified novel adenoviruses have demonstrated the significance of wild avian host species[5] as these wild hosts display varying feeding, roosting, and migration habitats, which may enhance adenovirus diversity. Additionally, avian hosts might assist viruses in gaining new gene combinations and overcome genetic barriers to infection in new host species.

This study was designed to investigate the genotype and percentage of diverse adenoviruses in natural populations of pigeons (*Columba livia domestica*), and the diversity of adenovirus in birds of the family Columbidae, including *Streptopelia decaocto* and *S chinensis*, was also evaluated in order to investigate the origin or spillover of these genotypes of PiAdV. The findings imply that the presence of diverse PiAdVs in the birds of the family Columbidae and the wild pigeons and wild turtledove birds may potentially serve as a natural source of these viruses.

Methods

Sample collection

A total of 194 fresh feces samples from meat-use pigeons (*C livia domestica*), homing pigeons (*C livia domestica*), wild pigeons (*C livia domestica*), and turtledoves (*S decaocto* and *S chinensis*) were collected using a noninvasive sampling collection technique from February 2022 through October 2023. These samples included 36 samples of meat-use pigeons, 49 samples of homing pigeons, 48 samples of wild pigeons, 41 samples of *S decaocto*, and 20 samples of *S chinensis*.

From November 2022 through December 2022, samples of meat-use pigeon feces were taken from the cages of a meat-use pigeon farm in Beijing, China, during an ongoing outbreak of infectious diseases in all 24 households. A total of about 80,000 adult pigeons aged 2 to 3 years and 120,000 young pigeons aged 1 to 2 months had a history of increased mortality during the outbreak. The first sign was inappetence, followed by sudden death, without any further signs or presentations in the next 2 to 5 days. This outbreak lasted for approximately 1 month, during which time the mortality rate for adult pigeons was approximately 25%, and for young pigeons it was approximately 40%. The 36 fecal samples were randomly collected, including 18 watery or soft feces and 18 solid feces.

From February through March 2022, 49 homing pigeon fecal samples were collected from a homing pigeon public house in Beijing, China. Overall, the pigeons were in good health, and the feces that were collected were all in normal clusters.

Eight turtledove and wild pigeon sampling sites were set up in 8 parks in Beijing, China, from July through October 2023. Corn, wheat, and other grains were placed on the sampling sites to attract the wild birds to come. By putting up portable monitoring equipment, the feces of the attracted birds could be gathered quickly, and the species of them could be identified at any time. At the sampling sites, a total of 108 fecal samples were collected: 48 samples came from wild pigeons, 41 from *S decaocto*, and 20 from *S chinensis*.

Deoxyribonucleic acid extraction and PCR

The 0.2-g fecal samples were pooled and mixed with 200 µL of PBS, followed by centrifugation (4,000 g for 10 minutes). The 200 µL of supernatants were then collected for nested PCR screening. A sensitive nested PCR screening was performed first using a set of highly degenerate primers that can detect the most conserved region of the adenoviral DNA-dependent DNA polymerase (pol) gene, and the PCR products were further sequenced. The amplicon sequence allows for a tentative genus classification of the identified adenoviruses by phylogenetic analysis. Pigeon adenovirus-2A and PiAdV-2B cannot be distinguished by the difference in pol sequence since they are so similar.[22] PiAdV-2's partial hexon gene was amplified, and its genotype was determined by sequence alignment. The sequences of referred degenerate nested PCR primers,[25] including a pair of outer primers and a pair of inner primers, were listed in **Supplementary Table S1**. DNAs were extracted from 200 µL of prepared supernatants using TaKaRa MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa Bio) according

to the manufacturer's protocol. The extracted DNAs were stored at −20 °C until PCR was performed. The PCRs were carried out in a PCR apparatus with PCR Reaction Mix (Vazyme) according to the manufacturer's instructions. For all PCRs, a final volume of 25 µL reaction mixture containing 2.5 µL of reaction buffer (10 X reaction buffer), 0.5 µL of dNTPs, 2.5 U of Taq-DNA-Polymerase (Vazyme), and 2 µL of template DNA was employed. The previously reported nested PCR procedures were used for partial pol gene screening.[25] For the first amplification, the concentrations of each forward primer polFouter and reverse primer polRouter were 1 µM, and the following setup was used: initial denaturation for 6 minutes at 95 °C, followed by 45 cycles at 94 °C for 30 seconds, 46 °C for 60 seconds, and 72 °C for 60 seconds and a final extension at 72 °C for 7 minutes. For the second round, 2 µL of product from the first reaction mixture and 1-µM concentrations of each forward primer polFinner and reverse primer polRinner were used to amplify the partial pol gene under the same conditions as in the first round. Based on previous studies,[22] the mixture included 500 nM of the appropriate forward and reverse primers in order to amplify the partial hexon gene of PiAdV-2. The sequences of primers were listed in Supplementary Table S1. The cycling protocol for the PiAdV-2 was 6 minutes at 95 °C and 35 cycles of 45 seconds at 95 °C, 1 minute at 47.3 °C, and 1 minute at 72 °C. A last elongation at 72 °C for 8 minutes marked the end of the PCR.

To check the results, 10-µL aliquots of the reaction mixtures were electrophoresed in 1% agarose gels. The amplicons were purified with NucleoSpin Gel and the PCR Clean-Up Kit (Macherey-Nagel). The expected lengths of the nested PCR products are 272 bp, and the partial hexon gene of PiAdV-2 are 392 bp. The purified products were sequenced by BGI Genomics (BGI, Shenzhen, China).

Primary sequence analysis and phylogenetic reconstructions

For sequence editing and aligning, the BioEdit (version 7.0.5.3; https://bioedit.software.informer.com/) software was applied. The sequences were compared to known sequences in the GenBank (National Center for Biotechnology Information), European Molecular Biology Laboratory, and Data Bank of Japan databases by use of TBLASTX (Version 2.1; http://www.clustal.org/clustal2/).

Gaps and primer sequences were trimmed off, and partial pol gene nucleotide sequences and deduced amino acid sequences were aligned by the ClustalW program with default parameters. Phylogeny reconstructions were visualized with Mega (Molecular Evolutionary Genetics Analysis, version 11; The Biodesign Institute) software using the neighbor-joining algorithm and the Jukes-Cantor distance model. Bootstrap support was assessed by 1,000 repetitive analyses. The phylogenetic tree shows the presently accepted species clustering. Similar adenoviruses are classified as members of a genotype.

Results

Diverse PiAdVs in pigeon population

Numerous adenoviruses were discovered to be present in pigeon populations by high-sensitivity nested PCR and subsequent sequencing. A total of 6 genotypes of PiAdV were detected in meat-use pigeons, homing pigeons, and wild pigeons, including PiAdV-1, PiAdV-2A, PiAdV-3, PiAdV-4, PiAdV-5, and a new, unclassified PiAdV (PiAdV-6). Pigeon adenovirus-1, PiAdV-2A, PiAdV-4, and PiAdV-5 were found in a large-scale meat-use pigeon farm, whereas PiAdV-1, PiAdV-3, PiAdV-4, PiAdV-5, and PiAdV-6 were found in wild and free-roaming homing pigeon flocks. **Table 1** displays the results of the adenovirus screening. Pigeon adenovirus-4 was the most prevalent genotype in fecal samples from various pigeon flocks. In meat-use, homing, and wild pigeon populations, the corresponding PiAdV-4–positive rates were 11.1%, 26.5%, and 31.2%. Pigeon adenovirus-5 was the second, with positive rates in meat-use, homing, and wild pigeon populations of 5.6%, 16.3%, and 12.5%, respectively. While the positivity rate of PiAdV-1 in meat-use pigeon fecal samples was 47.2%, the corresponding positivity rates in homing pigeon and wild pigeon samples were comparatively low at 2% and 2.1%, respectively. Pigeon adenovirus-2A and PiAdV-3 have also been found to have low positivity rates in fecal samples of various pigeon flocks.

Table 1—Results of the adenovirus screening.

	PiAdV-1	PiAdV-2A	PiAdV-2B	PiAdV-3	PiAdV-4	PiAdV-5	Unclassified
Meat-use pigeon (watery or soft feces)	3/18	1/18	0/18	0/18	2/18	1/18	0/18
Meat-use pigeon (solid feces)	14/18	1/18	0/18	0/18	2/18	1/18	0/18
Total meat-use pigeons	17/36	2/36	0/36	0/36	4/36	2/36	0/36
Total meat-use pigeon positivity (%)	47.2%	5.6%	0%	0%	11.1%	5.6%	0%
Homing pigeon	1/49	0/49	0/49	1/49	13/49	8/49	0/49
Homing pigeon positivity (%)	2%	0%	0%	2.1%	26.5%	16.3%	0%
Wild pigeon	1/48	1/48	0/48	1/48	15/48	6/48	1/48
Wild pigeon positivity (%)	2.1%	2.1%	0%	2.1%	31.2%	12.5%	2.1%
Streptopelia decaocto	0/41	0/41	0/41	0/41	5/41	4/41	0/41
S chinensis	0/20	0/20	0/20	0/20	3/20	1/20	0/20
Total turtledoves	0/61	0/61	0/61	0/61	8/61	5/61	0/61
Total turtledove positivity (%)	0%	0%	0%	0%	13.0%	8.2%	0%

PiAdV = Pigeon adenovirus.
The PiADV-2 subtype was determined by the sequence of the PCR product.

No PiAdV-2A was detected in carrier pigeons, and only 5.6% and 2.1% of meat-use and wild pigeon fecal samples tested positive for PiAdV-2A, respectively. Although PiAdV-3 was not found in meat pigeons, it was found at positive rates of 2.1% in homing pigeons and 2.1% in wild pigeons. Furthermore, an unclassified PiAdV was identified in wild pigeon feces samples based on partial pol gene sequencing and comparison with reference sequences in the database.

High proportion of PiAdV-1 in watery or soft feces

The pigeon fecal samples were collected from a poultry farm, and a suspected outbreak of PiAdV-associated courses of diseases was taking place in that farm during the sampling period. The genotypes of adenoviruses and the percentages of them were further calculated in these abnormal fecal samples as 18 of 36 of those collected (50%) were watery or soft. **Supplementary Figure S1** shows photos of normal and abnormal feces, including watery feces and soft feces. They differed significantly in the percentage of PiAdV-1 positive, with a positive rate of 77.8% (14 of 18) for these abnormal feces and 16.7% (3 of 18) for the solid feces collected in the same poultry farm. However, because of the small number of samples, PiAdV-2A, PiAdV-4, and PiAdV-5 could not provide a valid statistical comparison even if they were also detected in the watery or soft feces samples, and they did not differ significantly from those solid fecal samples.

Conserved partial pol genes of PiAdV

Once the partial pol genes of PiAdV were amplified, they were sequenced by 2-ended Sanger sequencing. Eighty-six sequences of 6 genotypes were obtained, including 19 of PiAdV-1, 3 of

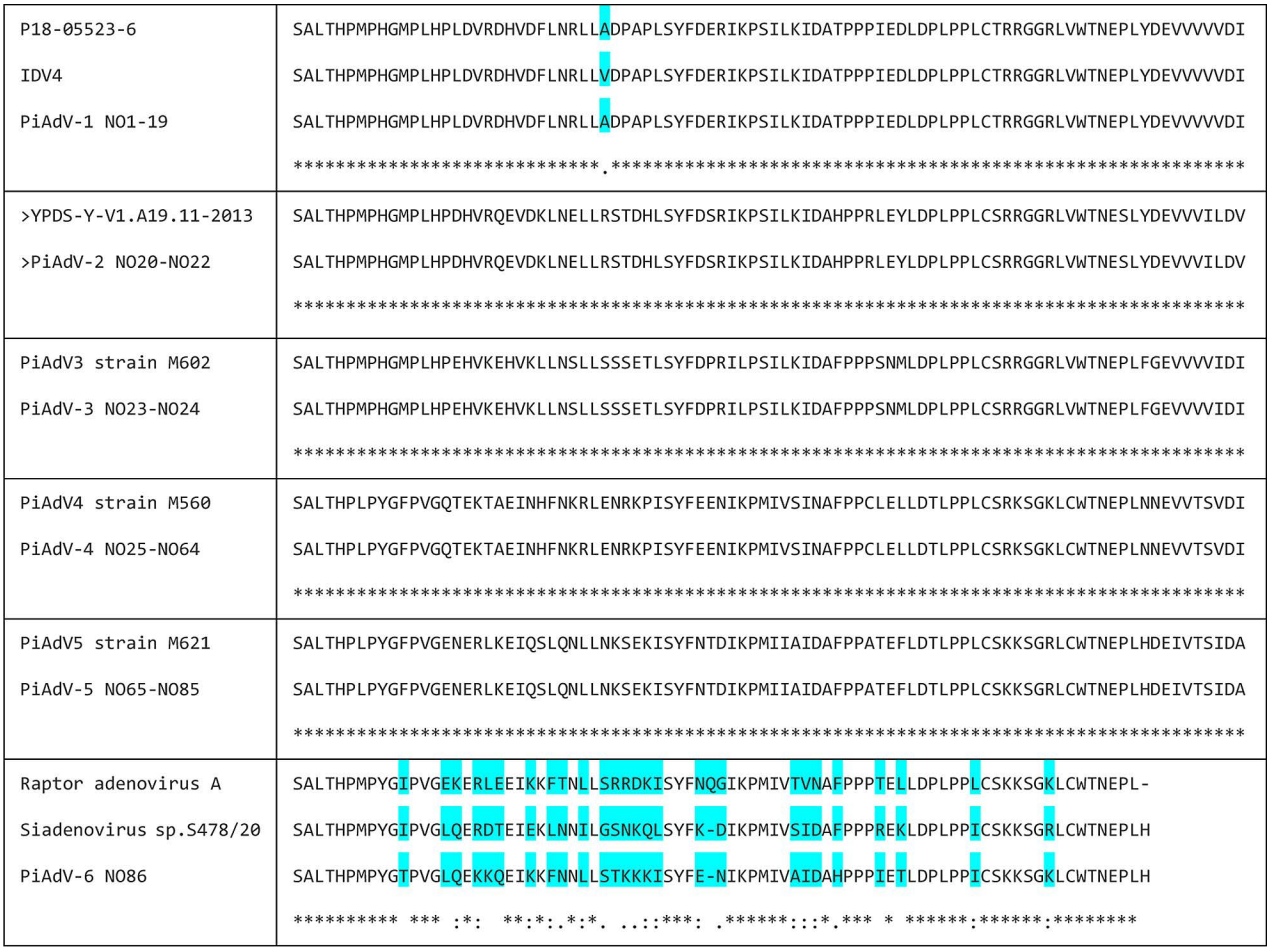

Figure 1—Differences of the amino acid sequence in the partial polymerase (pol) gene of pigeon adenoviruses (PiAdVs). Within the 90-amino-acid region, all 19 PiAdV-1 (PiAdV1 NO1 through NO19) pol protein sequences were the same, and they were consistent with the reference sequence of the P18–05523-6 strain (MW286325.1), whereas there was 1 amino acid difference with the IDV4 strain (NC_024474.1). All 3 PiAdV-2 (PiAdV2 NO20 through NO22), 2 PiAdV-3 (PiAdV3 NO23 and NO24), 40 PiAdV-4 (PiAdV4 NO25 through NO64), and 21 PiAdV-5 (PiAdV5 NO65 through NO85) partial pol amino acid sequences showed no amino acid diversity, and they all showed 100% consistency with the reference PiAdV-2 strain YPDS-Y-V1.A19.11–2013 (KX121164.1), PiAdV-3 strain M602 (KX555530.1), PiAdV-4 strain M560 (KX555530.1), and PiAdV-5 strain M621 (KX555530.1). As for the newly identified PiAdV (PiAdV-6 NO86), its amino acid sequence was 74.52% consistent with the Raptor adenovirus A (NC_015455.1) and *Siadenovirus* sp strain S478/20 (MW508338.2). The highlighted part of the figure shows the difference of their amino acids.

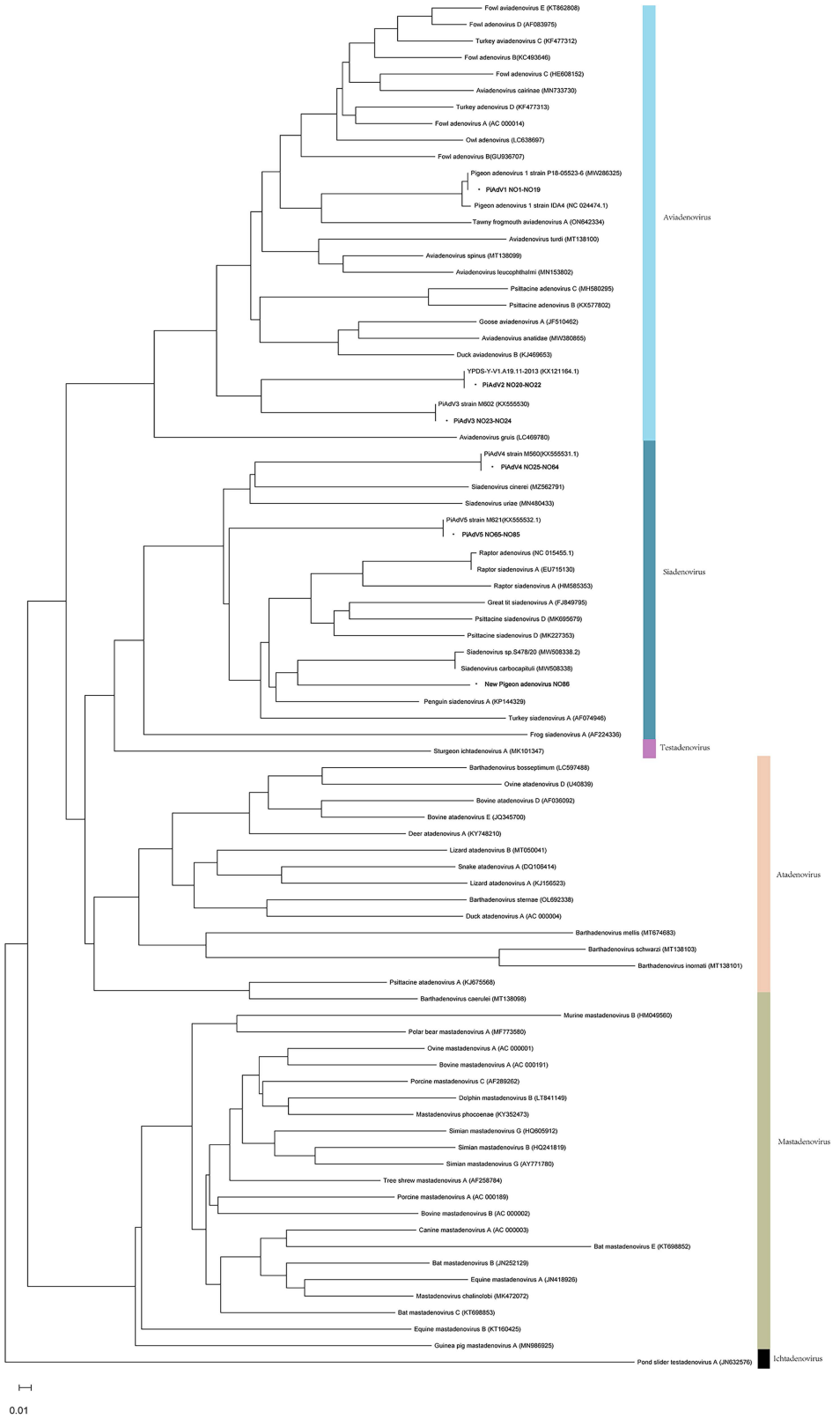

Figure 2—Phylogenetic relationship between PiAdV and other adenoviruses. The phylogenetic tree was generated based on amino acid sequences of the partial pol protein and using the neighbor-joining method. Pigeon adenoviruses were compared with adenoviruses of 6 genera. Scale bars indicate 0.01 amino acid substitutions per site. Bootstrap values are given at the respective nodes as determined for 1,000 iterations using the MEGA (version 11) software. The viruses are marked by the host name and type number omitting the abbreviation adenovirus. The highlighted color blocks represent the 6 genera for visualization.

PiAdV-2, 2 of PiAdV-3, 40 of PiAdV-4, 21 of PiAdV-5, and 1 of PiAdV-6. Although there were multiple sequences for each genotype, especially PiAdV-1, PiAdV-4, and PiAdV-5, their nucleotide sequence and predicted amino acid sequences show no differences or diversity. These sequences were also contrasted with those found in the GenBank database. **Figure 1** displays the comparison results between these sequences and the sequences in the GenBank database. Within the 90-amino-acid region with the name NO1 through NO90, all 19 PiAdV-1 (PiAdV1 NO1 through NO19) pol gene sequences were the same, and they were consistent with the reference sequence of the P18–05523-6 strain (MW286325.1), whereas there was 1 amino acid difference with the IDV4 strain (NC_024474.1). The partial pol amino acid sequences of the 3 PiAdV-2s (PiAdV-2 NO20 through NO22), 2 PiAdV-3s (PiAdV3 NO23 and NO24), 40 PiAdV-4s (PiAdV4 NO25 through NO64), and 21 PiAdV-5s (PiAdV5 NO65 through NO85) did not exhibit any amino acid diversity and were all 100% consistent with the referenced PiAdV-2 strain YPDS-Y-V1.A19.11-2013 (KX121164.1), PiAdV-3 strain M602 (KX555530.1), PiAdV-4 strain M560 (KX555530.1), and PiAdV-5 strain M621 (KX555530.1). As for the newly identified PiAdV (PiAdV-6 NO86), its amino acid sequence is 74.25% and 74.52% consistent with the Raptor adenovirus A (NC_015455.1) and *Siadenovirus* sp strain S478/20 (MW508338.2). The partial pol gene sequences of the newly identified PiAdV strain have been submitted to the National Center for Biotechnology Information GenBank database under the accession number PQ380003. The newly identified PiAdV was named PiAdV-6 for the first time, and it will not be found in other references.

Phylogenetic place of PiAdVs

Their genetic phylogeny was analyzed after building phylogenetic trees using their amino acid sequences and other additional reference sequences of typical adenoviruses retrieved from the GenBank database. The visible phylogenetic trees were in **Figure 2**. The phylogenetic tree revealed that PiAdV-1 (PiAdV-1 NO1 through NO19), PiAdV-2 (PiAdV2 NO20 through NO22), and PiAdV-3 (PiAdV3 NO23 and NO24) constitute a monophyletic group within the genus *Avidenovirus*, whereas PiAdV-4 (PiAdV4 NO25 through NO64), PiAdV-5 (PiAdV5 NO65 through NO85), and the newly identified PiAdV (PiAdV-6 NO86) belong to the genus *Siadenovirus*. Interestingly, they are not monophyletic as each of them appeared clearly in the separate clade.

Pigeon adenoviruses in turtledove populations

The 61 turtledove feces samples were screened to determine the genotype and proportion of various adenoviruses. Pigeon adenovirus-4 and PiAdV-5 were found in the turtledove fecal samples, with a positive proportion of 12% and 6%, respectively, which was lower than that in homing pigeons. In addition, no other PiAdV nor turtledove-specific adenovirus was detected. Remarkably, the PiAdV-4 and PiAdV-5 detected in pigeon and turtledove (*S decaocto* and *S chinensi*) fecal samples proved to be quite conserved, and their partial pol amino acid sequences were the same as those in pigeon populations.

Discussion

In addition to producing meat for human use, pigeon breeding is currently utilized for pigeon racing, sports, research, exhibition (display of fancy breeds), and other purposes. As the racing pigeon breeding industry grew, so did the frequency of interbreeding and commerce, and managing and preventing pigeon diseases became more and more important. At present, the quantity and diversity of adenovirus in pigeon flocks are poorly understood despite the fact that prior clinical studies[14] have identified 2 adenovirus-associated disease courses in pigeon flocks, named adenovirosis type I and adenovirosis type II. The adenovirus-associated diseases are still major infectious diseases threatening the pigeon breeding industry, according to professional veterinary feedback.[18,24] However, the viral genotype or serotype that causes the adenovirus-associated disease is still unknown.

Adenoviruses have a very complicated replicating mechanism, which results in a variety of virus release patterns.[26] Different sampling techniques and different sampling periods frequently produced significantly varied results due to the low sensitivity of the PCR method in the prior epidemiological screening of PiAdV variants.[21,27] In order to overcome the limited sensitivity of traditional PCR methods, the conserved pol gene fragment of the PiAdV was amplified in this study utilizing a highly sensitive nested PCR approach.[21,25] Combined with the subsequent Sanger sequencing method, it was able to detect the diversity of adenovirus in meat-use pigeons, homing pigeons, wild pigeons, and turtledoves. Some intriguing traits were discovered by screening the diversity of PiAdV in different pigeon flocks. Both homing and wild pigeon populations showed higher diversity and a higher positive percentage of PiAdVs compared to the meat-use pigeon group. Ignoring the PiAdV-1, PiAdV-4, and PiAdV-5 were found to be more distributed than other genotypes in all pigeon flocks in this study. Actually, the positive percentage of PiAdV-4 and PiAdV-5 was higher in free-roaming homing pigeons and wild pigeons than that in closed-farmed meat-use pigeon populations. Due to the breeding mode of racing homing pigeons, they usually face a higher frequency of trading, hybrid breeding, mixed training, and racing. This makes them more exposed to other populations, thus having an advantage over closed pigeon populations in terms of circulation and transmission of the PiAdVs. Closed ones are frequently more susceptible to outbreaks of a single virus because of their cramped, dense breeding conditions.

Prior epidemiological studies[21,22,28] on adenoviruses in homing pigeon flocks suggested that

PiAdV-2 exhibited a greater positive rate than other adenoviruses. Typically, the positive rate of PiAdV-2 was 13% to 30%. Nevertheless, the proportion of PiAdV-2 in all pigeon flocks examined in this study was much lower than that. The observed disparity may arise from distinct sample methodologies or disparate sampling sites. In this study, the samples were primarily collected from unstudied regions of northern China, and the feces samples were used instead of internal organs, like the liver and kidney, which are believed to be target organs of adenovirus.[18,21] The study complements the distribution of PiAdV-2 in pigeon populations in northern China, although the pathogenicity of PiAdV-2A has not yet been confirmed due to the lack of necessary clinical characterization.[22]

Pigeon adenovirus-1 was discovered to be significantly frequent in feces samples from meat-use pigeon farms where infectious necrotizing hepatitis adenovirus illness was ongoing during the sample collection phase. In contrast, it was less common in homing and wild pigeon populations, which gives reason to speculate that PiAdV-1 was spreading in this pigeon farms. In addition, another study[29] on the infectious disease outbreak in this flock was conducted; it provided supportive evidence that PiAdV-1 was the causative agent of the disease, and severe liver and kidney inflammatory injury was the cause of mortality. Obviously, the positive proportion of PiAdV-1 in the 2 types of feces was different as 16.7% of clumps feces and 77.8% of watery or soft feces samples were positive for PiAdV-1. From these data, PiAdV-1 appears to be associated with the presence of abnormal feces, consistent with previous reports.[29] In the pigeon flocks with necrotic hepatitis adenovirus disease, it appears that the shape of the feces is correlated with the health status of the pigeon, and the form of the feces may be utilized to identify infected and uninfected birds.

Nonrandom sampling may account for the low prevalence of PiAdV-1 in either wild or homing pigeon populations. It was challenging to get equivalent positive samples because most sick wild homing pigeons were anorexic, lethargic, or even died suddenly. They are also unable to travel foraging in groups with healthy pigeons. During the homing pigeon breeding process, once any subhealthy or sick pigeons were found, they were promptly quarantined or slaughtered in order to prevent the spread of infectious diseases. For this reason, positive PiAdV-1 samples were rarely found during routine farm sampling. However, the homing pigeons in public houses come from all over the country, and they are gathered together for training. Often, their training may involve flying thousands of kilometers across several provinces, and the frequency of interbreeding and commerce also boosts their mobility. This is undoubtedly the biggest source of virus transmission risk. In addition, wild and homing pigeons can transmit the virus directly through shared ecological niches. As natural PiAdV-1 reservoirs, homing or wild pigeons may pose a potential threat to meat pigeon farming.

In this study, a total of 6 genotypes of PiAdV were found, and multiple partial pol gene sequences were identified for each genotype except PiAdV-6. Nonetheless, each genotype's sequences remained unvaried and were also highly conserved when compared to the database reference sequences. Although PiAdV-4 and PiAdV-5 were found in different pigeon flocks and closely related turtledove flocks, their sequences were identical. In addition, only 1 amino acid difference in PiAdV-1 was found when compared with the IDV4 strain (NC_024474.1), which was isolated in the 1990s. Since pol genes are very well conserved, it is obviously reliable to classify new species based on them.[30,31] Considering that the newly discovered PiAdV PiAdV-6 exhibits more than 25% sequence differences at the nucleotide and amino acid levels when compared to *S carbocapituli* (MW508338) and Penguin siadenovirus A (KP144329), it should be identified as a new species.

Among the 6 genotypes, PiAdV-1, PiAdV-2A, and PiAdV-3 belong to the *Aviadenovirus* genus, whereas PiAdV-4, PiAdV-5, and PiAdV-6 are members of the *Siadenovirus* genus. The genus *Aviadenovirus* is believed to be avian-specific and to have coevolved with birds.[5,32] However, as consistent with the *Atadenovirus* and *Siadenovirus* genera, the evolution of the *Aviadenovirus* genus also reflects a combination of host switching and virus-host coevolution.[32] *Aviadenoviruses* originated from other wild birds, such as passerines and psittacines, which dominate in lineages 2 and 4 of the adenoviruses, respectively.[32] The identified PiAdV-1, PiAdV-2, and PiAdV-3 belong to lineage 1 and seem to be host-specific viruses. Even though they have not been detected in other species and their evolutionary relationship is unknown, this does not rule out the possibility of host switching. Although the hypothesis of the amphibian origin of *Siadenovirus* remains to be proven, it is true that this genus has been found in a variety of hosts, including frogs, great tits, penguins, raptors, parrots, skuas, turkeys, and so on.[5] This significant crossing between distantly related frog and avian hosts suggests that the major host switching event occurred in early *Siadenovirus* evolution. Frog siadenovirus A was thought to be the basal of all *Siadenoviruses* isolated from avian hosts.[32] It crossed the genomic barriers to infect psittacine birds (Psittacine siadenovirus F) firstly, and then Psittacine siadenovirus F appeared to coevolve with their host or evolve across the genomic barriers to infect multiple hosts, such as penguins, raptors, and skuas.[33] Based on the bootstrap network analysis using the available partial DNA pol gene sequences of known adenoviruses, 4 lineages within the genus *Siadenovirus* were identified. Lineages 2 and 3 compromised a range of diverse bird hosts, including psittacines, Columbiformes, poultry, and Strigiformes. Clearly, the identified PiAdV-4 and PiAdV-5 belong to lineage 2. The Penguin siadenovirus A (KP144329) and the newly identified PiAdV-6 constitute a monophyletic group, and they both belong to lineage 3. The detection of highly conserved PiAdV-4 and PiAdV-5 in turtledoves and

pigeons also provides evidence that they crossed genomic barriers to infect multiple hosts.

This study verified that pigeon and turtledove populations contain a variety of adenoviruses, particularly those with pathogenic genotypes, making them natural reservoirs. The risk of crossspecies transmission of the pigeon adenoviruses requires vigilance. Fortunately, the PiAdV-1, PiAdV-2A, and PiAdV-3 discovered in the investigation closely match the sequences refenced in the database, and the sequence's conservation may lower the possibility of changes in pathogenicity or the opportunities for cross-species transmission. Both turtledoves and pigeons belong to the family Columbidae, order Columbiformes. They are extremely closely related and have similar ecological niches in urban and suburban areas. It has been demonstrated that turtledoves serve as reservoirs for PiAdV-4 and PiAdV-5 and that virus spillover from their shared biological niche may lead to cross-species transmission between turtledoves and pigeons. Since PiAdV-1 and PiAdV-2 were not found in turtledove species, it is unclear if they can infect turtledove populations and establish a natural virus reservoir. Nevertheless, to find out if other PiAdVs were carried by other similar Columbiformes birds and coevolved with them, more epidemiological studies are still needed. In sum, these findings advance knowledge of the diversity of PiAdVs and offer fresh strategies for stopping and managing the spread of PiAdV disease. A deeper comprehension of the evolution and distribution of adenoviruses is also provided by this study.

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Supplementary Materials

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