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High Mortality in Terns and Gulls Associated with Infection with the Novel Gull Adenovirus

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ABSTRACT: High mortality in Caspian Terns (*Hydroprogne caspia*) and Great Black-headed Gulls (*Larus ichthyaetus*), was recorded on the northeastern shores of the Caspian Sea in June 2013. Retrospective high throughput sequencing of archived tissue samples conducted in 2018 revealed the presence of the recently identified novel gull adenovirus similar to that associated with mortality in gulls in the Netherlands in 2001. We suggest that that this gull adenovirus specifically can be considered as an emerging threat to the health and conservation of gulls and terns.

Key words: Adenovirus, Caspian Sea, Caspian Tern, Great Black-headed Gull, mortality.

Adenoviruses (AdVs) are nonenveloped DNA viruses possessing a linear double-stranded DNA genome and are divided into five genera (International Committee for the Taxonomy of Viruses 2018). Three genera, *Siadenovirus, Atadenovirus,* and *Aviadenovirus,* infect a wide range of bird species and some AdV species can cause a variety of clinical symptoms (McFerran and Smith 2000). Most of those infections are well-studied in domestic poultry and in wild bird species of the order *Anseriformes* (ducks and geese) (Zsak and Kisary 1984). However, little is known about AdVs in birds of the *Laridae* family (gulls and terns).

A mass mortality in Caspian Terns (*Hydroprogne caspia*) and sympatric Great Black-headed Gulls (*Larus ichthyaetus*) was reported (Sultangaliyev 2013) in the northern part of the Caspian Sea on the sandy island of Zuidwest Shalyga, Kazakhstan in June 2013 (Fig. 1). A mixed population of Caspian Terns (about 3,000 birds) and Great Black-headed Gulls (about 5,000 birds) breed on this island annually. The island has been designated as a nature reserve, and access for unauthorized people is prohibited. A comprehensive field investigation was conducted 7 d after the outbreak began to determine the cause of the mortality. An interview with the field rangers indicated that the mortality had started 1 wk earlier, appearing simultaneously in all age categories of both species, Caspian Terns and Great Black-headed Gulls; however, there was a noticeable predominance of juveniles among the affected birds.

The rangers estimated that approximately 70–75% of all the juveniles were affected. Approximately 90% of the carcasses were collected and incinerated by the rangers within the first week of the mass die-off. We found approximately 120 bird carcasses on the investigation day; 90% of them were moderately or highly decomposed. The proportion of dead birds from each species, Caspian Terns and Great Black-headed Gulls, was nearly 45% and 55% respectively. In addition, we found 10 sick birds, six gulls and four terns, with visible clinical signs, including depression and weakness, diarrhea, and uni- and bilateral paresis and paralysis of limbs and wings. According to our observations, signs of diarrhea were observed in 40% of gulls and 30% of terns, whereas paresis and paralysis were found equally in both species. However, the rangers reported that, paresis and paralysis were noticed more often in terns.

Four fresh carcasses, two terns and two gulls, were selected for post mortem examinations. Severe dehydration and lack of adipose tissue in the epicardium and intestinal mesentery were noted in both species. No other specific pathological lesion was found. Samples, 5×5 mm, from intestine, liver, spleen, and lung were collected from each of four necropsied birds and placed into viral

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FIGURE 1. Geographic distribution of an adenovirus outbreak causing mass mortality in Caspian Terns (*Hydroprogne caspia*) and Great Black-headed Gulls (*Larus ichthyaetus*) in 2013. (A) General map of Eurasia. The red square shows the location of sandy island of Zuidwest Shalyga, Kazakhstan, in the northern area of the Caspian Sea. (B) Photographs of the island from a helicopter.

transport medium containing Hank's balanced solution (Life Technologies, Paisley, UK), antibiotics (2,000 U/mL penicillin, 2 mg/mL streptomycin, 50 µg/mL gentamycin), antimycotic (50 U/mL nystatin), and 0.5% bovine serum albumin.

Investigation in 2013 produced negative results for Newcastle disease (using a reverse-transcription PCR, RT-PCR; Aldous et al. 2003) and avian influenza viruses (using an RT-PCR; Payungporn et al. 2004). Toxicological investigations by the environmental observation services also had negative results. Because the cause of the mass mortality was not identified, all samples were conserved at –80 C for in-depth analyses in the future.

In 2018, we carried out retrospective analysis of archived tissue samples using PCR, RT-PCR and high throughput sequencing to determine the real cause of the mass mortality. Samples from two terns and two gulls, including four lungs and intestines, two livers (one each from a tern and a gull), and one spleen (from a tern) were analyzed by PCR and RT-PCR using pan-genus and pan-family primers. Using RT-PCR with pan-PMV (*Paramyxoviridae* family) primers (Tong et al. 2008) and PCR with pan-herpesviruses primers (VanDevanter et al. 1996) produced negative results.

Molecular analyses of samples with PCR using primers targeting the conserved hexon gene of *Aviadenovirus* genus (Mase et al. 2009) showed positive results when analyzing all four intestines, one lung (from a gull), and one liver (from a gull) samples.

Using quantitative RT-PCR with pan-coronavirus (*Coronaviridae* family) primers (Muradrasoli et al. 2009) also produced positive results. Further massive parallel sequencing confirmed the presence of a nonpathogenic delta-coronavirus similar to variants isolated in the Middle East (Lau et al. 2018). This group of genetically close deltacoronaviruses found in various hosts including falcon, buzzard, and pigeon, has not yet been ratified by the International Committee on Taxonomy of Viruses. It has been suggested that this group of novel delta-coronaviruses originated from recombination between the International Committee for the Taxonomy of Viruses-ratified white-eye coronavirus HKU16 (WE-CoV HKU16) and magpie robin coronavirus HKU18 (MRCoV HKU18; Lau et al. 2018; Wille and Holmes 2020). These findings warrant further investigations.

Adenovirus- and coronavirus-positive lung and intestinal tissue samples from one tern and one gull were selected for further sequencing analyses. The frozen tissues were

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TABLE 1. Results of the BLAST search of the contigs obtained after the de novo assembly of all sequenced samples from Caspian Terns (*Hydroprogne caspia*) and Great Black-headed Gulls (*Larus ichthyaetus*) sampled following a mass mortality event in the northern part of the Caspian Sea, in Kazakhstan in 2013. The fragment size of the penton gene was 201 base pairs (bp), the hexon gene, 388 bp, and the polymerase gene, 454 bp.

Top hit	Coverage site (nt to nt)[a]	% Query coverage	E value	% Identity	Gene size (bp)	GenBank accession no.
Penton gene						
Gull adenovirus	562 to 762	100	5e-89	97.01	1,578	KC309440
Fowl aviadenovirus C	17543 to 17740	98	5e-39	82.41	1,578	MK572851
Hexon gene						
Gull adenovirus	1942 to 2329	100	0.0	98.71	2,818	KC309439
Fowl aviadenovirus E	22117 to 22487	95	4e-73	80.70	2,847	MK572857
Fowl aviadenovirus C	22283 to 22642	92	2e-70	80.61	2,814	HE608152
Polymerase gene						
Gull adenovirus	2414 to 2859	100	8e-160	89.91	3,411	KC309438
Fowl aviadenovirus D	7934 to 8352	94	3e-65	78.12	3,915	MN711789

[a] nt = nucleotide.

thawed and homogenized using a Tissuelyzer homogenizer (Qiagen, Hilden, Germany), then centrifuged. Supernatants were filtered through 0.22 µM filter (Membrane Solutions, Auburn, Washington, USA) and treated with a mix of nucleases: Benzonase (Sigma-Aldrich, St. Louis, Missouri, USA), Turbo DNAse (ThermoFisher, Vilnius, Lithuania), DNAse I (ThermoFisher), RNAse A (ThermoFisher), and RNAse T1 (ThermoFisher). Viral nucleic acid was extracted using the QIAamp Viral RNA Mini Kit (Qiagen).

For massive parallel sequencing, library preparation was conducted using the NEBNext Ultra RNA Library Preparation kit (New England Biolabs, Ipswich, Massachusetts, USA) according to the manufacturer's protocol. Library size selection was performed using Ampure XP beads (Beckman Coulter, Brea, California, USA). The size of libraries was checked on a Bioanalyzer 2100 instrument (Agilent Technologies, Boeblingen, Germany).

Sequencing was performed using the MiSeq Reagent version 3 kit on a MiSeq sequencer (Illumina, San Diego, California, USA). Obtained sequence data were trimmed and their quality was evaluated with FastQC (Andrews et al. 2010). In total, approximately 76,000 raw sequencing reads per sample were obtained. The obtained reads were assembled de novo using Geneious 11.0 software (Biomatters, Aukland, New Zealand) applying the installed "Geneious" algorithm with default parameters. Resulting contigs were subjected to BLASTn and BLASTx search in the local viral reference database as described in Metavisitor pipeline (Carissimo et al. 2017). Local BLAST hits with lengths >100 nucleotides (nt) were considered as significant at E value ≤10e-5, and the potential viral sequences were subjected to aligning with respective complete genome sequences downloaded from Genbank.

A BLAST search of the contigs from all sequenced samples revealed the presence of a virus belonging to the *Adenoviridae* family, using the cutoff E value of ≤10e-5 mentioned earlier. The final assembly, aligned to the reference gull AdV sequence, contained sequence fragments representing each of three main genes: polymerase, hexon, and penton genes (Table 1). The representative sequence of the 388 nt fragment of the hexon gene corresponding to nt 1942 to nt 2329 of previously described gull adenovirus (GenBank accession no. KC309439) is available (GenBank accession no. MT068636).

Multiple alignments were performed using the ClustalW algorithm in MEGA 7.0. software (Kumar et al. 2016) based on the fragments of nt sequences of the hexon (388

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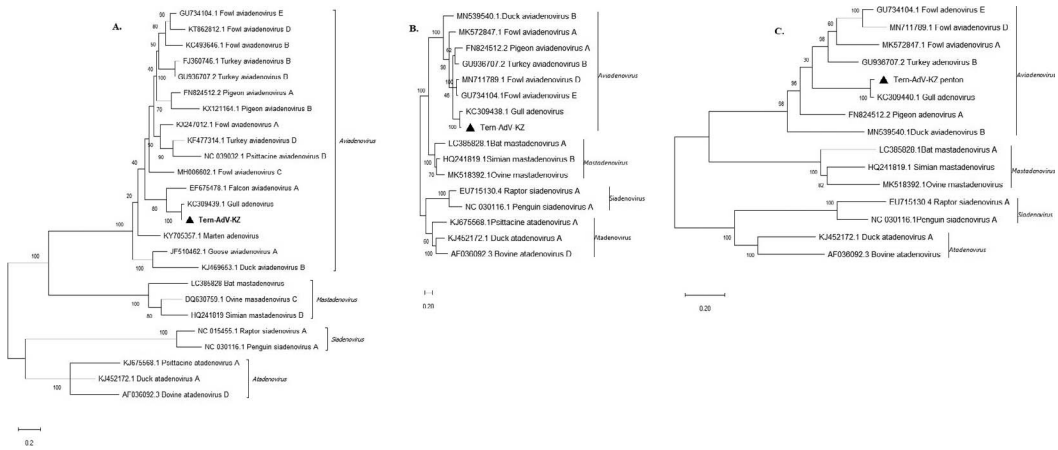

FIGURE 2. Phylogenetic trees for the partial (A) 388 nucleotide (nt) hexon, (B) 454 nt polymerase, and (C) 201 nt penton genes of the Tern-AdV-KZ adenovirus (indicated with a solid triangle) and other selected species of *Mastadenovirus*, *Siadenovirus*, *Atadenovirus*, and *Aviadenovirus* genera. The unrooted trees were constructed using the maximum-likelihood approach (MEGA 7.0 software) based on the Hasegawa-Kishino-Yano model. Numbers indicate the bootstrap values of 1,000 replicates. Scale bar refers to a phylogenetic distance of 0.2 nt substitutions per site. The Tern-AdV-KZ adenovirus was detected following a mass mortality event in Caspian Terns (*Hydroprogne caspia*) and sympatric Great Black-headed Gulls (*Larus ichthyaetus*) in the northern part of the Caspian Sea, on the island of Zuidwest Shalyga, Kazakhstan.

nt), penton (201 nt), and polymerase (454 nt) genes of the identified adenovirus. Phylogenetic trees (Fig. 2) were constructed using the "maximum likelihood" method and the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) suggested by ModelTest, implemented in MEGA 7.0.

To exclude bacterial infections, the 16S metagenome of bacteria was studied using lung and intestinal samples from gull and tern. The samples exclusively contained representatives of nonpathogenic microflora (Supplementary Material Fig. S1).

BLAST percentage similarity analysis and phylogenetic analysis demonstrated that we had identified a variant of gull adenovirus sharing 98.1% similarity on the nt across the hexon gene with gull Adenovirus that was described as associated with a mortality event in Herring Gulls (*Larus argentatus*) and Lesser Black-backed gulls (*Larus fuscus*) in the Netherlands in 2001; this was the only previous record of gull adenovirus (Bodewes et al. 2013). Gull AdV is a novel species in the *Aviadenovirus* genus that might have diverged from known AdVs. Genetically it is most closely related by hexon gene to Fowl AdV E and Fowl AdV C with identity of 80% on nt level. By penton gene, it is 82% similar to AdV C, and by polymerase gene is 78% similar to AdV D. Phylogenetically, Fowl AdV-D and Fowl AdV-E are closely related, but Fowl AdV-C represent a separate clade (Marek et al. 2016). Our detection of a closely related adenovirus, associated for a second time with mass mortality of terns and gulls, suggests that this virus is pathogenic and should be considered in the event of future mortality events in wild gulls or terns.

Gulls play an important role in both intra- and intercontinental transfer of many viruses (Benkaroun et al. 2016). Further surveillance of gull adenoviruses is very important to better understand the ecology, epidemiology, and influence of the virus on gull populations, particularly for Great Black-headed Gulls because they are listed in The Red List of Kazakhstan (1981).

Continued surveillance of wild and domestic birds in the region, and genome sequencing of adenoviruses isolated from any bird species, will help us to evaluate the potential effects of the newly identified virus on wild bird populations.

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SUPPLEMENTARY MATERIAL

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