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BRIEF REPORT

A novel cetacean adenovirus in stranded harbour porpoises from the North Sea: detection and molecular characterization

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Abstract Harbour porpoises (*Phocoena phocoena*) are the most prevalent cetaceans in the North Sea. The fecal viral flora of 21 harbour porpoises stranded along the Dutch coastline was analyzed by a metagenomics approach. Sequences of a novel cetacean mastadenovirus, designated harbour porpoise adenovirus 1 (HpAdV-1), were detected. The sequence of a 23-kbp genomic region, spanning the conserved late region, was determined using primer walking. Phylogenetic analysis indicated that HpAdV-1 is most closely related to bottlenose dolphin adenovirus and clusters with Cetartiodactyla adenoviruses. The prevalence of HpAdV-1 was low (2.6%) based on targeted PCR-screening of the intestinal contents of 151 harbour porpoises stranded between 2010 and 2013.

Harbour porpoises (*Phocoena phocoena*) are small cetaceans that live in the cooler coastal waters of the North Atlantic, North Pacific and the Black Sea [1]. They are the most prevalent cetaceans in the North Sea, where the population is currently estimated to consist of 250,000 porpoises, of which 85,000 may occur in the Dutch coastal waters [2–4]. Coinciding with the increasing population size in the southern North Sea, as observed since the 1990s, stranding numbers along the entire coastline increased to almost 900 in the Netherlands in recent years (http://www.walvisstrandigen.nl). Common causes of death determined upon necropsy of stranded individuals include by-catch, predation by grey seals (*Halichoerus grypus*), and various infectious diseases [5–9].

Some of the harbour porpoises stranded along the Dutch coastline since 2008 were subjected to necropsy at the Faculty of Veterinary Medicine, Utrecht University, The Netherlands. Necropsy was performed according to a standard protocol, described by Kuiken and Baker [10]. Samples from fresh cases were taken for histopathological analysis, fixed in formalin, and processed routinely. Samples for molecular analysis – including intestinal contents, if present – were stored at -20 °C. Intestinal contents from a total of 151 harbour porpoises were collected over a 5-year period (2010–2014). Metagenomic analysis was performed on the intestinal contents of 21 randomly selected harbour porpoises stranded dead in 2010 and 2011 (Supplementary Table 1).

Metagenomic analysis was performed as described previously [11, 12]. Briefly, intestinal contents were homogenized using a Fastprep24 tissue homogenizer (MP Biomedicals) in Hank's balanced salt solution. After centrifugation, supernatants were filtered and treated with Omnicleave Endonuclease (Epicentre Biotechnologies). Subsequently, viral RNA and DNA were extracted using a Nucleospin RNA XS Kit (Machery-Nagel) and a High Pure Viral Nucleic Acid Kit (Roche) according to the instructions of the manufacturer. Using random PCR in

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combination with next-generation sequencing with a 454 GS Junior instrument (Roche), random sequences of the DNA were obtained essentially as described previously [11]. Adaptor and primer sequences were removed from all reads, and the obtained reads were assembled using *de novo* assembly in CLC Genomics Workbench 5.5.1 (CLC Bio) and analyzed using nucleotide (contigs and singletons) and translated nucleotide BLAST. Sequences were classified into viruses, phages, bacteria, and eukaryotes based on the taxonomic origin of the best-hit sequence using MEGAN software. An E-value of e-10 was used as the cutoff value for significant virus hits for BLASTn and BLASTx.

Metagenomic analysis resulted in 156,314 reads, of which the hits against the virus database are shown in Supplementary Table 1. In samples from ten animals, no obvious viral sequences were detected. In porpoise UT353, adenovirus-like sequences were detected (132 reads, 5 contigs), with one of the contigs demonstrating 100% sequence homology with a partial nucleotide sequence from an adenovirus DNA polymerase gene. This sequence was obtained from the lymph node of a harbour porpoise stranded in the USA in 2008 (strain MH08-032; GenBank JN377908.1). The aim of this study was to characterize the adenovirus-like sequences obtained from the intestinal contents of the investigated harbour porpoises, the presence of which might point towards a novel cetacean mastadenovirus. Several other virus-like sequences were detected in the intestinal contents of these animals (Supplementary Table 1) and will be the focus of future studies.

Adenoviruses are non-enveloped, medium-sized DNA viruses with a linear, double-stranded genome of 26-45 kbp [13–15]. They are taxonomically divided into five genera, infecting members of all major vertebrate linages. Only adenoviruses belonging to the genera *Mastadenovirus* and *Atadenovirus* infect mammals. In immunocompetent mammals, most adenoviruses generally do not cause severe disease in their natural hosts [16]. In immunosuppressed and non-natural hosts, adenoviruses can cause a wide range of disease, including disease of the respiratory and gastrointestinal system. In pinnipeds, adenoviruses have specifically been linked to hepatitis and ocular lesions [17, 18]. In bottlenose dolphins, adenovirus infection was associated with gastroenteritis [19].

The adenovirus-like contigs were mapped against the full genome sequence of the closely related bovine adenovirus type 2 (GenBank NC_002513.1). Specific primers were designed to amplify the genomic region spanning from the E1B 55k to the pVIII gene. Amplified PCR fragments (Phusion Hot Start II High-Fidelity DNA Polymerase, Thermo Fisher Scientific) were cloned in the pJET1.2/blunt vector (Thermo Fisher Scientific), and for each position, at least three clones were sequenced using primer walking (Macrogen; sequences available upon request). From the 454 reads, a 22,920-nt fragment was obtained, comprising the nearly complete conserved late (L) region as well as parts of the E1 region of the genome (Fig. 1A). BLAST homology searches showed that the average amino acid sequence identity to the bottlenose dolphin AdV was 75% ([20], GenBank KR024710). The sequenced genome region displayed an organization typical for those of mastadenoviruses. This includes the presence of the IX and V structural protein coding genes as well as the presence of introns in the genes for IVa2, DNA-dependent DNA polymerase and the terminal protein precursor (pTP) [14]. Interestingly, the IVa2 gene contained an alternative splice acceptor site (CGGA) preceded by an additional methionine in the same frame (position 3314 on the leftwards-transcribed *l* strand), as well as a well-conserved donor site (AGGT) of a 4-amino-acid leader. Although splicing in the IVa2 gene is regarded as a universal phenomenon for members of the genus *Mastadenovirus* [14], the amount of experimental evidence is scarce and is mostly based on human AdVs. In the genus *Atadenovirus,* no indications for splicing are present for ruminant AdVs, while sequence data of lizard AdVs do imply the presence of such introns [21, 22]. The weak acceptor signal may suggest a lack of splicing, and direct translation from the preceding methionine may occur; experimental data on this are needed. In the predicted amino acid sequence of the penton base, a fiber-binding site could be identified (RLNNLLG), whereas the integrin-binding motifs RGD and LDV are, similar to most non-human AdVs, not present. Protease cleavage sites are present in all precursor proteins (marked with p), out of which the second one in pVII is of type II, a trait of members of the genus *Mastadenovirus* [14, 23]. For pX, only one protease cleavage site is predicted, unlike in most mastadenoviruses, but similar to other mastadenoviruses of the clade Cetartiodactyla, a group formed by cetaceans, ruminants, hippos, pigs, and camelids within the mammalian superorder Laurasiatheria [24] (Fig. 1B).

Amino acid sequence alignments of the complete polymerase and penton base protein sequences were generated using ClustalX2 [25]. Representatives of the genera *Aviadenovirus*, *Siadenovirus*, *Atadenovirus* and *Mastadenovirus* were included. Model selection was done using Prottest v3.2, based on both the Akaike and Bayes information criteria [26]. Maximum-likelihood calculations were performed using the PhyML-aLRT online platform, and the reliability of the topology was tested by both bootstrap and aLRT-Shimodaira-Hasegawa-like testing [27]. For the polymerase-based tree, the LG+I+G+F model was indicated to be the most appropriate, with a $_{p}$inv value of 0.68 and an α parameter of 0.695, whereas LG+I+G was indicated with $_{p}$inv

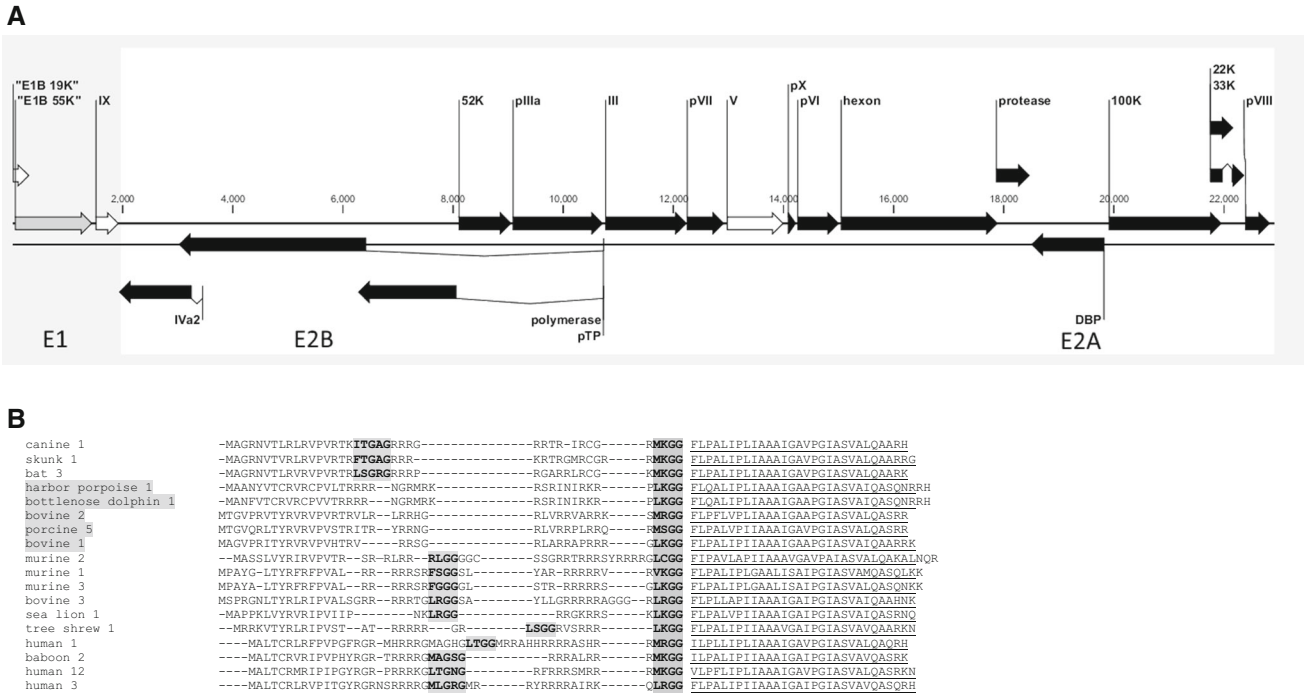

Fig. 1 Partial HpAdV-1 genome layout and analysis of pX of mastadenoviruses. **A** The genome organization of a nearly 23-kbp-long fragment of the harbour porpoise adenovirus 1 genome is schematically depicted. The sequenced region shows typical characteristics of members of the genus *Mastadenovirus.* Genes that only occur in members of this genus are depicted in white. E1B 55K has the only homologue in the genus *Atadenovirus*, family *Adenoviridae,* and is depicted with a grey background. **B** Alignment of the amino acid sequence of pX of various mastadenoviruses, with protease cleavage sites indicated by a grey background. The conserved transmembrane motif of the mature protein is underlined. Even-toed ungulate adenoviruses are indicated by grey shading. Both harbour porpoise adenovirus 1 and bottlenose dolphin adenovirus 1 lack one cleavage site in pX, similar to all Cetartiodactyla adenoviruses to date. Bovine adenovirus 3 is an exception to this and is believed to be more closely related to primate adenoviruses

= 0.7 and $\alpha = 0.614$ for the penton-based one (Fig. 2). In both trees, the novel harbour porpoise AdV, herein designated HpAdV-1 (deposited under GenBank accession number KY35247), groups together with the other cetacean bottlenose dolphin AdV and the AdVs of the genus *Mastadenovirus* that are found in hosts belonging to the Cetartiodactyla clade.

In order to investigate the prevalence of HpAdV-1 among stranded harbour porpoises in the Netherlands, the intestinal contents of 151 animals were subjected to DNA extraction and PCR analysis. Fecal samples were mixed 1:5 with Stool Transport and Recovery buffer (Roche Diagnostics) and incubated for 30 min at -20 °C. After thawing, samples were incubated for 15 min at 95 °C, followed by centrifugation for 3 min at $8000 \times g$. DNA was extracted from 200 µl of supernatant using a High Pure PCR Template Preparation Kit (Roche Applied Science). Other tissues from harbour porpoises of which the intestinal contents tested positive for HpAdV-1 were subjected to DNA extraction. For this, 37.5 g of tissue was homogenized in Magna Lyser green bead tubes, using a Magna Lyser (Roche), and DNA was extracted using a QIAGEN DNeasy Blood & Tissue Kit. PCR was performed using primers Phph.AdV.DNApol.F01 (5'-CCCAAGTTGATACAGCGAGC-3') and Phph.AdV.DNApol.R01 (5'-GCAGACCTGGTGAAAACGTT-3') targeting a 385-bp fragment of the conserved DNA polymerase gene of HpAdV-1. Taq DNA polymerase (Thermo Fisher Scientific) was used for diagnostic PCR, with the thermal profile being a denaturation step of 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The reaction was carried out in a T100 Thermal Cycler (Bio-Rad). PCR products were analyzed by 2% gel electrophoresis, and fragments of the expected sizes were analyzed by Sanger sequencing (Macrogen).

HpAdV-1 was detected in the intestinal contents of 4 out of 151 samples from harbour porpoises (2.6%, animal identification numbers: UT353, UT803, UT1002 and UT1025). The positive animals were three juvenile males and one juvenile female that stranded between 2010 and 2013 at various places along the Dutch coastline. Known causes of death were bycatch (n = 2) and emaciation (n = 1). The cause of death of one of these animals remained unknown due to an advanced state of decomposition. Available organ samples (for most animals including

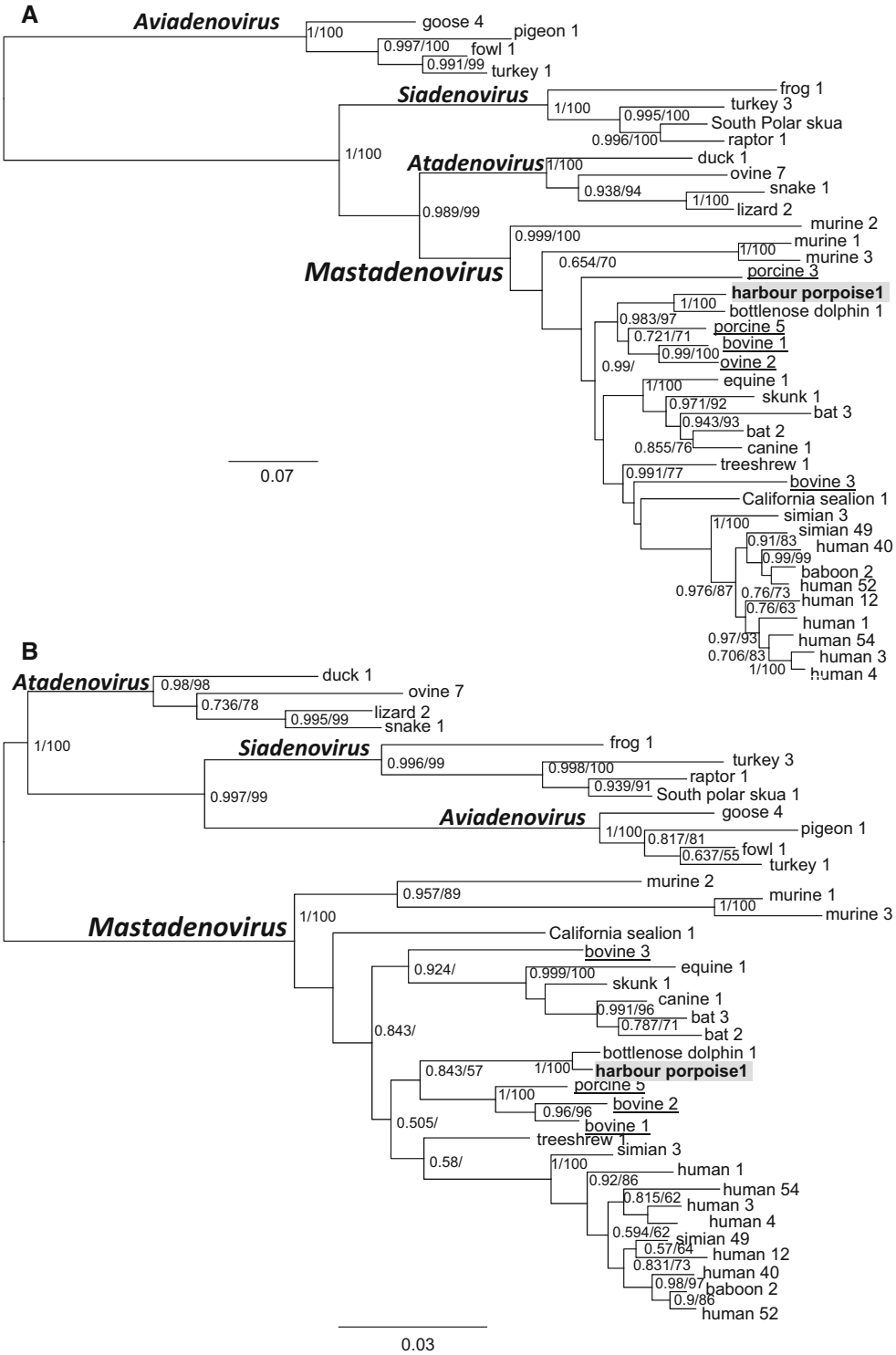

Fig. 2 Maximum-likelihood phylogeny inference of the DNA-dependent DNA polymerase and the penton-base protein of harbour porpoise adenovirus. Analysis of **A** polymerase and **B** penton base protein indicate that hpAdV-1 is monophyletic with bottlenose dolphin adenovirus 1 (highlighted in bold). Both viruses are included in the clade containing the other even-toed ungulate mastadenoviruses (underlined). The topology of both trees was tested by aLRT-Shimodaira–Hasegawa-like test and bootstrap analysis, and these values are presented as node labels. Only aLRT-SH-like values over 0.5 and bootstrap values over 50 for 100 repeats are shown

cerebrum, cerebellum, lung, lymph nodes, liver, kidney, spleen, and bladder) all tested negative for HpAdV-1 by PCR, except for the intestine of animal UT1002. None of the animals demonstrated macroscopic or microscopic lesions commonly associated with gastrointestinal virus infection.

Adenoviruses have been isolated from gastrointestinal samples of several healthy marine mammals, including a sei whale (*Balaenoptera borealis*) [28], two bowhead whales (*Balaena mysticetus*) [29] and a beluga whale (*Delphinapterus leucas*) [30]. More recently, a novel adenovirus was detected in blood and fecal samples and was

linked to self-limiting gastroenteritis in four captive bottlenose dolphins (*Tursiops truncatus*) [19]. Members of the family *Adenoviridae* have long been suggested to have an apparent co-evolutional history with their host species [13]. This is further supported by our results, as both the evolutionary relationships implied by the phylogenetic reconstructions and the organization of the pX protease cleavage sites mirror the evolutionary relationships of the Cetartiodactyla [31]. Events of presumed host-switches, however, further increase our understanding of the evolution of adenoviruses. Bovine adenovirus 3, yet another adenovirus of a cetartiodactylan host, contains both pX cleavage sites and is believed to be related to primate AdVs, supported by both phylogeny and serology [32]. These observations and the detection of HpAdV-1 in similar samples from multiple harbour porpoises suggest that porpoises are the natural hosts of this virus. The clinical impact of HpAdV-1 is unclear, since no typical adenovirus lesions were found in the four harbour porpoises that tested positive for HpAdV1 in their feces. It is likely that HpAdV-1 is a common but low-prevalence virus and that disease only develops if predisposing factors are present. Similarly, the harbour porpoise from the US that stranded in 2008 and in which HpAdV-1 was detected did not show any evidence of adenovirus-associated disease upon histopathological examination. Detecting HpAdV-1 in harbour porpoises from both European and North American Atlantic coastal waters demonstrates that HpAdV-1 is widespread.

In summary, we used metagenomic analysis to explore the viral flora of intestinal contents of stranded harbour porpoises along the Dutch coastline. The nearly complete genomic sequence of the conserved central genome region of HpAdV-1 was determined, revealing that this region displays divergence higher than 5–10% from that of the bottlenose dolphin AdV. HpAdV-1 therefore seems to be not only a novel AdV type but also a member of a new species. Phylogenetic analysis indicated a close evolutionary relationship to even-toed ungulate adenoviruses of members of the Cetartiodactyla, which is in line with long co-evolution of these adenoviruses with their respective hosts. Although the prevalence of HpAdV-1 among Northwestern European harbour porpoises is low (2.6%), the detection of HpAdV-1 sequences in a North American harbour porpoise suggests a wide geographic range. In contrast to the recently reported bottlenose dolphin adenoviruses, no adenovirus-associated lesions were found upon histological examination of HpAdV-1-positive harbour porpoises. Further detection and characterization of viral strains is required to increase our understanding of the biology and evolution of cetacean adenoviruses.

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Compliance with ethical standards

Harbour porpoise necropsies were funded by the Dutch Ministry of Economic Affairs (Grant Number 140000353). All authors declare that they have no conflict of interest. All applicable International, National, and/or Institutional Guidelines for the care and use of animals were followed.

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