

Species-Specific Identification of Human Adenoviruses in Sewage

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Abstract

Human adenovirus (HAdV) diversity in sewage was assessed by species-specific molecular methods. Samples of raw sewage were collected in 14 sewage disposal systems from January to December 2011, in Poland. HAdVs were detected in 92.1% of the analysed sewage samples and was significantly higher at cities of over 100 000 inhabitants. HAdV DNA was detected in sewage during all seasons. The most abundant species identified were HAdV-F (average 89.6%) and -A (average 19.6%), which are associated with intestine infections. Adenoviruses from B species were not detected. The result of the present study demonstrate that human adenoviruses are consistently present in sewage in Poland, demonstrating the importance of an adequate treatment before the disposal in the environment. Multiple HAdV species identified in raw sewage provide new information about HAdV circulation in the Polish population.

Key words: adenoviruses' detection, sewage, species-specific identification

Introduction

Human adenoviruses (HAdVs) are members of the *Adenoviridae* family, group of medium-sized viruses characterized by a nonenveloped icosahedral nucleocapsid and a double stranded DNA genome. The *Adenoviridae* family consists of five genera, of which the genus *Mastadenovirus* includes human viruses classified into 57 serotypes clustered into 7 subgroups (A-G). Human adenoviruses have different organ tropisms, causing a wide variety of clinical manifestations including respiratory tract infections, acute conjunctivitis, cystitis, gastroenteritis and systemic infections in immunocompromised patients. Subgroup A, F and G species primarily infect the gastroenteric tract, but only types 40 and 41 (subgroup F) have been strongly associated with gastroenteritis. Enteric adenoviruses multiply in the gastrointestinal tract, and are excreted in large numbers in the faeces of infected persons (even more than 10^8 copies/g of stool). The main source of adenoviruses in the environment is human faecal matter. Viruses cannot replicate outside their host's tissues and therefore cannot multiply in the environment; however, they can survive in the environment for extended periods of time and have good tolerance for changing environmental conditions. HAdVs are extremely common in wastewater throughout the year, with

concentrations between 10^3 and 10^7 genome copies/L reported in municipal wastewater from different geographical areas (Katayama *et al.*, 2008; Fong *et al.*, 2010; Kishida *et al.*, 2012; Rodriguez *et al.*, 2013;). In seawater, the enteric adenoviruses have been shown to be substantially more stable than either polio 1 or hepatitis A virus (Enriquez and Gerba, 1995). Many studies have suggested that HAdV is a good candidate as a fecal pollution indicator because of its known stability and persistence in aquatic environments compared to other enteric viruses (Pina *et al.*, 1998; Hundesa *et al.*, 2006; Albinana-Gimenez *et al.*, 2009; Fong *et al.*, 2010; Hewitt *et al.*, 2013).

During the last years, more attention has been focused on the sewage virological quality, the risk of virus-associated waterborne illness, the need for routine monitoring viral contamination and the environmental surveillance through the analysis of sewage. The presence of enteric viruses in sewage and hence in environmental surface waters reflects the infectious status of the population and constitutes a public health risk. The aim of this study was species-specific identification of human adenoviruses in sewage from Poland. Sewage samples from 14 sewage disposal systems in Poland (located in 13 towns) were examined for the presence of adenoviruses by molecular methods. Predominant adenovirus species in sewage were determined.

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Experimental

Materials and Methods

Sewage samples. Samples of raw sewage were collected in 14 sewage disposal systems from January to December 2011, one sample a month. A total of 163 sewage samples were processed according to the protocol described earlier (Zurbriggen *et al.*, 2008). To describe the process briefly, AlCl_3 (final concentration, 0.5 mM) was added to 500 ml of sewage sample, and the pH was adjusted to 3.5. Following the addition of 250 μl of a SiO_2 slurry, the samples were stirred for 30 min., followed by centrifugation at room temperature and $1500 \times g$ for 5 min. to pellet the SiO_2 . The virus was recovered by rocking the pellet for 20 min. with 3 ml of 50 mM glycine (pH 9.5) containing 3% (wt/vol) beef extract. After centrifugation for 5 min. at 4°C and $1500 \times g$, the concentrates were used to DNA extraction.

DNA extraction and PCR. Viral DNA was extracted from 100 μl of concentrated sewage using spin columns (QIAamp DNA stool kit – Qiagen) following the manufacturer's instructions. PCR was carried out using human adenovirus group-specific primers complementary to regions of the hexon gene (ADV-F, ADV-R) and HAdV species-specific primers (Table I). PCR amplification was performed in 25 μl volumes containing 23 μl of reaction mixture (Platinum PCR SuperMix with additions of 200 nM of each primer) and 2 μl of DNA extract. Amplification with group-specific starters started with an initial denaturation at 94°C for 5 min., followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. Reaction mixtures were then held at 4°C . Amplifica-

tion with species-specific starters started with an initial denaturation at 94°C for 5 min., followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 56°C for 60 s, extension at 72°C for 120 s., and final extension at 72°C for 5 min. Reaction mixtures were then held at 4°C . Amplification products were analysed in 1% agarose gels, GelRed-stained and viewed with the Molecular Imager Gel Doc system (BioRad Laboratories Inc.).

Statistical analysis. To identify statistical significance, a Student's *t* test analysis was performed online (<http://www.physics.csbsju.edu/stats/>). Results with *p* values of less than 0.05 were considered significant.

Results

Sewage samples were collected each month from each sampling site. In total, 163 samples of raw sewage were investigated by molecular methods. Out of the 163 samples analysed, 150 were positive for human adenoviruses using HAdV group-specific primers (92.1%). The percentage of PCR-positive samples in different sampling months was 76.9% (October) to 100% (February, March, June, July, August, November) (Fig. 1). The percentage of PCR-positive samples at the different sampling sites range between 75% and 100% and was significantly higher at cities of over 100 000 inhabitants ($p = 0.027$) (Fig. 2).

A total of 163 sewage samples were examined for the presence of A, B, C, D, E and F species of human adenoviruses by molecular methods using species-specific primers. Adenoviruses from species A were found in 32 specimens (19.6%). The percentage of positive samples in different sampling months was 7.1% (June, July, September) to 57.1% (March) (Fig. 1), with the highest

Table I
Oligonucleotide primers used in this study.

Target	Name	Sequence (5'→3')	Amplicon size (bp)	References
Human adenovirus	ADV-F	GCCACGGTGGGTTTCTAAACTT	131	Gunson <i>et al.</i> , 2009
	ADV-R	GCCCCAGTGGTCTTACATGCACATC		
HAdV species A	AdA1	GCTGAAGAAMCWGAAGAAAATGA	1444–1537	Xu <i>et al.</i> , 2000
	AdA2	CRTTTGGTCTAGGGTAAGCAC		
HAdV species B	AdB1	TSTACCCYTATGAAGATGAAAGC	670–772	Xu <i>et al.</i> , 2000
	AdB2	GGATAAGCTGTAGTRCTKGGCAT		
HAdV species C	AdC1	TATTCAGCATCACCTCCTTCC	1988–2000	Xu <i>et al.</i> , 2000
	AdC2	AAGCTATGTGGTGGTGGGGC		
HAdV species D	AdD1	GATGTCAAATTCCTGGTCCAC	1205–1221	Xu <i>et al.</i> , 2000
	AdD2	TACCCGTGCTGGTGTAAAATC		
HAdV species E	AdE1	TCCCTACGATGCAGACAACG	967	Xu <i>et al.</i> , 2000
	AdE2	AGTGCCATCTATGCTATCTCC		
HAdV species F	AdF1	ACTTAATGCTGACACGGGCAC	541–586	Xu <i>et al.</i> , 2000
	AdF2	TAATGTTTGTGTTACTCCGCTC		

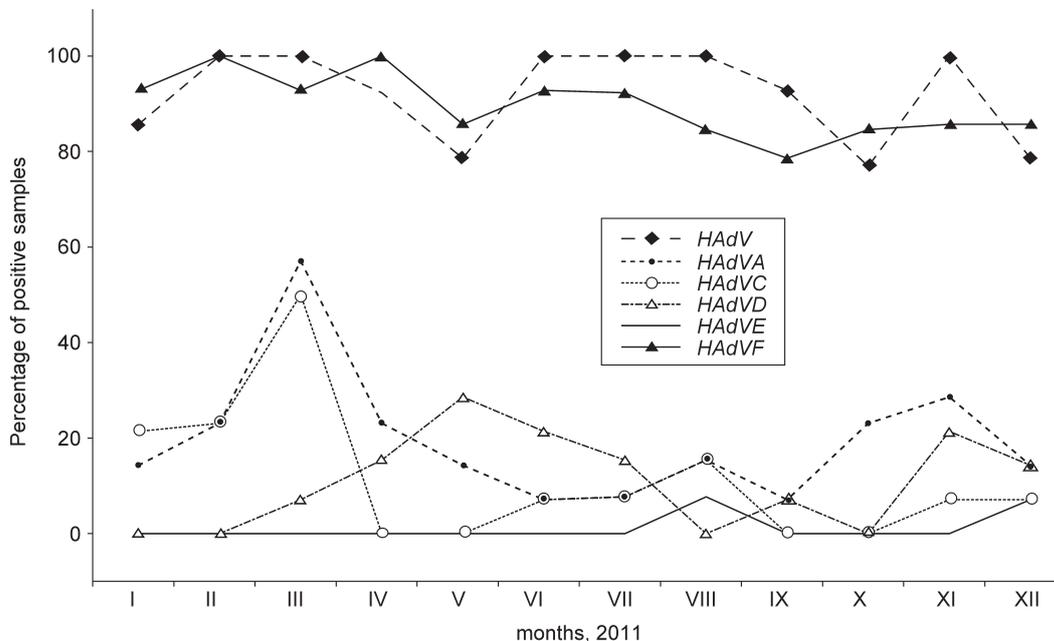


Fig. 1. Adenovirus positive sewage samples depending on season.

detection rate in the winter and early spring. The percentage of samples positive at the different sampling sites ranged between 0% and 58.3% and was higher at cities of over 100 000 inhabitants (not significantly different). Adenoviruses from B species were not detected in sewage. Adenoviruses from species C were found in 19 specimens (11.6%). The percentage of positive samples in different sampling months was 0% (April, May, September, October) to 50.0% (March) (Fig. 1). The percentage of samples positive at the different sampling sites ranged between 0% and 33.3% and was higher at cities of over 100 000 inhabitants, this difference is not significant. Adenoviruses from species D

were found in 18 specimens (11.0%). The percentage of positive samples in different sampling months was 0% (January, February, August, October) to 28.6% (May) (Fig. 1). The percentage of samples positive at the different sampling sites ranged between 0% and 37.5% and was higher at cities of over 100 000 inhabitants (not significantly different). Adenoviruses from species E were found only in two specimens (1.2%) collected in August and December from two different cities. Adenoviruses from species F were the most prominent species detected in sewage, they were found in 146 specimens (89.6%). The percentage of positive samples in different sampling months was 78.6% (September) to 100% (February, April) (Fig. 1). The percentage of samples positive at the different sampling sites ranged between 50% and 100% and was significantly higher at cities of over 100 000 inhabitants ($p = 0.017$) (Fig. 2).

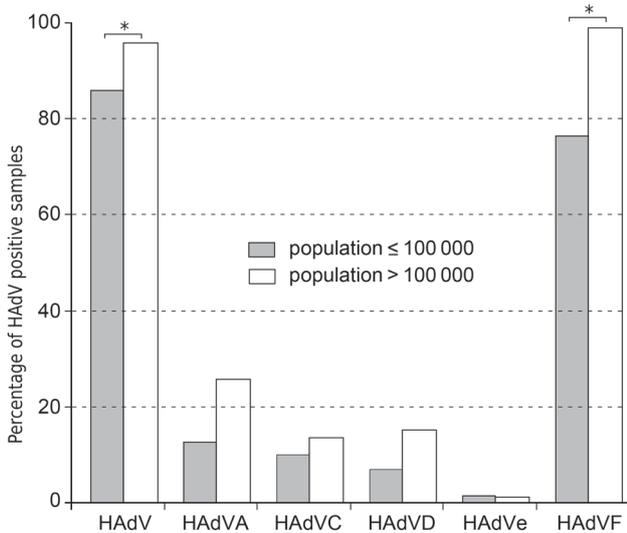


Fig. 2. Percentage of adenovirus positive sewage samples at cities with a population over and below 100 000 inhabitants. The asterisk indicates a statistically significant difference ($p < 0.05$).

Discussion

Adenoviruses have been detected in sewage worldwide. Recent metagenomic studies have shown that multiple types of viruses can be found in raw sewage (Cantalupo *et al.*, 2011; Ng *et al.*, 2012). In this study, sewage samples were used to detect of diversity of HAdVs in wastewater. Samples of sewage were collected from several locations around Poland. Samples were evaluated by PCR assay for the presence of human adenoviruses. Our investigation confirmed that sewage is a rich source of adenoviruses. HAdVs were detected in 92.1% of the analysed sewage samples and was significantly higher at cities of over 100 000 inhabitants.

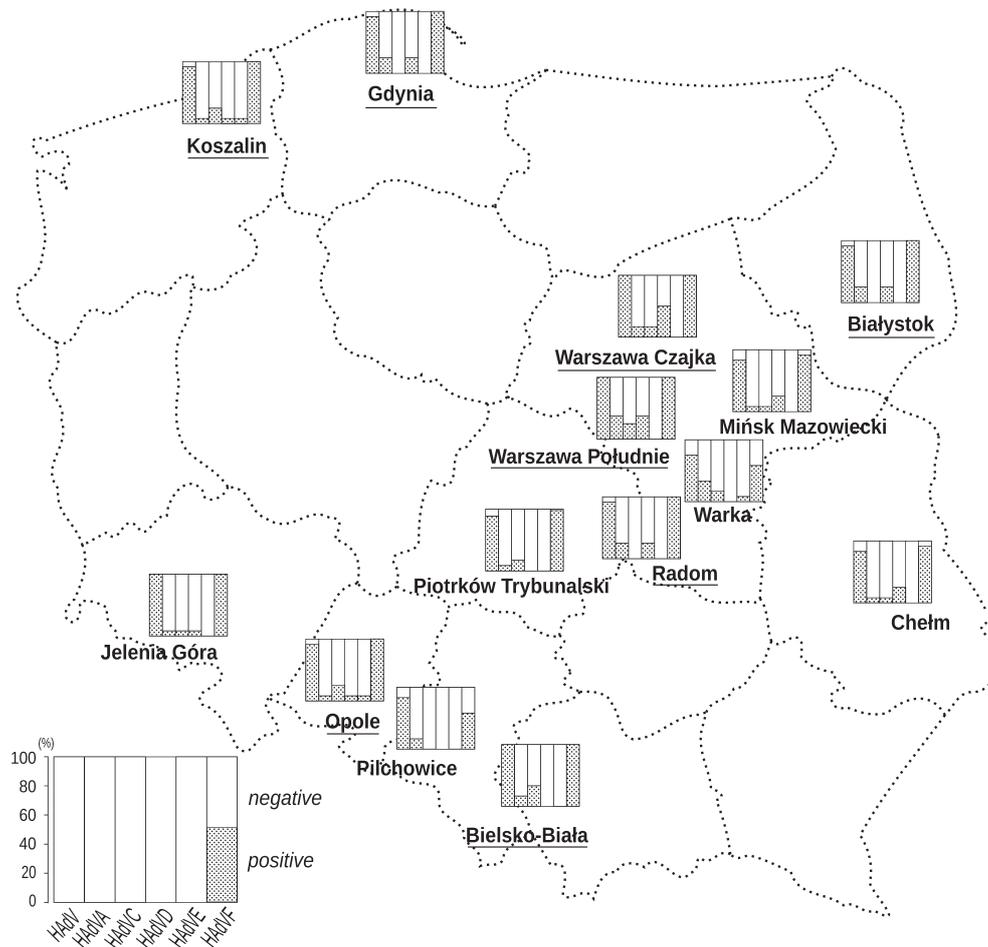


Fig. 3. Proportion of adenovirus positive and negative samples depending on sampling site (names of cities with population over 100 000 are underlined).

Our results are in agreement with Puig *et al.* (1994), reporting detection of HAdVs in 100% of sewage. In other studies, HAdVs were detected in 56 and 20% of sewage and treated effluent samples, respectively (Pusch *et al.*, 2005). Katayama *et al.* (2008) detected HAdVs in 100 and 99% sewage and treated effluent, respectively. Adenoviruses were detected in sewage during all seasons. Adenovirus infections have been observed to occur throughout the year with little or no seasonal variation in shedding. After infection, HAdV excretion by the host can last from months to years (Jiang, 2006) and the consequent lack of a seasonal pattern for this viruses in sewage has been confirmed by several studies (Jiang, 2006; Katayama *et al.*, 2008).

All samples were subjected to species-specific PCR for detection human adenovirus species A, B, C, D, E, and F. Species F adenoviruses mainly, but also A, C, D, G cause diseases of intestine. HAdV-A was identified as the etiologic agent of a diarrhea outbreak in a hematology hospital ward in London (Jalal *et al.*, 2005). Recently, HAdV-D and -C were detected in faeces of children with diarrhea in Bangladesh, Kenya and Brazil (Jarecki-Khan *et al.*, 1993; Filho *et al.*, 2007; Magwalivha

et al., 2010). In this study, species F was identified as the predominant in sewage (89.6%), followed by species A, C, D and E. We detected all species of human adenoviruses associated with intestine infections. Seasonal differences in detection were observed for species A, C (peak in March) and D (peak in May). Probability of detection of species F in sewage was determined by population size. Adenoviruses from species F were detected in 99% samples at cities over 100 000 inhabitants. This trends agrees with results from other environmental studies. Barrella *et al.* (2009) detected the presence of human adenovirus from species F in 82% of sewage samples. Fong *et al.* (2010) isolated adenoviruses from species F (63%), A (29%), B (3%) and C (3%) from raw sewage and primary effluents. Santos *et al.* (2004) isolated adenoviruses 40 and 41 (species F) from 62 of 69 sewage and surface water samples collected in San Paulo, Brazil, over a 3-year period. In South Africa, species F, D and C (serotype 2) were isolated from treated drinking water and river water, human adenovirus species D isolates were predominant in treated drinking water (van Heerden *et al.*, 2005). Adenovirus species F has been identified as one of the most prevalent

viruses globally in the etiology of childhood gastroenteritis. The HAdV serotypes 40 and 41 (species F) have long been recognized as the main etiological agents of 1–20% of acute viral gastroenteritis in children (Cruz *et al.*, 1990; Jothikumar *et al.*, 2005; Shimizu *et al.*, 2007). Shimizu *et al.* state that 50% of all adenoviruses found in stool specimens are types 40 and 41 (species F). HAdV-40 is known to be widespread in the European population, where it can cause outbreaks of gastroenteritis, mostly in children during winter. After infection, HAdV types 40 and 41 can cause mortalities as much as 50% in immunocompromised individuals (Echavarría, 2008). Considering that these viruses are shed for extended periods in faeces, urine, and respiratory secretions of infected persons (Jiang *et al.*, 2006), their high prevalence in this study suggests a high incidence of species F infections in the host population. Several authors have suggested that the enteric serotypes 40 and 41 (genus F) dominate overall HAdV serotypes in sewage. Although some recent studies have demonstrated (Sinbanda and Okoh, 2012; Bibby and Peccia, 2013) that HAdV-C were more frequent detected in sewage and environmental samples than HAdV-F. In the present work HAdV-C were detected in 11.6 % of the sewage samples, but study results differed depending on sampling site (from 0 to 33%) and sampling month (from 0 to 50%). Bibby and Peccia (2013) identified species C in 78% of sewage samples. These results do not seem to be divergent taking under consideration that Bibby and Peccia collected ten samples in winter months from five wastewater treatment plants, served population ranging from 100 000 to 1 000 000 people.

In the present study, human adenoviruses in sewage were detected by direct nucleic acid amplification from sewage concentrates by PCR. A number of studies have demonstrated that PCR is more rapid and sensitive than cell culture for adenovirus detection and species-specific identification by PCR is a popular method used to conduct adenovirus characterization (Xu *et al.*, 2000). Nevertheless serotyping of adenoviruses on the basis of sequencing of PCR products is potentially more informative. Methods used for the identification of human adenoviruses in raw sewage have many limitations, mainly due to the nature of the samples examined. Raw sewage usually contains organic compounds at high concentrations, which may inhibit PCR reaction.

In conclusion, this research demonstrates that human adenoviruses are consistently present in sewage in Poland. Human adenovirus species F was the most prominent adenovirus species detected in sewage, but molecular analyses indicated the presence of additional adenovirus species. HAdV species A, C, D and E were found in sewage samples, demonstrating the occurrence of multiple HAdV species in sewage. The presence of adenovirus species in sewage may repre-

sent a public health risk particularly taking in account immunocompromised individuals. Raw wastewater represent a significant source of pathogens that has a potential to contaminate aquatic environments and very important is adequate treatment before the disposal in the environment.

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