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Prevalence of Respiratory Adenovirus Species B and C in Sewage Sludge

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Abstract

Human adenovirus diversity in sewage sludge was assessed by Ion Torrent sequencing and annotation of partial adenovirus hexon genes. The most abundant species identified were HAdV-C (average 78%) and -B (average 20%), which are associated with respiratory infections. These findings reinforce the necessity to consider aerosol exposure to sewage-derived pathogens.

Human adenoviruses are among the most common human pathogens ¹ and cause variable infectious symptoms based on species, including respiratory, gastrointestinal, and ocular infections, summarized in Table 1 ^{2, 3}. Due to the emerging importance of adenoviruses in the environment, the U.S. Environmental Protection Agency included adenoviruses on the final Contaminant Candidate List (CCL3) ⁴. Sewage sludge represents the concentrated waste from thousands to millions of human inputs and human pathogens can be excreted through either feces or urine ⁵. Human adenoviruses are often considered the most abundant human viral pathogen in sewage sludge ^{6, 8}, and adenovirus has been proposed as an improved indicator of sewage sludge fecal pollution ⁶. The primary disposal method for sewage sludge is through land application as fertilizer, which may expose residents to the pathogens present in the sewage sludge through inhalation, ingestion, or direct contact ⁹. In a previous risk assessment of residential exposure to biosolids-derived aerosols, adenoviruses accounted for significantly higher predicted risk than enteroviruses ⁹, even though enteroviruses are the only viruses considered in U.S. land application regulations. Thus, understanding the infectious properties of adenoviruses present in sewage sludge is necessary to ensure the protection of public health. The goal of this study was to determine the distribution of adenovirus species in untreated and anaerobically digested sewage sludge (commonly termed biosolids), using an amplicon sequencing approach. A comprehensive review of sequencing approaches for viral pathogen identification is provided within reference ¹⁰. Prior adenovirus wastewater research has focused primarily on HAdV-F and gastroenteritis ^{6, 11, 12}, or adenoviruses as a whole, with no discrimination of individual species ^{6, 8}. Previous sequencing efforts of the adenovirus hexon gene in wastewater, while informative, have been limited by number of sequences and have also cultured viruses prior to sequencing, potentially hindering insights into overall species distribution ^{13, 14}. However, different species have highly divergent pathogenic characteristics, and knowledge

of species distributions would improve our understanding of the consequences of human exposure and infectious risk in the environment.

Untreated (IN) and anaerobically digested (OUT) sewage sludge was sampled from five wastewater treatment plants geographically distributed throughout the continental U.S. resulting in a total of ten samples. Sampling was conducted during winter months, and all plants received residential wastewater, served populations ranging from 100,000 to 1,000,000 people, and produced U.S. EPA designated class B biosolids. DNA was extracted directly from sewage sludge using a Mobio Powersoil DNA kit (Mobio, California, USA), as previously described⁸, and amplified with previously developed hexon gene targeted primers^{15, 16} that were amended with a 5' sample-specific barcode. Adenovirus hexon genes are known to contain variable regions that are useful for identifying species and types^{1, 17-19} and the primers used have previously been shown to be able to amplify and differentiate adenovirus species A-F¹⁵. A table detailing species-specific primer mismatches is included in the supplementary information (Table S1), an analysis of primers and primer target genes for adenovirus species A, B, C, D, E, and F, demonstrated no statistical difference ($p < 0.05$) in the number primer-target mismatches between any of the species. PCR amplicons were ~150bp in length. All 10 samples were PCR-positive for adenovirus. It has previously been estimated that approximately 0.1% of adenoviruses detected by molecular methods would be detected by cell culture methods²⁰; however, it is not known what percentage is infectious in an environmental context. PCR products were gel-verified, purified using a Mobio PCR clean-up kit (Mobio, California, USA), and sequenced on an Ion Torrent 314 chip at GeneWiz (New Jersey, USA). Raw sequences are deposited in MG RAST²¹ under accession number 4501936.3. Sequences underwent initial QC using DynamicTrim from the solexaQA toolkit²² for a minimum phred score of 15 and were split using the pyrosequencing pipeline on the RDP website²³ with a minimum read length of 100 bp. Trimmed reads underwent initial annotation through a BLASTn search of the NCBI viral genome database with a maximum E-value of 0.001. This E-value has been commonly used in metagenomic studies, and has previously been shown to reduce false negatives⁷. A table summarizing read count data is included in the supplementary information (Table S2). The database contains representatives from HAdV-A to -F. Addition of adenovirus 52, the only member of species G, to the database did not result in any species G identifications. Approximately 99.8% of annotated reads were annotated as adenovirus. Comparison with the NCBI "nt" database was not practical due to the high number of adenoviral vectors in the database that also match these sequences. Adenovirus species annotations are summarized in Figure 1.

The most abundant adenoviral species was C in nine out of ten cases, with an average relative abundance of $78\% \pm 17\%$ standard deviation, followed by adenovirus species B, with an average relative abundance of $20\% \pm 17\%$. Although most of the emphasis in prior environmental monitoring literature is placed on gastrointestinal adenovirus species F, the average relative abundance of adenovirus species F was only $0.7\% \pm 1.7\%$, with a maximum of 5.5%. However, it is important to note that due to varying infectious characteristics, higher abundance of certain species may not necessarily correlate with higher infectious risk from environmental exposure. There was no treatment effect on species distribution. For example, average abundance of species HAdV-C decreased from $84\% \pm 15\%$ in untreated

samples to $71\% \pm 18\%$ in anaerobically digested samples; however, these differences were not statistically significant.

It is interesting to observe that these findings in sewage sludge reflect previous findings of clinical adenoviral infection in the general public. Gray et al. reported the most abundant clinical adenovirus species in the US were HAdV-C (57.6%) and -B (34.6%) in the general population²³. Madelboim et al. reported similar findings from clinical presentations in Israel, for adenovirus species C (57%) and B (14%)²⁴. As sewage sludge is the concentrated waste from the general population and adenoviruses are excreted into this waste stream, the relative abundance of adenoviruses in sewage may mirror that in the general population. In addition to supporting our species distribution results in sewage sludge, these clinical observations highlight the importance of respiratory adenoviruses in human populations and suggest the potential for population disease monitoring through sewage.

As an alternative means of interpreting the data, and to obtain a phylogenetic, rather than taxonomic, assessment of adenovirus diversity, a subset of sequences were aligned using clustalX²⁶, and an unrooted tree was produced and formatted in MEGA²⁷ (Figure 2). None of the sequences included in this subset were annotated as HAdV-F. This independent analysis again highlights the abundance of HAdV-C, and also demonstrates that the read length produced provides the resolution necessary to show taxonomic and phylogenetic diversity of human adenoviruses.

Conclusions

Overall, these findings demonstrate the importance of considering respiratory HAdV-C and -B in environmental exposure assessments and demonstrates a case where sewage sludge pathogen diversity reflects the clinical presentation of disease. Adenovirus species F gastrointestinal pathogens^{11, 12} or broad spectrum adenovirus assays targeting all species^{6, 8} have traditionally been the focus of environmental monitoring. However, the prevalence of respiratory adenoviruses in wastewater reinforces the notion that consideration of alternative disease outcomes and exposure routes is necessary to ensure protection of human health and to produce adequate environmental regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Relative abundance of adenovirus species identified by partial hexon gene sequencing for untreated (IN) and anaerobically digested (OUT) sewage sludge from five major US wastewater treatment plants.

Figure 2.

Neighbor-joining tree of aligned adenovirus hexon gene sequences. High quality reads were selected from multiple samples using a minimum phred score of 20 and a minimum 100 bp BLAST alignment with reference database sequences to minimize the effects of insertions and deletions caused by the sequencing platform. Representatives of each adenovirus species were included in the alignment (HAdV-B AB243118.1, HAdV-C AB330083.1, HAdV-D AB053166.1). Full circles represent bootstrap values >50% (500 iterations). Scale bar is for 0.01 changes per base.

Table 1Infectious symptoms associated with the major adenovirus species ^{2, 3}

Adenovirus Species	Infectious Symptoms
HAdV-A	Gastrointestinal
HAdV-B	Respiratory, Urinary
HAdV-C	Respiratory
HAdV-D	Ocular, Gastrointestinal
HAdV-E	Respiratory
HAdV-F	Gastrointestinal
HAdV-G	Gastrointestinal