

Molecular detection of human adenovirus in urban wastewater in Egypt and among children suffering from acute gastroenteritis

Elmahdy M. Elmahdy, Nehal I. Ahmed, Mohamed N. F. Shaheen, EL-Chaimaa B. Mohamed and Samah A. Loutfy

ABSTRACT

Incidence of enteric viruses in sewage, the efficacy of wastewater treatment plants to remove these viruses, and health effects from their release into the surface water are very important environmental issues in the microbiology field. One of the most pathogenic enteric viruses is adenovirus which can cause a serious disease such as gastroenteritis with low grade fever and mild dehydration in humans. In this study we performed qualitative polymerase chain reaction (PCR) analysis of HAdV on 60 stool samples from children with acute gastroenteritis admitted to Abu-Rish hospital and 96 environmental samples (32 raw sewage, 32 treated sewage, 32 sewage sludge) collected from Zenin wastewater treatment plant (WWTP). HAdV were detected in 17 (28.3%) of stool, 27 (84.4%) of raw sewage, 16 (50%) of treated sewage and 25 (78%) of sludge samples. The viral concentrations were in the range of 2.02×10^6 – 7.23×10^6 , 8.7×10^5 – 4.3×10^6 , 1.22×10^4 – 3.7×10^6 and 1.48×10^6 – 1.77×10^7 GC/mL in stool, raw sewage, treated sewage, and sludge, respectively. HAdV was detected throughout the whole year of sample collection. Moreover, our results suggested that males were more susceptible to adenovirus infections than females. The results indicate that the high incidence of HAdV in the treated sewage may cause adverse health effects.

Key words | adenovirus, gastroenteritis, real time PCR, sewage, WWTP

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INTRODUCTION

Discharge of urban wastewaters into surface waters represented a source of environmental bacterial and viral contamination where people infected with enteric viruses can shed 10^{10} copies of pathogenic viruses per gram of stool, leading to high viral loads in sewage (Kuo *et al.* 2010). A public health hazard exists in wastewater recycling for irrigation, shellfish cultivation or industrial purposes due to the presence of many types of pathogenic viruses that can be transmitted by the fecal–oral route (Katayama *et al.* 2002). The morbidity and mortality risks due to diarrheal

diseases increase in developing countries such as Africa and southeast Asia according to the World Health Organization (WHO) (Babaniyi 1991). Human adenoviruses (HAdV) are a major cause of clinical infections including gastroenteritis, conjunctivitis, ocular, respiratory diseases, haemorrhagic cystitis and chronic systemic infections in immunosuppressed people (De Jong 2003; Van Heerden *et al.* 2003; Selvaraj *et al.* 2006). HAdV within the family Adenoviridae have linear double-stranded DNA (Stewart *et al.* 1993) and include five serotypes, which have been classified in seven species (A, B, C, D, E, F, G). It has been reported that HAdV, particularly types 40 and 41, are the second most important viral pathogens of infantile gastroenteritis after rotavirus (Jothikumar *et al.* 2005; Fong *et al.* 2010).

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doi: 10.2166/wh.2019.303

Many HAdV types can be shed in high concentrations (ranging from 10^5 to 10^{15} viral particles per gram of stool) in the stool of an infected person for months (Wold & Horwitz 2007). Fecal indicator bacteria are commonly used for water quality measurements, however they can fail to predict the presence of other pathogens such as human enteric viruses that are more environmentally tolerant than the fecal indicator bacteria (Hewitt *et al.* 2013). HAdV are more tolerant to chemical/physical parameters and UV irradiation than other water-borne pathogens such as enteric viruses and fecal indicator bacteria (Nwachuku *et al.* 2005). Many studies have been conducted to investigate HAdV incidence in clinical and environmental samples, but few have simultaneously studied the communal level incidence of HAdV in fecal, raw wastewater, treated wastewater and sludge samples, which represent different nodes in the fecal–oral route of transmission of enteric pathogens. However, it should be emphasized that this study is not focused on source tracking of HAdV in the fecal and environmental samples. In this study, HAdV was detected in clinical stool samples and wastewaters by conventional polymerase chain reaction (PCR) and then the viral concentrations were determined in some positive samples by real time RT-PCR.

MATERIALS AND METHODS

Wastewater and sludge samples

During the period January–December 2017, a total of 64 sewage samples (32 of raw sewage and 32 of treated sewage) and 32 sewage sludge samples from a secondary treatment step were collected from Zenin wastewater treatment plant (WWTP) to cover the seasonality of HAdV occurrence in wastewater and sludge. Eight samples from each study site were collected per season. The capacity of this wastewater treatment plant is $330,000 \text{ m}^3/\text{day}$ and the finally treated wastewater is discharged into the Nahia effluent system and then to the River Nile.

Clinical samples

In 2017, a total of 60 stool samples were collected from children suffering gastroenteritis who were admitted to Abulreesh

hospital (Sayeda Zeinab, Cairo Governorate). Samples were collected in clean containers then transferred to the laboratory within 3 hours of collection for viral examination.

Concentration of sewage samples

Sewage samples (raw and treated wastewater) were concentrated according to previously published protocol (USEPA 2001) yielding a mean recovery efficiency of 75%. Briefly, 2.5 mL of 1 M magnesium chloride (Merck-Schuchardt, Germany) was added to a 1 L sample to increase the stability of the viruses during the transportation process (APHA 2005). The samples were adjusted to pH 3.5 by 1 N HCL, and then concentrated by filtration using a nitrocellulose membrane filter (0.45 μm pore size, and 142 mm in diameter) on a stainless-steel filter holder. The adsorbed viruses on membrane filter were eluted in 100 mL of 3% beef extract-0.05 M glycine solution, pH 9.5. All samples were reconcentrated using an organic flocculation method (USEPA 1996). Finally, these samples were stored at -20°C until used.

Concentration of sludge samples

One hundred grams of sludge was dissolved in 300 mL of (10% beef extract, 1.34% $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 12% citric acid). The mixture was stirred for 30 min at pH 7 and then centrifuged at 4,500 rpm for 15 min. The supernatant was adjusted to pH 3.5, stirred for 30 min and centrifuged at 4,500 rpm for 15 min, finally the obtained pellet was dissolved in 0.15 M Na_2HPO_4 and stored at -20°C until analysis (USEPA 1984).

Concentration of clinical stool samples

Approximately 0.2 g of stool samples were weighed, diluted by 1 mL PBS (20% wt/v), and vortexed for 30 s. Samples were clarified by centrifugation at 7,000 rpm, 4°C for 10 min, and then the supernatant was taken and archived at -80°C until use.

Extraction of viral nucleic acid

Viral nucleic acid was extracted from 300 μL of concentrated samples using a DNA extraction kit (Patho

Gene-spin™, Korea) according to the manufacturer's instructions. Nucleic acids were eluted in 60 µL elution buffer and stored at -20 °C. Standard precautions were followed to avoid contamination.

Detection of human adenoviruses using the PCR technique

The PCR was performed as previously described by Puig *et al.* (1994) using primers specific for the hexon region of the HAdV genome. Briefly, 5 µL of extracted DNA was added to 25.5 µL of DEPC treated water, 5 µL (10×) PCR Buffer, 4 µL MgCl₂ (25 mM), 4 µL of dNTPs mix (BIO-LINE, 10 mM, Cat. No. BIO-39044), 0.5 µL (5 U/µL) Go Taq DNA polymerase (Promega, Cat. No. M7805), 3 µL (20 pmol/µL) of each of forward and reverse primers (5'-GCCCGCAGTGGTCTTACATGCACATC-3' sense primer, 3 µL (20 pmol/µL) of 5'-CAGCACGCCGCGGATGTCAAA GT-3') For PCR conditions, an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 94 °C for 90 s, 55 °C for 90 s and 72 °C for 2 min, and an additional extension at 72 °C for 10 min. Ten microliters of PCR product mixed with 2 µL of loading dye and subjected to electrophoresis on 1.5% agarose gel stained with ethidium bromide 10 mg/mL in TAE (Tris Acetate EDTA) buffer at 100 V for 30 min amplified products were visualized using transilluminator (UV light) comparing to a 100 bp DNA ladder (SibEnzyme, Cat. No. M27, Russia).

Real time PCR for quantification of human adenoviruses

Some positive samples as detected by conventional PCR were selected for further quantitative PCR analysis. Real time PCR assay was performed for HAdV by using SYBER GREEN (Applied Biosystems StepOne™ Real time PCR system Thermal Cycling Block, Singapore). Extracted viral DNA from stool, sewage, and sludge were subjected to amplification according to Grondahl *et al.* (1999) using forward 5'ATGACTTTTGGAGGTGGATCCCATG'3 HAdV F1 and the reverse 5'GCCGAGAAGGGCGTGCGCAGGTA'3 HAdV R1 primers. The reaction mixture contained 12.5 µL of Maxima SYBR Green/Rox q PCR master mix (2×) (Fermentas, California, USA), 3 µL (30 pmol/µL) of each forward and reverse primers, and 6.5 µL (100 ng/µL) of

extracted DNA in a final volume of 25 µL reaction mixture. The HAdV thermal cycling protocol consisted of four steps: step 1: 95 °C for 10 min for DNA denaturation; step 2: three temperature cycles, repeated 40 times, at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; step 3: final extension at 72 °C for 7 min; step 4: melting curve 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s and finally cooling at 4 °C forever. The genome copies for adenovirus was determined by comparison with a standard curve constructed using ten-fold serial dilution (10⁻¹ to 10⁻⁶ copies/mL) by cloning the PCR amplicon into a plasmid (pBR22 Invitrogen USA).

Physicochemical characteristics of raw treated wastewater

The physico-chemical parameters such as pH, total chemical oxygen demand (COD_{tot}), soluble chemical oxygen demand (COD_{sol}), biochemical oxygen demand (BOD_{tot}), total suspended solids (TSS), total Kjeldahl nitrogen (TKN), ammonia, nitrite-nitrogen (NO₂-N), nitrate-nitrogen (NO₃-N), and total phosphorus (TP) of the sewage samples were analyzed. All these parameters were characterized using standard methods (APHA 1989).

Statistical analyses

To evaluate the possible correlation between viral detection in samples from each point of collection, a Pearson correlation and linear regression test were performed using GraphPad Prism 5.0 (USA); data were considered statistically significant at $P \leq 0.05$.

RESULTS

Physicochemical characteristics of raw treated wastewater

The physico-chemical analysis of sewage samples was measured for sewage samples. The average values of all parameters were calculated for raw and treated sewage (Table 1). Of the physicochemical parameters, only COD_{tot}, COD_{sol}, BOD_{tot}, and TSS showed a statistically significant reduction after treatment; in fact, the average values of

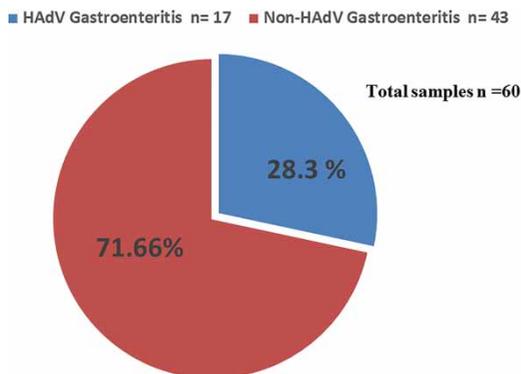
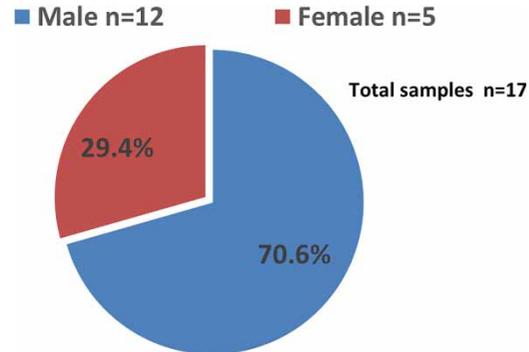
Table 1 | Average values with standard deviations of physico-chemical of sewage samples

Parameter	Units	Raw sewage	Treated sewage
pH	–	7.5 ± 0.3	7.7 ± 0.3
COD _{tot}	mg/L	354 ± 78	148 ± 47.8
COD _{sol}	mg/L	168 ± 51	72.9 ± 23.8
BOD _{tot}	mg/L	207 ± 53	80.3 ± 28.3
TSS	mg/L	185 ± 44	71.5 ± 26.1
TKN	mg/L	48 ± 9.5	40.5 ± 10
Ammonia	mg/L	24 ± 4.2	19.8 ± 4
NO ₂ -N	mg/L	0.09 ± 0.2	0.15 ± 0.3
NO ₃ -N	mg/L	0.22 ± 0.3	0.5 ± 0.5
Phosphorus	mg/L	1.9 ± 0.7	1.1 ± 0.5

COD_{tot}, COD_{sol}, BOD_{tot}, and TSS decreased to 58.2, 56.6, 61.2 and 61.3% respectively. Of the TKN and ammonia, 15.6 and 17.5% were eliminated in the final effluent. Meanwhile, the phosphorus content was not significantly reduced in the final effluent, and nitrite-nitrogen (NO₂-N), nitrate-nitrogen (NO₃-N) underwent a noteworthy increase, from 0.09 mg/L in raw sewage to 0.15 mg/L in treated sewage for nitrite and from 0.22 mg/L in raw sewage to 0.5 mg/L treated sewage for nitrate.

Presence of HAdV in stool samples

Adenovirus was detected in 28.3% (17/60) of clinical specimens, as shown in Figure 1, with higher incidences in males 12 (70.6%) than females 5 (29.4%), as shown in Figure 2. The viral concentrations in positive clinical samples ranged from 2.02×10^6 to 7.23×10^6 GC/mL.

**Figure 1** | Distribution of RV gastroenteritis among children with acute gastroenteritis.**Figure 2** | Distribution of RV diarrhoea in children according to their gender.

Presence of HAdV in environmental samples

Sewage samples were taken bi-monthly from January to December 2017, in the Giza Governate, Egypt. Thirty-two samples of influent (raw sewage, prior to treatment), 32 effluent (post-treatment sewage) and 32 sewage sludge samples were analyzed for the presence of HAdV by conventional PCR and some positive samples were tested by real time PCR. HAdV was detected in 27 out of 32 (84.4%) influent samples, 16 out of 32 (50%) effluent samples, and 25 out of 32 (78%) of activated sludge. The viral concentrations in raw sewage, treated sewage, and sewage sludge were in the range of 4.3×10^5 – 8.7×10^6 , 1.22×10^4 – 3.7×10^6 and 1.48×10^6 – 1.77×10^7 GC/mL, respectively. The WWTP was efficient to remove 59% (16/27) of HAdV.

Seasonal variation of HAdV in sewage

We analyzed the occurrence rates of HAdV in sewage samples throughout the year of sample collection. A total of 16 sewage samples (eight raw sewage and eight treated effluents) were collected each season. HAdV was detected throughout the whole year with a peak in winter 100% (16/16), followed by autumn 68.7% (11/16), spring 62.5% (10/16), and summer 37.5% (6/16), as shown in Figure 3.

DISCUSSION

Diarrheal illnesses remain a major public health concern and represent the third leading cause of death, globally. In

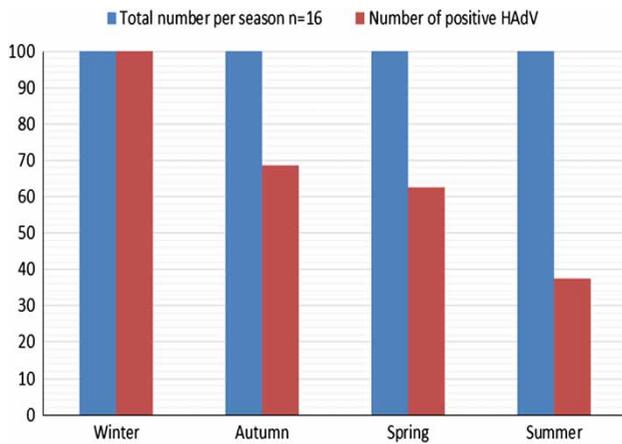


Figure 3 | Seasonal variation HAdV in wastewater; 16 samples (eight raw and eight treated sewage) were collected per season. Total number per season was set as 100%.

recent years, viral diarrhea has gradually increased in infants and children in both developing and developed countries (Basu *et al.* 2003; Ifeanyi *et al.* 2009; Khoury *et al.* 2011).

Several serotypes of human adenovirus, but mainly enteric types 40 and 41, have been strongly associated with gastroenteritis in childhood (Grimwood *et al.* 1995). Enteric HAdV can be transmitted via the fecal–oral route, with contaminated water and food as possible vectors (Mickan & Kok 1994). HAdV has been suggested as a suitable indicator for the emerging viral contaminants of human origin, as they have been reported in various types of water worldwide throughout the year, being more tolerant to sewage treatment processes (Bofill-Mas *et al.* 2006; Okoh *et al.* 2010; Schlindwein *et al.* 2010).

In the present study, we studied the incidence of HAdV in both clinical and sewage samples in a community. The percentage of HAdV in the clinical stool samples was 28.33% (17/60). This finding is higher than those in studies performed in Egypt where HAdV was detected in 6.8% of children with acute gastroenteritis (Zaghloul *et al.* 2013) and was also higher than those reported by Zaki & El Kheir (2017), where HAdV was detected in 20% of children with acute gastroenteritis. In comparison with other countries such as Australia, Brazil, Indonesia, Saudi Arabia, Korea, Iran, UK, Turkey, Hungary and Sweden, the detection rates of enteric adenovirus ranged from 1 to 96.3% of diarrheal patients (Uhnnoo *et al.* 1984; Wood *et al.*

1989; Grimwood *et al.* 1995; Akhter *et al.* 1999; Saderi *et al.* 2002; Subekti *et al.* 2002; Koh *et al.* 2008; Bányai *et al.* 2009; Tekin 2010; Costa *et al.* 2017). Differences in frequency rates in the current research as compared to other studies may be attributed to several reasons such as the diagnostic technique used for detection, level of economic status, the difference of age of the studied children, and/or geographical region of the study area. Moreover, our results showed that males were more frequently infected with HAdV than females, as seen by other researchers (Aminu *et al.* 2007; Motamedifar *et al.* 2013; Jaff *et al.* 2015), suggesting that males may be more susceptible to HAdV infection than females and further studies are required to understand this issue.

The role of WWTPs as possible sources of pathogenic microbes in receiving watersheds is highly critical, especially in different parts of the world facing water scarcity due to the increasing need for water reuse (Okoh *et al.* 2010). The activated sludge system is the best process applied in biological wastewater treatment, removing both suspended solids and soluble biodegradable compounds. For this reason, the efficiency of WWTP is generally based on the measurements of chemical parameters such as COD, BOD, and nutrient removal and its sustainability can be assessed by considering many potential effects such as global warming or eutrophication (Rodriguez-Garcia *et al.* 2011). Several reports have identified various environmental factors such as retention time, pH, oxygen concentration, rainfall amount, and temperature, affecting plant performance (Doorn *et al.* 2006). Our results suggested a negative correlation between occurrence of HAdV in sewage and physico-chemical parameters.

WWTPs at optimum performance can reduce viral and bacterial pathogens by ~2 log units (Carducci *et al.* 2009; Petrinca *et al.* 2009; Wen *et al.* 2009). In fact, the disinfection step generally depends upon various factors, including the type of disinfectant used, its contact time with treated wastewater, temperature, pH, and pathogen type. In recent years, an increasing number of studies have been carried out to investigate enteric viruses as causative agents of water-borne and food-borne outbreaks (Brunkard *et al.* 2011; EFSA & ECDC 2011) to assess the viral removal by WWTPs (Thompson *et al.* 2003; Carducci *et al.* 2009; Nordgren *et al.* 2009; Kuo *et al.* 2010). However, few studies

have been conducted to investigate the incidence of HAdV in clinical and community water samples simultaneously.

In the present study, HAdV was detected in 67% (43/64) of total collected wastewater samples, mostly in the winter and autumn months. These findings may be attributed to favorable environmental conditions, such as lower temperature compared to other seasons. Lipp *et al.* (2001) reported that low temperature enhances the survival of enteric viruses for longer periods in the water environment. A high prevalence of HAdV in the winter and autumn months has also been observed in other epidemiological studies (Gagnon *et al.* 2010; Rigotto *et al.* 2010). In contrast, HAdV were detected in higher rates during the summer season than the winter season (Haramoto *et al.* 2007; Elmahdy *et al.* 2016a, 2016b); this may be due to change of atmosphere and difference in geographical area. Moreover, our results suggested that the WWTP were effective at removing HAdV as the percent positive sample decreased from 84.4% (27/32) to 50% (16/32) for raw and treated wastewater, respectively. Therefore, WWTP accounts for only a 34.4% decrease in the incidence of viral genomes and this may be considered high risk as the wastewater treatment process was not able to significantly decrease the HAdV load.

Our results also reveal that the activated sludge samples collected from the WWTP are polluted with higher viral genome concentrations than those detected in sewage samples. Based on previous studies, samples of sewage sludge can contain a viral load of at least 1–2 log₁₀ units higher than the raw sewage (Fumian *et al.* 2011; Prado *et al.* 2012), which may be due to the high sorption capacity of HAdV to solids (Wong *et al.* 2013; Elmahdy *et al.* 2016b).

Real time PCR was used for detection of adenovirus in several studies and HAdV was detected in 30.6% (22/72) of Tyume River in South Africa with a viral load ranging between 6.54×10^3 and 8.49×10^4 GC/L (Sibanda & Okoh 2012). This was lower than our result because of the highest viral load are known to be present in wastewater and stool. In another study, HAdV was detected in final effluent of wastewater samples at concentrations ranged from 1.10×10^4 to 2.37×10^5 GC/L (Osuolale & Okoh 2015) but our results showed that the viral load of HAdV in the treated effluent ranged from 1.22×10^4 to 3.7×10^6 GC/ml, such a

difference could be attributed to the efficiency of the wastewater treatment plant.

In conclusion, the presence of HAdV in final treated effluent is a huge public health concern. The levels of viral contamination in treated effluent and sewage sludge of the WWTP must be considered by the regulatory and local authorities for improving the efficiency of conventional WWTP for the removal of pathogens.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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