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RESEARCH ARTICLE

Molecular detection and genotypic characterization of enteric adenoviruses in a hospital wastewater

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Abstract

Hospital wastewater (HWW) represents a major source of the diffusion of many antibiotics and some toxic pathogenic microorganisms in the aquatic environment. Sanitation services play a critical role in controlling transmission of numerous waterborne pathogens, especially enteric human adenoviruses (HAdVs) that can cause acute gastroenteritis. This study intended to evaluate the human adenoviruses (HAdVs) detection rates, to determine the genotype of these viruses and to assess the efficiency of HAdVs removal in hospital pilot wastewater treatment plant (PWWTP) in Tunis City, Tunisia. Therefore, hospital wastewater samples (n = 102) were collected during the study year from the two biological wastewater treatment techniques: natural oxidizing ponds and the rotating biological disks or biodisks. Nested polymerase chain reaction (Nested PCR) was used to evaluate the HAdVs detection rates. The genotype of HAdVs positive samples was achieved by the sequencing of the PCR products. HAdVs were detected in 64% (65/102) of positive wastewater samples. A substantial increase in the frequencies of HAdVs was observed at the exit of the two wastewater treatment techniques studied. The typing of HAdVs species F showed the occurrence of only HAdVs type 41. This data acquired for the first time in Tunisia showed high persistence and survival of HAdVs in the two biological wastewater treatment techniques experienced, and mainly highlighted the poor virological quality of the treated wastewater intended for recycling, agriculture reuse, and discharges into the natural receiving environments. Consequently, tertiary wastewater treatment appeared necessary in this case to decrease the load of enteric viruses flowing in the water environment.

Keywords Enteric human adenoviruses · Hospital wastewater · Wastewater treatment plant · Nested-PCR · Waterborne diseases · Environmental virology

Introduction

Hospital wastewater (HWW) contains an important quantity of many kinds of pollutants such as harmful chemical compounds, radioactive elements, pharmaceutical waste, and biological products (infectious viruses and bacteria). These pollutants are generated by the laboratory and research activities (Uhrbrand et al. 2017; Souza and Féris 2016; Le et al.

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2016). HWW play a main role in both the appearance of these types of pollutants and their spread in the receiving waters, and it constitutes a potential risk for public health and aquatic ecosystems (Uhrbrand et al. 2017). The discharge of HWW in urban sewage systems without treatment represents one of the main environmental problems. In Tunisia, this wastewater was rejected in the principal sewage network, which was further mixed and treated with domestic sewage in the wastewater treatment plant (WWTP). The absence of specific HWW treatment techniques on the premises resulted in the increase in the number and the rate of gastroenteric viruses in this particular water environmental (Prado et al. 2011; Uhrbrand et al. 2017). Virological analysis of HWW may produce important data on the circulation of viral strains in the human population. This information would interestingly support epidemiological studies, giving an idea about the possible clinical and subclinical infections in the population. The discharge of unsuitable and contaminated treated HWW in natural receiving environment, its reuse in agriculture, and its recycling for shellfish farming could lead to the diffusion and propagation of enteropathogenic viruses such as enteric human adenovirus (HAdVs) (Prado et al. 2011). In addition, HAdVs 40/41 have been detected in different aquatic environments such as sewage, surface, recreational and drinking water, rivers and sea water (Grøndahl-Rosado et al. 2014; Kuo et al. 2015; Lin and Singh 2015; Vergara et al. 2016; Kaas et al. 2016; Adefisoye et al. 2016; La Rosa et al. 2017). These viruses are of real concern to public health. They were placed by the U.S. Environmental Protection Agency in its contaminant candidate list for drinking water (Jothikumar et al. 2005). Numerous studies have suggested that HAdVs could be a better candidate as a fecal pollution indicator because of its known stability and persistence in aquatic environments than other enteric viruses (Fong et al. 2010; Hewitt et al. 2013; Rames et al. 2016).

Human adenoviruses (HAdVs) are non-enveloped, double-stranded DNA viruses, in the *Mastadenovirus* genus and *Adenoviridae* family. Seven known HAdVs species (A–G) have been reported. Historically, HAdVs were classified by hemagglutination and serum neutralization reactions into more than 64 serotypes (Seto et al. 2011). HAdVs are present at a greater frequency in wastewater than are other enteric viruses. A high number of these viruses are excreted with important rates from infected people (up to 10^{11} viral particles per gram of feces) (Fong et al. 2010). HAdVs species F types 40 and 41 are enteric pathogens that are considered to be related to 5–20% of acute gastroenteritis cases in the pediatric population (Sdiri-Loulizi et al. 2009; Khoshdel et al. 2015; Zhang et al. 2016). HAdVs types 41 were the predominant type of enteric adenoviruses in pediatric gastroenteritis worldwide (Sdiri-Loulizi et al. 2009; Khoshdel et al. 2015; Zhang et al. 2016). Recently, more focus has been made on the wastewater virological quality, the risk of virus-related waterborne diseases, the requirement for the routine surveillance of viral pollution and the environmental intensive care through the sewage analysis. The occurrence of enteric viruses in sewage and hereafter in environmental surface waters reveals the infectious status of the population and establishes a public health hazard. In Tunisia, the identification of HAdVs is well documented in clinical samples (Fodha et al. 2007; Sdiri-Loulizi et al. 2009). However, the first Tunisian environmental study showed the presence of low detection rates (0.4%) in sewage samples (Sdiri-Loulizi et al. 2010). The objectives of this work were to (i) evaluate the HAdVs detection rate, (ii) determine the genotype of circulating enteric HAdVs, and (iii) assess the efficiency of HAdVs removal in urban and hospital wastewater treatment plant in Tunis City, Tunisia.

Materials and methods

The pilot plant and sample collection

During the year 2011, 102 wastewater samples ($n = 102$) were collected from the two biological treatment processes situated in a semi-industrial pilot wastewater treatment plant of El Menzeh 1 (PWWTP). This pilot plant is located in the Mutuel-Ville, eastern suburbs of Tunis City, Tunisia. This plant previously described by Ibrahim et al. (2016) simultaneously receives essentially a municipal and a hospital wastewater coming from three neighboring clinics. Only two biological techniques were studied in this work: (i) the natural oxidizing ponds or natural lagoons and (ii) the rotating biological disks or rotating biodisks. The main physical and geometric characteristics of these two treatment techniques and the timing of wastewater sampling were reported in the latest study of Ibrahim et al. (2016). At six different points of the two treatment processes, wastewater samples were collected at the output of each basin (B_1, B_2, B_3, B_4, and B_5) of natural oxidizing pond process and at the output of the rotating biodisks process (Fig. 1). At each sampling point, 1 l of wastewater was sampled in sterile glass bottle, stored at 4 °C and transported to the laboratory for immediate physicochemical and microbiological analysis. Fecal coliform and streptococci concentrations were assessed by the most probable number methods (MPN) as recommended by Rodier (1978), and the main physicochemical parameters of wastewater samples were performed following the standard methods for the examination of water and wastewater, the AFNOR guide (AFNOR 1992).

Methods of viruses extraction

The adapted method of beef extract and $AlCl_3$ described by the USA Environmental Protection Agency protocol was used for the extraction of viruses from wastewater (EPA 1992; Ibrahim et al. 2015, 2016). Concentration of viruses was

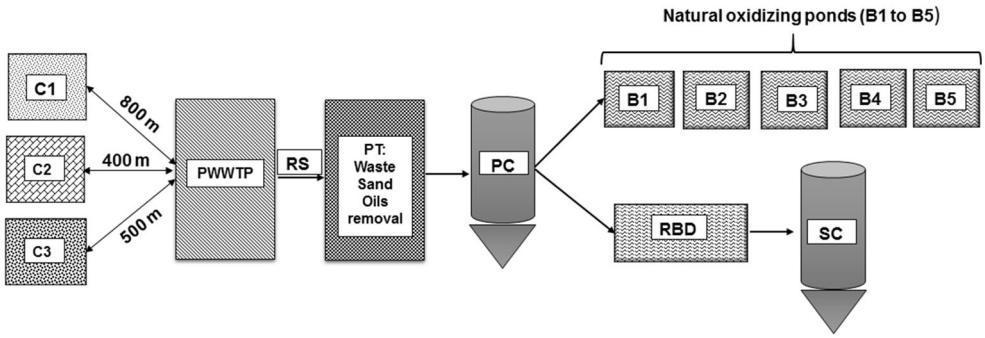

Fig. 1 Schematic plan of the two wastewater treatment techniques considered: the natural oxidizing ponds and the rotating biodisks

achieved by the polyethylene glycol 6000 (PEG 6000) (Lewis and Melcaft 1988). The decontamination of the viral particles extract was done by the filtration using a mixed cellulose ester membrane filter of pore size of 0.22 μm.

Viral DNA extraction and nested PCR reaction

Viral DNA was extracted via 800 μL of wastewater extract using an automatic extractor NucliSENS® EasyMag™ platform (bioMérieux, Marcy L'Etoile, France) to obtain a final volume of 110 μL, according to the manufacturer's instructions, then stored at − 40 °C until further analysis. A nested PCR was performed with two primer pairs (Adv-Hex1DEG/Adv-Hex2DEG; Adv-Hex3DEG/Adv-Hex4DEG) to amplify the gene segment coding for the adenoviruses 2 hexon polypeptides mentioned by Akusjärvi et al. (1984) and according to the protocol of Allard et al. (2001).

A primary PCR was performed using 50 μM of each primer (Adv-Hex1DEG and Adv-Hex2DEG) in a total reaction volume of 50 μL consisting of 2 μL of DNA samples, 10× PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP, Taq polymerase, dimethylsulfoxyde (DMSO), and water (H_2O). The amplification was carried out in a thermocycler under the successive cycling conditions: 94 °C for 3 min, followed by 35 cycles (94 °C in 30 s for denaturation, 55 °C in 30 s for annealing, and at 72 °C in 1 min for extension), and an ultimate extension at 72 °C for 5 min. We added 5 μL of the amplicons as a template for the nested PCR by using the same mixture as primary PCR and 50 μM of each inner primer (Adv-Hex3DEG and Adv-Hex4DEG) and amplified for 35 respective cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, with an ultimate extension cycle at 72 °C for 5 min. The amplicons were visualized by electrophoresis in 2% agarose gel added with ethidium bromide in TAE buffers.

DNA sequencing and phylogenetic analysis

For molecular characterization, the obtained amplicons of all positive wastewater samples were purified using Amicon Column (Ultra 30 K. Millipore, reference UFC503096), following the manufacturer's recommendations. DNA sequencing was performed using the ABI 3100 automated sequencer (PE Biosystems) and the ABI Prism® BigDye® terminator cycle sequencing ready reaction Kit (Applied Biosystems). The Codon Code Aligner software was used for the chromatogram analyses. For phylogenetic analysis, the HAdVs sequences were compared to previous enteric adenoviruses (Types 40 and 41) strains available in Gen Bank using the Basic Local Alignment Search Tool (BLAST) program. Phylogenetic tree was built using the neighbor-joining method based on the Kimura two parameter model (Kimura 1980) by using the MEGA software (Kumar et al. 1994) and the PHYLIP package (Felsenstein 1993). Confidence values of the internal nodes were calculated by performing 1000 bootstrap replicates. The conception of the tree was made by the Tree View program (Page 1996). The HAdVs sequences found in the present study from wastewater samples have been submitted to the International Centre for Biotechnology Information and received the following accession numbers KT369590—KT369610.

Statistical analysis

Differences in HAdVs detection rates were tested by the least significant difference, according to the Waller-Duncan a, b test with the SPSS software program (SPSS for Windows, version 19; SPSS Inc., Chicago, IL, USA).

Results

Physicochemical and bacteriological analysis

The mean values of the main physicochemical and bacteriological characteristics carried out for all wastewater samples collected from the two biological wastewater treatment techniques such as natural oxidizing ponds and rotating biodisks are reported in Table 1. The main physicochemical and bacteriological parameters tested on the collected wastewater samples in this study were electrical conductivity (EC), suspended solids (SS), chemical oxygen demand (COD), biological oxygen demand (BOD_5), ammonium nitrogen ($N\text{-}NH_4$), ortho-phosphates ($P\text{-}PO_4$), fecal coliforms (FC), and finally fecal streptococci (FS).

Table 1 The results of physicochemical and bacteriological analysis in two biological wastewater treatment procedures: natural oxidizing ponds and rotating biodisks

Parameters/Procedures	pH	Temp (°C)	EC (S/cm)	SS (mg/l)	COD (mgO_2/l) (mgO_2/l)	BOD5 (mgO_2/l)	N-NH4 (mg/l)	P-PO4 (mg/l)	FS (NPP / 100 ml)	FC (NPP/ 100 ml)
Natural oxidizing ponds (B_1 to B_5)	8 ± 1	18 ± 16	1530 ± 360	60 ± 2	198 ± 86	134 ± 75	20 ± 6	23 ± 12	$10^5 \pm 10^2$	$3\ 10^4 \pm 10^2$
Rotating biodisks (D)	8 ± 1	18 ± 16	1.5 ± 0.3	30 ± 12	30 ± 12	35 ± 15	14 ± 11	12 ± 5	$4\ 10^5 \pm 2\ 10^1$	$5\ 10^4 \pm 10^1$

Temp temperature, *EC* electrical conductivity, *SS* suspended solids, *COD* chemical oxygen demand, *BOD_5* biological oxygen demand, *N-NH4* ammonium nitrogen, *P-PO4* ortho-phosphates, *FS* fecal streptococci, *FC* fecal coliforms

The results of the analysis of the physicochemical parameter analysis showed an important reduction in the five different basins from natural oxidizing ponds techniques, and at the exit of the rotating biodisks procedure. Similarly, the bacteriological result obtained revealed a substantial decrease of the mean values of fecal coliforms and fecal streptococci at the exit of the two wastewater treatment techniques considered in this work (Table 1).

Molecular HAdVs detection

The wastewater sample analysis revealed 65 positive samples for HAdVs from the 102 samples tested (64%), of which 54/102 (53%) were from the five different natural basins (B_1 to B_5) and 11/102 (11%) were from the rotating biodisks. The distribution of positive samples of HAdVs in each basin (B_1 to B_5) of the natural oxidizing ponds procedure showed the following HAdVs detection rates: 15% ($n = 4/26$), 29% ($n = 6/21$), 80% ($n = 12/15$), 50% ($n = 6/12$), and 87% ($n = 14/16$) in B_1, B_2, B_3, B_4, and B_5, respectively (Fig. 2).

At the exit of the rotating biodisks process, the HAdVs detection rate was about of 75% ($n = 9/12$) (Fig. 1). Differences in HAdVs detection rates during the year 2011, as well as among the natural oxidizing ponds and the rotating biodisks techniques were statistically different ($P < 0.05$) (Fig. 2, Table 2).

Resistance of HAdVs to the biological treatment

The HAdVs detection results showed lower viral detection rates in the first two basins B_1 and B_2 as compared to the three other ponds (B_3, B_4, B_5), and at the exit of the rotating biodisks. In fact, HAdVs detection was present with low frequencies of 15 and 29% in the first two basins, B_1 and B_2, respectively. However, in the three other basins, B_3, B_4, B_5, and at the exit of the rotating biodisks procedure, HAdVs detection rate was almost 20 to 70 times higher than those recorded for the two basins, B_1 and B_2 (80% in B_3, 50% in B_4, 87% in B_5, and 75% at the exit of the rotating biodisks). The HAdVs detection rates recorded in the effluent of the two lines of treatment, the natural oxidizing ponds and the rotating biodisks process, showed a substantial increase in the HAdVs detection frequencies. This important increase was observed from the first (B_1) to the last basins (B_5) of the natural oxidizing lagoons and at the exit of the rotating biodisks that showed a great resistance or survival of HAdVs at the two biological techniques tested in this study ($P < 0.05$) (Table 2; Fig. 2).

Seasonal variation of HAdVs detection

The monthly distribution of HAdVs detection rates showed their presence during the entire sampling period of the year

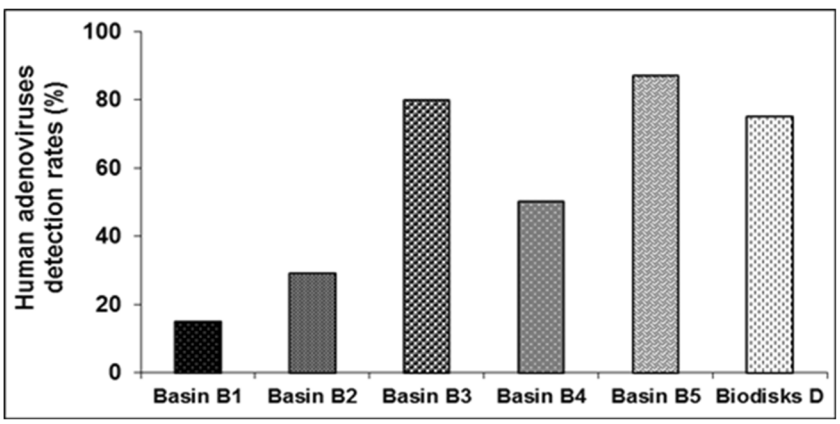

Fig. 2 Molecular human adenovirus detection rates at the exit of the two wastewater treatment techniques: the natural oxidizing ponds and the rotating biodisks

Table 2 Molecular human adenovirus detection rates at the output of the two studied wastewater treatment methods: natural oxidizing ponds and rotating biodisks

Human adenovirus detection rates													
Basins/months	December	January	February	March	April	May	June	July	August	September	October	November	Total
Basin B_1	0 a; α	1 a; α	1 a, b; α	2 a; α	1 a; α	1 a; α	1 a; α	2 a; α	0 a; α	0 a; α	0 a; α	1 a; α	10 a
Basin B_2	2 b,c; α	0 a; α	2 b; α	1 a; α	1 a; α	1 a; α	1 a; α	1 a; α	0 a; α	1 a; α	1 a; α	0 a; α	11 a
Basin B_3	2 b,c; α	0 a; α	0 a; α	1 a; α	2 a; α	2 a; α	1 a; α	1 a; α	0 a; α	1 a; α	1 a; α	1 b; α	12 a
Basin B_4	1 a,b; α, β	0 a; α	0 a; α	1 a; α, β	2 a; β	2 a; β	0 a; α	1 a; α,β	0 a; α	0 a; α	0 a; α	0 a; α	7 a
Basin B_5	3 c; γ, δ	0 a;α	2 b; β, γ	0 a; α	4 b; δ	3 a; γ, δ	0 a; α	1 a; α,β	0 a; α	1 a; α,β	0 a; α	0 a; α	14 a
Biodisks	2 b,c; α	1 a; α	2 b; α	1 a; α	2 a; α	1 a; α	0 a; α	1 a; α	0 a; α	1 a; α	0 a; α	0 a; α	11 a
Total	10 γ, δ	2 α, β	7 β, γ, δ	6 α, β, γ,δ	12 δ	10 γ, δ	3 α, β	7 β, γ, δ	0 α	4 α, β, γ	2 α, β	2 α, β	65

a, b, c… within a column means followed by the same letter are significantly different according to Waller-Duncan *a*, *b* test at $P < 0.05$; α, β… within a line means followed by the same letter are significantly different according to Waller-Duncan *a*, *b* test at $P < 0.05$

2011, with a peak frequency during the cold seasons (winter and spring) (Table 2, Fig. 3). A peak HAdVs detection frequency is observed during the winter and spring with 12% in April and 10% in December and in May. However, during the summer and autumn, the frequencies of HAdVs detection were of the order of 3 and 2% in June and in October–November, respectively (Table 2, Fig. 3). The comparison of HAdVs rates recorded over the different seasons in the five basins of natural oxidizing ponds and at the exit of the rotating biodisks processes showed a significant difference ($P \leq 0.05$) (Table 2, Fig. 3). Thus, HAdVs encountered in the two basins, B_1 and B_2, appeared at low frequencies during the seasons of winter, of spring, of autumn, and of summer (2% in December and in February, 1% in July and in September in the basin B_2 (Table 2)). In the three other basins (B_3, B_4, and B_5) and at the exit of the rotating biodisks, HAdVs are recorded with moderate frequencies during the cold months (winter and spring) (3% in December and 4% in April in the last basin B5) and in the warmer season (autumn and summer) (1% at July and September in B_5) (Table 2).

Phylogenetic analysis of HAdVs

The molecular typing of HAdVs strains was performed on all positive samples by nested-PCR technique, but for a technical consideration only the genotyping of 21 samples could be identified. The phylogenetic analysis revealed only the circulation of HAdVs type 41 in hospital wastewater in the area of study. The phylogenetic tree, represented in Fig. 3, indicated that all 21 obtained strains belonged to type 41 of enteric HAdVs and clustered together within the same group (Fig. 4). Nucleotide and amino acid sequence identity analyses achieved between the obtained HAdVs strains and others published in GenBank showed nucleotide similarity varying from 98 to 100%, in type 41 clusters.

Discussion

In this study, we provide conclusive evidence of the occurrence of HAdVs in some wastewater sampled in Mutuelleville a suburb area of Tunis City, Tunisia. This wastewater was of a particular quality. Indeed, it was collected at a wastewater pilot

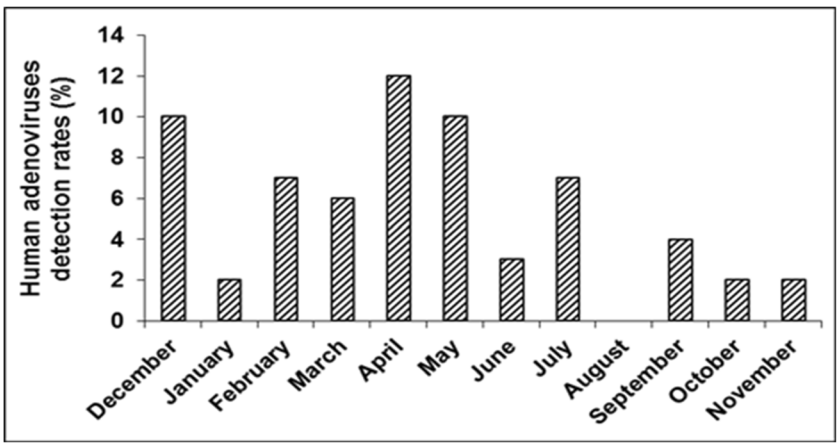

Fig. 3 Seasonal variation of human adenoviruses detection rates of the two wastewater treatment techniques: the natural oxidizing ponds and the rotating biodisks

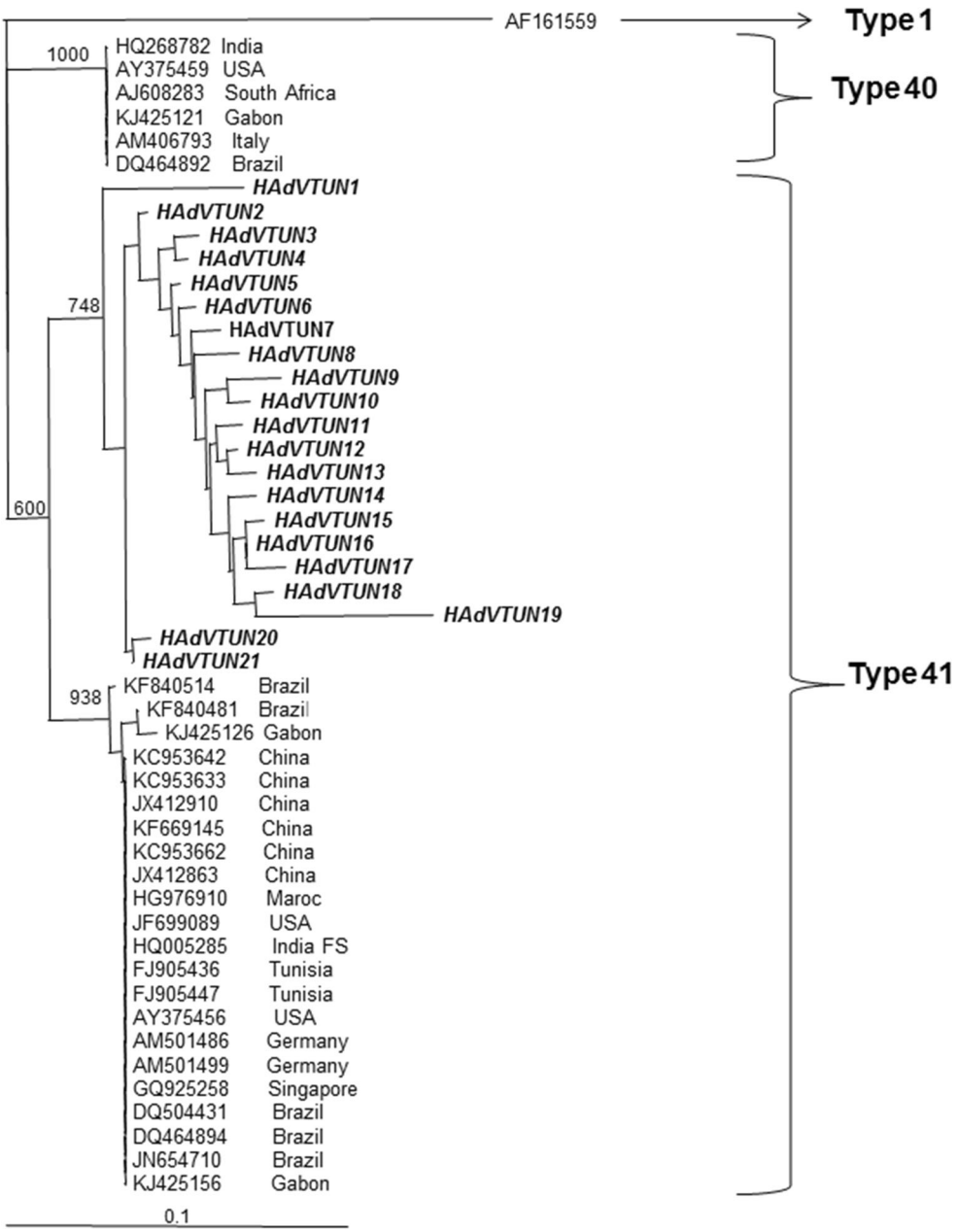

Fig. 4 Phylogenetic trees of enteric human adenoviruses based on the comparison of sequences in the hexon genes from the 21 viral strains isolated in Tunisia and some published nucleotide sequences in GenBank of different genotype of human adenoviruses. The tree was constructed using the neighbor-joining method and the human adenovirus type 1 (AF161559) sequence as outgroups. Numbers on the branches show bootstrap statistical analysis values (1000 replicates). Tunisian strains described in this work are in bold italic

plant, which receives sewage from mainly three neighboring hospitals. Hence, there is a need to study the movement and the circulation of enteric viruses such as enteric adenovirus 40/41 in this kind of specific wastewater to control the microbiological quality of the wastewater and to evaluate eventual gastroenteritis outbreaks that could be caused by these types of viruses in Tunis City. The first molecular epidemiology studies of HAdVs serotypes 40/41 in Tunisia reported the occurrence of these serotypes in 2.3% of fecal samples tested between the years 2003–2007 (Sdiri-Loulizi et al. 2009). HAdVs was detected in 0.4% of sewage samples collected in Tunisia between 2003 and 2010 (Sdiri-Loulizi et al. 2010). Thus, these two studies revealed low frequencies of the molecular detection of HAdVs in feces and in sewage (Sdiri-Loulizi et al. 2009, 2010). Oppositely, findings of the present study indicate high HAdVs molecular detection frequency. Out of 102, 65 from (64%) samples collected during the 2011 were positive for HAdVs in an important residential area of Tunis City. This high rate of HAdVs detection (64%) recorded in the present work is similar to those recorded in two other environmental studies carried out in Italy (60%) and in South Africa (62.5 and 64%) (Iaconelli et al. 2017; Adefisoye et al. 2016; Osuolale and Okoh 2015). Similarly, important HAdVs detection frequencies were noted in various regions of the world, such as Brazil (100%), Norway (92%), Greece (76.9 to 92.3%), and Poland (92.1%) (Fumian et al. 2013; Grøndahl-Rosado et al. 2014; Kokkinos et al. 2015; Wieczorek et al. 2015). However, a low HAdVs prevalence

was detected in wastewater samples in Morocco (45.5%), in Taiwan (27.3%), and in river water in Italy (21.6%) (Amdiouni et al. 2012; Lim et al. 2015; La Rosa et al. 2017). The presence of infectious HAdVs at high rates in treated wastewater emphasizes the urgent need for wastewater treatment surveillance. This study corroborates other studies achieved in developed countries concerning DNA viruses like adenoviruses as good markers of human fecal contamination (Fumian et al. 2013; Rames et al. 2016). The significant HAdVs detection rates in domestic and hospital wastewater point the importance of environmental virology monitoring as a tool to detect new genotype and to study the epidemiology of enteric HAdVs circulating in the human population. In addition, the important HAdVs frequencies detected in this study could be explained by the particular quality of wastewater circulating in the semi-industrial pilot plant, which comes from three different neighboring clinics in the residential area of study.

The present work assesses the performance of treatment of two kinds of biological techniques, namely, the natural oxidizing ponds and the rotating biodisks at the scale of a semi-industrial pilot plant located in an important residential and business area of Tunis City. The results of the physicochemical and the bacteriological analyses showed a substantial decrease of the average value of these two types of parameters at the exit of the two biological wastewater treatment techniques assessed in this study. However, the mean values of tested physicochemical parameters of treated wastewater by natural lagoon techniques and the mean values of bacteriological characteristics of treated effluents by rotating biodisks techniques were not in compliance with the Tunisian standard of water discharges NT 106-02 (COD = 90 mg/l, BOD_5 = 30 mg/l, fecal coliforms = $2{,}10^3$/100 ml, fecal streptococci = 10^3/100 ml). Therefore, a poor physicochemical and a good bacteriological quality of the treated water by natural oxidizing pond technique were observed. However, the opposite results were registered in the rotating biodisk procedure with an excellent physicochemical water quality and with a poor bacteriological one. Similar results were reported in previous Tunisian environmental studies conducted by Hassen et al. (1994) and Ibrahim et al. (2015, 2016, 2017a, b). The effectiveness of natural oxidizing ponds in fecal bacteria removal was explained by many factors such as solar radiation, the activity of the microbial flora, the abundance of organic matter, the growth of algae, the oxygen rate, and the slow residence time, which contribute and ensure the microbial inactivation (Hassen et al. 1994; Ibrahim et al. 2015, 2016, 2017a, b). Indeed, the fecal pollution indicator bacteria, such as fecal coliforms and fecal streptococci, were commonly used to evaluate the bacteriological quality of wastewater. The enumeration of these bacteria is very easy and of low-cost to monitor the microbiological water quality. These specific bacteria could be suggested as virological markers of fecal contamination of water (Lin and Ganesh 2013). In addition, their occurrence in treated wastewater reflects the presence of enteric viruses such as noroviruses, rotaviruses, enteric adenoviruses, astroviruses, Aichi viruses, enteroviruses, hepatitis A, and E viruses in these polluted waters (La Rosa et al. 2010; He et al. 2011; Adefisoye et al. 2016).

The virological results of these two treatment techniques showed a significant increase in the HAdVs prevalence from one point to another in the natural oxidizing pond and at the output of the rotating biodisks process. These findings document for the first in Tunisia the ineffectiveness of these two types of biological treatment for the removal of enteric adenoviruses in wastewater. These results confirmed the high resistance of HAdVs to the biological treatment adopted in this study. The persistence in the treated wastewater would qualify this type of virus as a virological indicator of the viral contamination of the environment. Similarly, other environmental studies showed the high persistence of HAdVs at high concentrations in various water environments during wastewater treatment of different countries such as Italy, the USA, Brazil, South Africa, and Poland (La Rosa et al. 2010; Fong et al. 2010; Prado et al. 2011; Vecchia et al. 2012; Osuolale and Okoh 2015; Adefisoye et al. 2016; Wieczorek et al. 2015; Iaconelli et al. 2017). Indeed, the virological results of this study showed a high resistance of HAdVs type 41 to the two secondary biological treatment techniques. This result is similar to that obtained in a recent study by Ibrahim et al. (2017a) showing the resistance of Aichi viruses to the same treatment techniques: the natural lagoon and the rotating biodisks. Similarly, another recent environmental study revealed the permanent presence of HAdVs in the treated effluents and the high resistance of this virus to the activated sludge procedure found in two wastewater treatment plants in the Eastern Cape Province in South Africa (Adefisoye et al. 2016). Also, an important HAdVs concentrations were recorded in raw and in treated sewage by activated sludge process in five different wastewater treatment plants situated in central Italy (La Rosa et al. 2010). Furthermore, this study indicated the high resistance of this type of virus (HAdVs) to the biological treatment as compared to that determined for other enteric viruses, such as noroviruses GI, noroviruses GII, and enteroviruses (La Rosa et al. 2010; Vecchia et al. 2012). Osuolale and Okoh (2015) reported the high HAdVs contents in the final effluents treated by activated sludge process and by tertiary chlorination in five wastewater treatment plants of the Eastern Cape in South Africa. By the same, the high resistance of HAdVs to biological and tertiary treatment was described in two different hospital wastewater treatment plant located in Rio de Janeiro, Brazil. The mean values of HAdVs viral loads obtained in filtered effluents from anaerobic process and in chlorinated effluents from the activated sludge process were of around 2.8×10^3 and 1.4×10^3 genome copies/ml; respectively (Prado et al. 2011). Indeed, the high HAdVs concentrations

and frequencies reported in this study and in other environmental works showed that conventional and standard wastewater treatment techniques such as natural lagoons, rotating biodisks, and activated sludge were inadequate and ineffective for enteric adenoviruses removal. The phenomenon of aggregation and adsorption of the viral particles to the particulate matter induced viral particle aggregation that may contribute to the protection of these viruses during their transport in the sewage network and during the processes of wastewater treatment (Enriquez et al. 1995; Carter 2005). These adsorption and aggregation, as well as their physicochemical properties (high resistance to pH variations and many chemicals) were responsible for the HAdVs persistence in sewage and their resistance to tertiary treatment (UV, chlorine, ozone…) (WHO 2005; Carducci et al. 2009). The findings of this study revealed the occurrence and the persistence of HAdVs in treated effluents used for recycling, agriculture reuse and release into the receiving water environments in the area of Tunis City, Tunisia. Thus, this result could denote a significant public health danger to humans in direct or indirect contact with these effluents.

On the other hand, in earlier Tunisian studies, Ibrahim et al. (2015, 2016) revealed the sensitivity of Norovirus GII and Rotaviruses A to the two wastewater treatment techniques. In Spain, a recent environmental study indicated that the lagoon system was a good natural technique for biological wastewater treatment concerning mainly the pathogen removal. The number of fecal indicator bacteria log showed a decrease of 2.58 U-log for *Escherichia coli* (EC), and 1.65 U-log for *intestinal enterococci* (IE) (Fernandez-Cassi et al. 2016). The viral loads of human adenovirus (HAdV), JC polyomavirus (JCPyV), and human noroviruses (NoV GI and GII) were log declined by 1.18 genomic copies (Fernandez-Cassi et al. 2016). The effectiveness of the activated sludge biological process coupled to tertiary treatment of chlorination in the wastewater treatment plant situated in Michigan showed lesser than 2 logs $_{(10)}$ (99%) of HAdVs removal (Fong et al. 2010).

In addition, our study showed a high prevalence of HAdVs during the cold seasons of winter and spring (10 and 12% in December and in April, respectively), and conversely, a low rate of HAdVs during the hot seasons of summer and autumn (3 and 2% in June and October, respectively). This seasonal variation of HAdVs in wastewater revealed a significant difference between the HAdVs prevalence recorded over the different seasons. The high HAdVs detection rate registered during the cold months was explained by the climatic and environmental conditions, such as low temperature that contributes in promoting the survival and the persistence of these viruses for prolonged periods as compared to other warm months of the year. Some other climatic factors such as pH, salinity, solar, and ultraviolet radiation affected seriously the microbial survival. For example, a higher temperature allowed the inactivation of enteric adenoviruses by the denaturation of their proteins and their nucleic acids (Lipp et al. 2001; Adefisoye et al. 2016). Similarly, a recent African environmental study reported by Adefisoye et al. (2016), revealed that high concentrations of HAdVs recorded in sewage sampled from five wastewater treatment plants were detected in the winter. In discordance with our results, a recent environmental study conducted in Taiwan reported the monthly distribution of HAdVs over a year and showed no significant seasonal variations in wastewater (Kuo et al. 2015). In addition, HAdVs were detected at a regular rate in sewage in Poland during all seasons (Wieczorek et al. 2015). This monthly distribution of HAdVs over the year is in general linked to the variation and the specificity of the climate in each country.

The 21 Tunisian HAdVs strains and of serotype 41 showed a similar nucleotide sequence of 98 to 100% with the reference strains published in the GenBank, and isolated from fecal samples such as: KF840514, KF840481, AY375456, and DQ504431 in Brazil; KC953642, KC953633 KC953662, KF669145, JX412910, and JX412863 in China; HQ005285 in India; FJ905447 and FJ905436 in Tunisia; KJ425126 and KJ425156 in Gabon; HG976910 in Morocco; and AM501486 and AM501499 in Germany (Reis et al. 2016; Liu et al. 2014; Dey et al. 2011; Sdiri-Loulizi et al. 2009; Soares et al. 2004; Filho et al. 2007). In addition, the following nucleotide sequences JF699089, GQ925258, JN654710, and FJ905453 were found in sewage samples in the USA, in Singapore, in Brazil, and in Tunisia, respectively (Sibley et al. 2011; Aw and Gin 2010; Fumian et al. 2013; Sdiri-Loulizi et al. 2010). Finally, the nucleotide DQ464894 of HAdVs strain was detected in river water in Brazil (Miagostovish et al. 2008). The phylogenic analysis of all HAdVs strains detected in this study established the only detection of HAdVs species F serotypes 41 in wastewater. This finding was in concordance with two previous studies conducted by Sdiri-Loulizi et al. (2009, 2010) that have reported the dominance of HAdVs serotypes 41 in clinical and in sewage samples in the region of Monastir, Tunisia. Indeed, the first Tunisian environmental study showed the only HAdVs strain detected in raw sewage was of type 41 that possessed a high identity nucleotide sequence of 100% compared to a Brazilian strain (DQ464894) isolated from river water (Sdiri-Loulizi et al. 2010; Miagostovish et al. 2008). Consequently, these findings confirmed that the HAdVs serotype 41 was documented as the enteric HAdVs predominant serotypes in wastewater worldwide. This dominance and the high prevalence of HAdVs species F serotype 41 in wastewater may confirm that this species and this serotype of enteric HAdVs were the most frequent in the human population. Additionally, this enteric HAdVs serotype 41 is detected and its high resistance to two biological wastewater treatment techniques (natural oxidizing ponds and rotating biodisks) is proven for the first time in Tunisia. Similarly, other studies showed that the most abundant species and serotypes identified were HAdVs species F (HAdVs-F) and

HAdVs serotype 41 (HAdVs- 41) in wastewater samples in diverse regions of the world such as Michigan, Poland, Norway, South Africa, Taiwan, Germany, Italy (Fong et al. 2010; Wieczorek et al. 2015; Myrmel et al. 2015; Osuolale and Okoh 2015; Kuo et al. 2015; Iaconelli et al. 2017).

Conclusion

The present work reported the high HAdVs prevalence and the only detection of HAdVs serotype 41 in wastewater samples discharged directly in hospital wastewater treatment, pilot plant located in the residential and business zone of Mutuelleville, a suburb of Tunis City. In addition, this study exemplified the first documentation in Tunisia showing the high resistance of HAdVs serotype 41 to two secondary biological treatment techniques positioned in this pilot plant. Thus, tertiary treatment of disinfection is necessary for the abatement of these viruses. Our results demonstrate the advantages of environmental surveillance and investigation as an important tool to study the molecular epidemiology of the virus community circulating in the population. We highlight the necessity of environmental surveillance programs in countries, such as Tunisia with the inadequate and the problematic epidemiological surveillance system and no environmental surveillance system currently in action**.** This study highlights the urgent need for environmental control programs and the establishment of innovative and effective wastewater treatment plants in hospitals, which are merely dumping the wastewater in the municipal sewage system.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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