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ORIGINAL PAPER

Molecular Typing of Enteroviruses, Adenoviruses, and Hepatitis A Viruses in Untreated and Treated Sewage of a Biological Treatment Plant in Greece

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Abstract The effluents of a sewage treatment plant may contain infectious human viruses representing a major public health issue. In the present study, an 8 months survey was conducted in order to evaluate the presence of enteroviruses (EV), adenoviruses (AdV), and hepatitis A viruses (HAV) in untreated and treated sewage samples collected from a primary treatment municipal wastewater plant, located in the northeastern Greece. Reverse transcriptase-polymerase chain reaction (RT-PCR) and nested polymerase chain reaction techniques have been applied for viral nucleic acid detection. Positive samples were confirmed by sequencing, and comparative phylogenetic analysis was performed on the isolated viral strains. EVs, AdVs, and HAV have been detected in 40% (10/25), 40% (10/25), 4% (1/25) of the samples collected from the plant's inlet, and in 12% (3/25), 44% (11/25), 0% (0/25) of the samples collected from the plant's outlet. Adenovirus types 3 (Ad3), 10 (Ad10) and 41 (Ad41), and hepatitis A virus type H2 have been recognized, while for enteroviruses Coxsackie type A2 and Echovirus types 27 and 30 have been recorded. The results suggest that treated sewage may still contain human viruses and thereby represent a potential health hazard. Moreover, their possible reuse in agriculture or elsewhere must be considered with concern. Furthermore, this study shows the usefulness of molecular methods for virus detection, typing and virological quality analysis of sewage treatment plants.

Keywords Wastewater · Enterovirus · Adenovirus · Hepatitis A virus · Virus detection

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Introduction

It has been documented that numerous different pathogens may even be present in the final treated effluents of wastewater treatment plants. For this reason, a few regulations have been issued in Europe to control the microbiological quality of treated effluents (Petrinca et al. 2009). Although controls of the microbial pollution of treated wastewater are currently required by Greek regulations (FEK.2089/t.B'/9-10-2008), microbiological monitoring is only limited to bacterial parameters, even though wastewater treatment plants effluent discharged into surface waters can be a severe source of environmental viral contamination and constitute a major public health problem (Villar et al. 2007; Pinto et al. 2007; Carducci et al. 2008). Large numbers of viruses are excreted in human feces and urine, which even at low concentrations may cause illness when ingested (Albinana-Gimenez et al. 2006; Stoner et al. 1996; Tonry et al. 2005). The enteric viruses found in human stool belong to more than 140 types of which enterovirus (EV), adenovirus (AdV), hepatitis A virus (HAV), norovirus (NoV) genotype I and II, and rotavirus (RV) are those most often detected in the environment. These viruses are responsible for a large number of epidemics because of their presence in the aqueous environment or food (Papadopoulos et al. 2006; Carducci et al. 2009; Petrinca et al. 2009; Sinclair et al. 2009; Vantarakis et al. 2009).

In an attempt to better understand the viral contamination and resistance to various treatments to assess the virological quality of wastewaters and to estimate the risks related to wastewater release to surface waters, many studies have been reported recently. In a study performed by Carducci and colleagues, the efficiency of viral removal by an urban sewage plant was evaluated by screening inlet

and outlet for the presence of HAV and HAdV. The consistent presence of viruses, an abatement rate of about 2 Log_{10} and the inadequacy of bacterial indicators for assessing the presence or removal of viruses was recorded (Carducci et al. 2008). In another consecutive study, the monitoring was extended for an additional 6-month-period, and the presence of HAdV, Torque Teno virus (TTV), HAV, RV, EV, NoV genogroups I and II has been confirmed, while the best indicator for virus inactivation in recycled waters seemed to be HAdV (Carducci et al. 2009). Myrmel et al. (2006) detected HAdVs in 96% of inlet and 94% of outlet samples, supporting the potential of these viruses as indicators of viral contamination from sewage. In addition, in another study, the presence of different types of viruses such as hepatitis A viruses, adenoviruses, rotaviruses, astroviruses, noroviruses and enteroviruses in 67% of sewage samples was detected. During evaluation of the removal efficiency of different treatment stages, primary treatment was found to be less effective in the removal of viruses (Anastasi et al. 2008).

Human enteroviruses are subclassified into polioviruses (PV, serotypes 1–3), coxsackieviruses group A (CAV, serotypes 1–22 and 24), coxsackieviruses group B (CBV, serotypes 1–6, echoviruses (ECV, serotypes 1–7, 9, 11–27, and 29–33) and enteroviruses 68–71 (EVs, 4 serotypes) (Sano et al. 2004; Ehlers et al. 2005; Carducci et al. 2006; Petrinca et al. 2009). Human AdVs are divided into six subgroups (A to F) comprised of 51 different serotypes (1–51). Out of the six subgroups, AdVs of subgroup F (enteric serotypes 40 and 41) are estimated to be associated with 5–20% of acute gastroenteritis cases among infants and young children (Haramoto et al. 2007). Many researchers have reported the qualitative detection of human AdVs in various kinds of water, such as raw sewage, river water, seawater, and tap water (Carducci et al. 2006; Myrmel et al. 2006; Haramoto et al. 2007; Carducci et al. 2008; Mena et al. 2009; Petrinca et al. 2009). Hepatitis A virus (HAV) is the main cause of acute hepatitis worldwide and has been associated with many outbreaks linked to sewage contamination of shellfish or to contaminated water, used for drinking, irrigation, vegetable washing or recreational use (Morace et al. 2002; Vantarakis et al. 2009). Data from many studies reflect the persistent circulation of HAV in the Mediterranean region and the need for monitoring polluted environmental samples to prevent diffusion of the virus into the population (Myrmel et al. 2006; Pinto et al. 2007; Carducci et al. 2008; Petrinca et al. 2009).

Only very few studies concerning with the determination of untreated and treated sewage virological quality, have been performed in Greece. In one of these studies, analyzing sewage samples from four biological treatment plants (two in the city of Athens and two in the city of Patras) EVs and AdVs were detected (Komninou et al. 2004). To enrich the poor existing data on the virological quality of the influents and effluents of wastewater treatment plants in Greece, an 8 months survey was conducted to examine the EVs, AdVs, and HAV presence and eventual reduction in sewage samples collected from a primary treatment wastewater plant, located at Alexandroupoli, in the north eastern Greece. The present study has been focused on the detection and typing of three types of viruses, EVs, AdVs, and HAV in raw and treated sewage samples.

Materials and Methods

Wastewater Treatment Plant and Sampling

The wastewater plant of the present study receives urban sewage from the city of Alexandroupolis, the capital of Evros prefecture. The city has approximately 50,000 inhabitants and is located in northeastern Greece. The plant is officially registered as a secondary treatment plant with aerobic digestion of the sludge. It is located at the western part of the city near the airport of Alexandroupoli. The plant receives only urban and not industrial sewage. It treats 9,000 m^3 of sewage from the city of Alexandroupoli and 500 m^3 of sewage derived from villages located close to the city, per day. The drainage system of the city is connected to the plant at a percentage of about 70%. Military campus and new build areas are excluded and have not yet been connected to the plant. The wastewater effluents are discharged into the Thracean Sea, and their quality is of interest for swimmers and tourists visiting beaches which are located in close distance from the treatment plant outlet. Moreover, shellfish cultivation facilities are also located nearby.

From 1 May 2007 to 30 December 2007, 50 samples (25 untreated samples from the inlet and 25 treated samples from the outlet) were collected weekly from the municipal sewage treatment plant. A 100 ml sample was collected in 500 ml plastic bottles during each sampling. The samples were delivered to the laboratory in portable refrigerators at the same day of collection and they were immediately subjected to virological analysis for the detection of human AdVs, EVs and HAV.

Sample Concentration, Viral Extraction, and Biomolecular Analysis

Samples kept at 4°C were concentrated within 24 h to a final volume of 1 ml PBS after centrifugation at 220,000×g for 1 h according to previously published protocols (Girones et al. 1993; Vantarakis and Papapetropoulou 1998). Viral nucleic acids were extracted from

concentrated samples using the QIAamp RNA mini-kit (Qiagen, USA) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) and nested PCR techniques have been used for the detection of EVs, human AdVs and HAV, according to previously published protocols (Girones et al. 1993; Vantarakis and Papapetropoulou 1998). The amplification products were analyzed on 2% agarose gel containing ethidium bromide and were visualized under UV illumination. All samples were tested in parallel, as neat and diluted (1:10) and the positive samples were recorded. In addition, randomly chosen negative samples were tested again after being diluted and were confirmed to be negative.

Sequence Analysis—Phylogenetic Tree Analysis of Viral Sequences

Positive PCR products were purified using the QIAquick PCR purification kit (Qiagen, USA), according to manufacturer's recommendations, and confirmed by sequencing. Nucleotide sequencing reaction of the positive samples was performed in both directions. The obtained nucleotide sequences were analyzed by BLAST program at the NIH website (NCBI, National Centre for Technology Control, NIH, USA), and were compared with each other and with other published sequences. Multiple alignments were performed with the Clustal X program. The neighbour-joining method has been applied for the phylogenetic tree analysis, the reliability of which was assessed by bootstrap resampling (1,000 pseudoreplicates), using MEGA 4.0.2 program (Saitu and Nei 1987; Thompson et al. 1997; Kumar et al. 2001).

Results

Virus Detection

In total, viruses have been detected in 60% of the untreated samples collected from the plant entry and in 52% of the treated samples collected from the plant's outlet. In two cases, no viruses have been detected after analysis of untreated samples, while they have been detected in the corresponding treated. Moreover, EVs and human AdVs have been detected to be contemporaneously present in 15% of the samples analyzed. EVs, have been detected in 40% (10/25), of the samples collected from the inlet, and in 12% (3/25) of the samples collected from the outlet. The enteroviruses detected were Coxsackie type A2 and Echovirus types 27 and 30. Human AdVs were present in 40% (10/25) of the samples collected from the plant entry, and in 44% (11/25) of the samples collected from the plant outlet. Adenovirus types 3 (Ad3), 10 (Ad10) and 41 (Ad41) have been recognized. HAV of H2 strain has been detected in 4% (1/25) of the samples collected from the plant entry and in none of the samples collected from the outlet (Fig. 1).

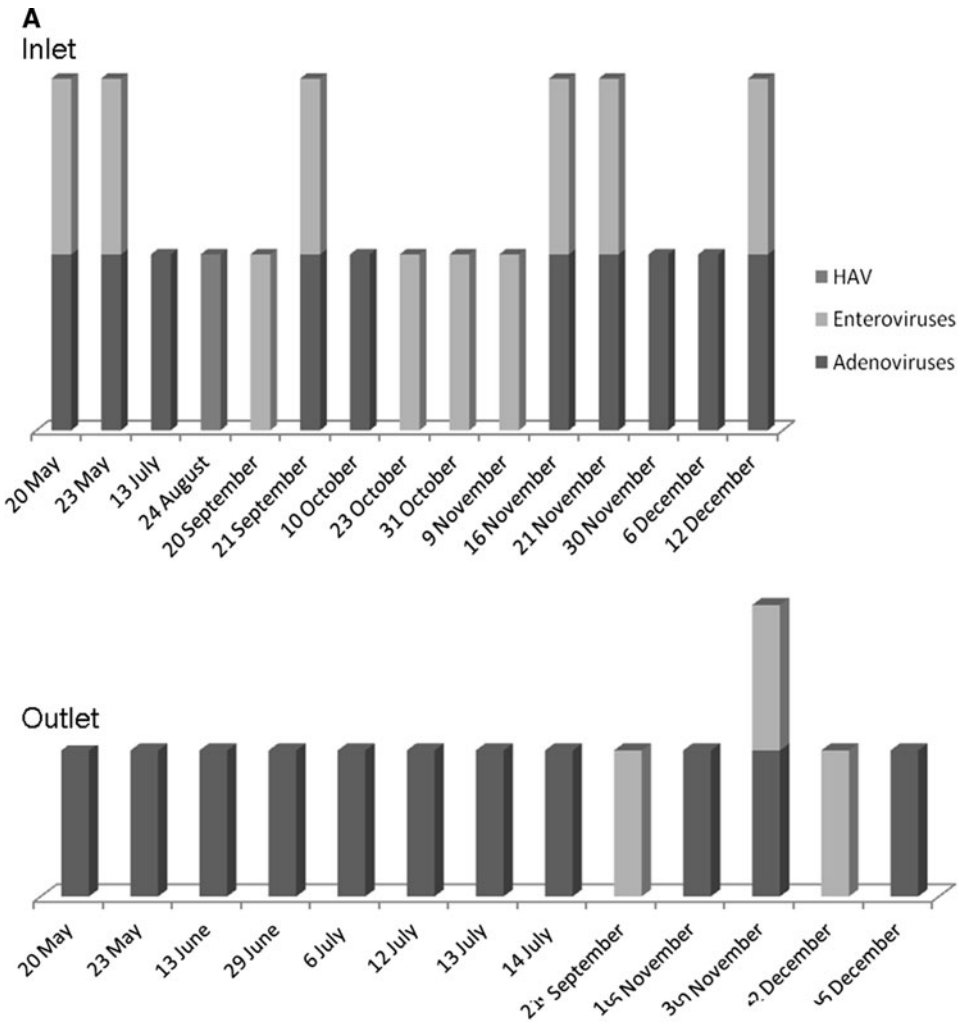
Phylogenetic Analysis of Viral Sequences

Figure 3 shows the phylogenetic tree analysis of nucleotide sequences from the positive HAV sample, and twelve (12) selected HAV strains. Two of the 12 reference sequences (Clin1Alex, Clin2Alex) derived from one of our studies (Vantarakis et al. 2009). During this study, we have characterized isolated strains from a HAV outbreak, by analyzing clinical samples from hospitalized patients in the hospital of Alexandroupoli. The outbreak was occurred in the interested region during the same time period of the present research work (Vantarakis et al. 2009). Analysis of the sequences revealed a high degree of identity. Identities among the nucleotide sequence of sewage isolate and the reference isolates varied between 94 and 98%, while compared to the clinical isolates the identity percentage was 97%. Figure 4 shows the phylogenetic tree analysis of a human AdV nucleotide sequence of the study and the nucleotide sequences of 12 selected reference strains. Percentage identities of the sewage AdV isolate and the reference isolates ranged between 93 and 98%. Figure 2 shows the phylogenetic tree analysis of nucleotide sequences of 3 EVs sewage isolates along with nucleotide sequences of 16 reference strains.

Discussion

The vast majority of the wastewater treatment plants in Greece are primary or secondary treatment plants. It is well known that primary and secondary sewage treatment processes do not efficiently reduce the virus concentration, in contrast with tertiary treatment processes (Lodder and de Roda Husman 2005, Anastasi et al. 2008). Depending on the applied processes, treated sewage discharged onto surface waters may significantly enhance the virus concentrations in the environment. Water may be contaminated by more than 140 serotypes of viruses via wastewater (Gantzer et al. 1998). Inadequately treated sewage in Greece is mainly discharged to the sea, and this may significant affect important national economic sectors such as the touristic industry.

Concerns about health risks have renewed interest in the effects of wastewater treatment on pathogens (Godfree and Farrell 2005). Recent research on microbial resistance to treatment and disinfection demonstrates that the outer surfaces and the nature of the genome are critical to our



B

Inlet

Date / Virus	20/05	23/05	13/07	24/08	20/09	21/09	10/10	23/10	31/10	09/11	16/11	21/11	30/11	06/12	12/12
AdV	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+
EV	+	+	-	-	+	+	-	+	+	+	+	+	-	-	+
HAV	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-

Outlet

Date / Virus	20/05	23/05	13/06	29/06	06/07	12/07	13/07	14/07	21/09	16/11	30/11	02/12	06/12
AdV	+	+	+	+	+	+	+	+	-	+	+	-	+
EV	-	-	-	-	-	-	-	-	+	-	+	+	-
HAV	-	-	-	-	-	-	-	-	-	-	-	-	-

AdV: Adenovirus, EV: Enterovirus, HAV: Hepatitis A Virus

Fig. 1 AdV, EV, and HAV presence in the inlet and outlet of the wastewater treatment plant

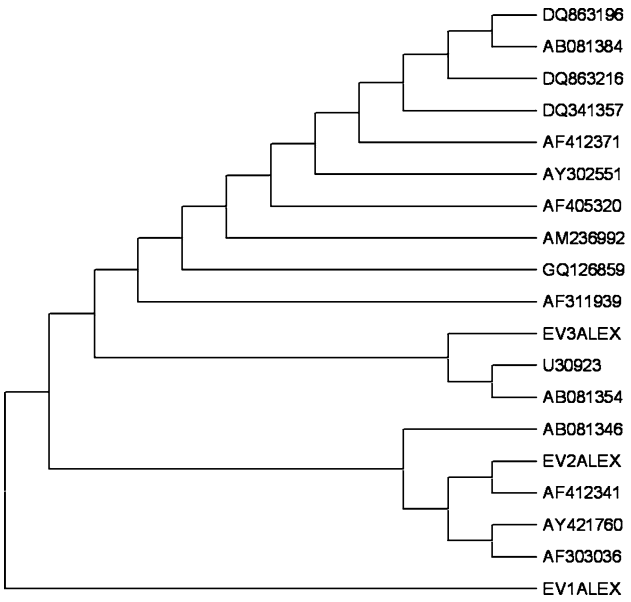


Fig. 2 Phylogenetic tree analysis of three Enterovirus (EVs) nucleotide sequences (EV1ALEX, EV2ALEX, EV3ALEX) of Greek strains isolated from the sewage treatment plant of the city of Alexandroupoli. Reference sequences were selected from GenBank database under the accession numbers indicated in the figure

understanding of resistance to disinfectants and removal by physical methods (Nwachcuku and Gerba 2004). The upgrade of wastewater treatment by the application of tertiary treatment processes will certainly affect virological quality of the treated sewage. UV disinfection technology is of growing interest in the water industry, representing a potential tool for a more effective sewage treatment (Hijnen et al. 2006; Gomila et al., 2008).

During the last years, more attention has been focused on the sewage virological quality, the risk of virus-associated waterborne illness, and the need for routine monitoring viral contamination (Morace et al. 2002; Villar et al. 2007; Carducci et al. 2009). To enrich existing information, an 8 months survey was conducted to examine the EVs, AdVs, and HAV presence in raw and treated sewage samples collected from a primary treatment wastewater plant, located at the city of Alexandroupoli. EVs have been included in the present study because it is the only referred viral group in the Greek legislation for sewage (inlet and outlet) analysis (Vantarakis and Papapetropoulou 1998). In the present study, EVs have been detected in 40% of the samples collected from the plant inlet and in 12% from the outlet. EVs were typed as Coxsackie type A2 and Echovirus types 27 and 30 (Fig. 1). Analyzing wastewater samples, Wullenweber and Agbalika (1984) detected Coxsakievirus B (25%) and Echovirus (7%), while Petrinca et al. (2009) identified Coxsackie viruses B1 through B5, Coxsakievirus A9 and Echovirus 1. Ehlers and colleagues (2005) detected EVs in 42.5% of sewage samples and commented that the prevalence of these viruses particularly in sewage was probably higher than detected, because of the fact that the detection techniques used were more efficient for treated than untreated sewage. EVs are shed in large number in sewage and their detection in our untreated samples reflects their circulation inside the local human population. Moreover, although the virus reduction from 40 to 12% is a significant criterion for the efficiency of the primary treatment plant studied, the presence of EVs in the treated samples may still represent a real public health hazard.

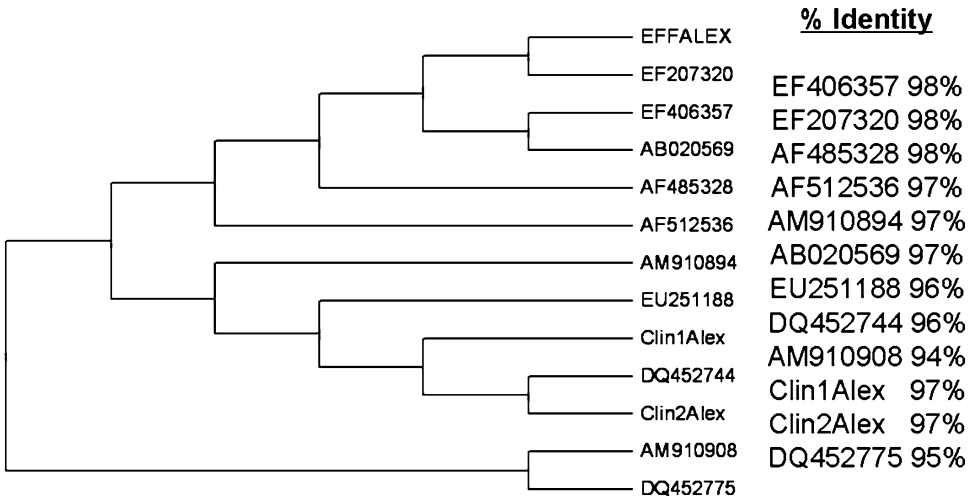


Fig. 3 Phylogenetic tree analysis of one Hepatitis A virus (HAV) nucleotide sequence isolated from the influent of the sewage treatment plant of the city of Alexandroupoli (EFFALEX). Reference sequences were selected from GenBank database under the accession numbers indicated in the figure. The sequences of two HAV strains isolated from two hospitalized patients during a HAV outbreak in the interested region are also included in the study. Percentage identity values of the unique HAV nucleotide sequence of the current study compared to the other sequences included for the construction of the phylogenetic tree are also presented and range from 94 to 98%

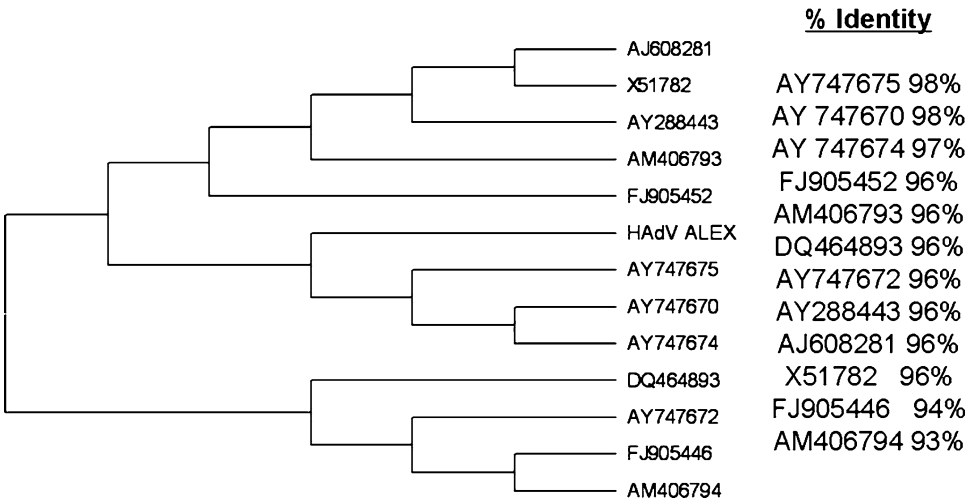


Fig. 4 Phylogenetic tree analysis of a human Adenovirus (hAdVs) nucleotide sequences (hAdV ALEX), isolated from the sewage treatment plant of the city of Alexandroupoli. Reference sequences were selected from GenBank database under the accession numbers indicated in the figure. Percentage identity values of a hAdV nucleotide sequence of the current study compared to the other sequences included for the construction of the phylogenetic tree are also presented and range from 93 to 98%

The presence of HAV was also assayed in raw and treated sewage samples collected from the sewage treatment plant of our study. HAV has been detected only in 4% (1/25) of the samples collected from the inlet and has not been detected in any of the samples collected from the plant's outlet. The isolated strains have been recognized as type H2. The low rate of HAV positivity in our study is in agreement with the results of other studies (Myrmel et al. 2006; Carducci et al. 2008). However, the low rate of HAV positivity noticed in the present study is in disagreement with data from other studies where elevated rates have been reported, as for example in Brazil (32%), in India (24.4%), and in Cairo city in Egypt (71%) (Vaidya et al. 2002; Villar et al. 2007). These differences in HAV prevalence are due to the different endemicity of HAV infection in these different geographical areas, as well as to the methods used to concentrate and detect HAV in the sewage samples (Villar et al. 2007).

For the phylogenetic tree analysis of the nucleotide sequence of the positive HAV sample, 10 reference nucleotide sequences have been incorporated, along with 2 sequences (Clin1Alex, Clin2Alex) of HAV strains isolated from clinical samples derived from hospitalized patients in the Alexandroupoli hospital, during a HAV outbreak described in the Prefecture of Evros (Fig. 3). The HAV outbreak occurred during the same time period of the current study (Vantarakis et al. 2009). In this outbreak, more than 38 HAV cases were recorded in the area from June to September. Because of the absence of a surveillance system for Hepatitis A in Greece and of the possibility of asymptomatic cases, higher number of cases in the community was expected. Analysis of the nucleotide sequences revealed a high degree of identity. Identities among the nucleotide sequence of the sewage isolate and the reference isolates varied between 94 and 98%, while compared to the clinical isolates the identity percentage was 97%, probably depicting the same strain. A close genetic relationship was observed among sewage and clinical viral isolates showing that viral strains could have been disseminated into the environment. Thus, they could be considered as a reference for risk assessment (Carducci et al. 2006; Villar et al. 2007). The analysis of nucleotide sequence variations in environmental isolates can provide useful information for addressing epidemiological questions, such as pathways for viral spread, since several studies have suggested that different sequences may be related to the geographical origin of the virus (Morace et al. 2002).

Many researchers have reported the qualitative detection of human AdVs from various kinds of water by using polymerase chain reaction (PCR). Human AdVs are excreted with the feces of the infected patients at a concentration of up to 10^{11} viral particles per gram. Human AdVs of various serotypes may be discharged into aquatic environments via feces and have been detected in various waters worldwide including wastewater (Haramoto et al. 2007). The possible role of human AdV as an indicator of viral contamination has already been proposed by several authors, because of its high stability under environmental stress and sewage treatment procedures (Albinana-Gimenez et al. 2006; Carducci et al. 2009). However, our incomplete understanding of the relations of the amount of human AdVs DNA with infectivity represent an obstacle to the application of this measure as indicator of virus presence (Carducci et al. 2009).

In a 6 months study of a wastewater treatment facility, it has been demonstrated that the plant did not perform well for the removal of fecal indicator bacteria, human enteric viruses, or parasite cysts. Supplementary treatment and disinfection were recommended to protect public health (Payment et al. 2001).

The detection of viruses by molecular techniques is useful for the detection of emergent viruses in community

wastewaters and water supplies. Quantification of HAdV using PCR (QPCR) may be useful for evaluating virus removal efficiency in water treatment plants and as an index of the virological quality of water and of the potential presence of human viruses (Albinana-Gimenez et al. 2006).

The virus types isolated from the raw sewage demonstrated the virus types circulating in the community. In addition, the results of our study confirm the high stability of viruses in raw sewage and final effluent and the difficulty of viral removal by sewage treatment. This constitutes an important source of viral dissemination in the environment. Primary treatment can remove only small percentages of viruses, thus underlining the need for more effective treatments (secondary, tertiary, or novel treatment techniques) to achieve a higher level of public health protection. European regulations of wastewater effluents do not mainly contain microbiological standards to be fulfilled. Present European regulatory directives must be expanded in order to cover the microbiological quality of waste water effluents.

Conclusion

The results of the present study clearly indicate that treated sewage from the studied treatment plant contains different human pathogenic viruses. Sewage viral load is discharged to the environment and constitutes a serious hazard of public health. Moreover, the advantage of a more advanced sewage treatment is demonstrated, as the primary treatment was ineffective in eliminating the viral load. It is evident that risks based only on bacterial standards may seriously underestimate the risk of virus associated waterborne illness. The virological monitoring is a critical component of the evaluation of sewage quality and should be seriously considered to be added to the routine testing performed for wastewater plant management. Finally, the need for more integrated and detailed studies of the viral sewage quality of treatment plants in Greece is evidenced, because of the poor existing data.

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