Analysis of adenoviruses and polyomaviruses quantified by qPCR as indicators of water quality in source and drinking-water treatment plants

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Abstract

Three drinking-water treatment plants were analyzed for the presence of human adenoviruses (HAdV) and JC polyomavirus (JCPyV), previously suggested as viral contamination indicators, in order to define their water quality in relation to the presence of viral pathogens and the efficiency of the treatments applied.

The 90% of the river water samples had positive results of HAdV (10¹⁰ genome copies (GC)/L); and 48%, of JCPyV (10⁰–10³ GC/L). Lower concentrations of HAdV and JCPyV were found in different treatment steps of the plants in absence of bacterial standards. Virus removal efficiencies were higher than 5 logs in plants 1 and 3 and could be quantified as >2 logs in plant 2. However, three post-chlorinated samples from plants 2 and 3 (11%) were found to be positive for HAdV by qPCR, but did not show infectivity in the cell cultures assayed. Simple methods based on the adsorption–elution of viruses from glass wool give low-cost and efficient virus recovery from source water and large-volume water samples. Quantification of JCPyV and HAdV using qPCR is useful for evaluating virus removal efficiency in water treatment plants, identification of Hazard Analysis and Critical Control Points (HACCP) and as a molecular index of the virological quality of water. Though infectivity is not guaranteed when using qPCR techniques in water treated with disinfection processes, the quality of source water, where viruses have proved to have infective capabilities, and the removal efficiency of viral particles may be efficiently quantified.

1. Introduction

Infected individuals shed large quantities of viral particles in feces and urine and many viruses transmitted by the fecal–oral route are widely prevalent in the environment (IAWPRC, 1983). Viruses such as JC polyomavirus and some human adenovirus strains establish persistent infections and viral particles may be shed in feces for months or even years (Shah, 1995; Wadell et al., 1988). Thus, high viral loads are detected in sewage, (Bofil-Mas et al., 2000; Katayama et al., 2008; Pina et al., 1998) and lower concentrations in river water (Albinana-Gimenez et al., 2006; Choi and Jiang, 2005; Haramoto et al., 2005), sea water (Calgua et al., 2008; Haramoto et al., 2007) and even drinking water (Lambertini et al., 2008; Van Heerden

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Several studies confirm that infectious water-related diseases are not only a primordial cause of mortality and morbidity worldwide, but also that both the spectrum and incidence of many water-related diseases are increasing (WHO, 2003).

The general demand for high-quality water has increased the pressure on environmental and public health policies to ensure the microbiological safety of water. To reduce human health risk from waterborne and water-related illness, water quality standards are established by the WHO and are adopted by most nations worldwide. Classic microbiological indicators such as fecal coliform and enterococcus are the most commonly used indicators to evaluate the removal of fecal contamination in water purification processes. However, the adequacy of these bacteria for indicating the occurrence and concentration of human viruses and protozoa cysts has been questioned in recent years (Formiga-Cruz et al., 2003; Lipp et al., 2001; Tree et al., 2003).

There is information available about some water-borne pathogens, but the improvement in molecular technology for detecting viruses in water has focused attention on new groups of viruses that could in general terms be considered emergent pathogens in diverse geographical areas (Bofill-Mas et al., 2000; Pina et al., 1998). Human Polyomavirus JC and human adenoviruses are two groups of viruses that are being detected more often in the environment by molecular methods (Albinana-Gimenez et al., 2006; Bofill-Mas et al., 2000; Haramoto et al., 2007; Pina et al., 1998) and have been suggested as indicators of viral contamination of human origin (Bofill-Mas et al., 2000; Pina et al., 1998).

JC polyomavirus (JCPyV) is a human virus classified in the Polyomaviridae family that persists indefinitely in individuals and is excreted regularly in urine by healthy individuals (Shah, 1995). The initial infection occurs in childhood and is not apparent (Major et al., 1992). The virus infects a large number of the population worldwide and, consequently, its presence in water causes no health risk for most of the population. The pathogenicity of the virus is commonly associated with immunocompromised states but has attracted more attention due to AIDS-linked immunosuppression (Berger et al., 1987). JCPyV is associated with progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease (Berger et al., 1987). Some laboratories have suggested an association between JCPyV and human cancer such as colon cancer (Enam et al., 2002; Hori et al., 2005). In previous studies, JC polyomavirus was found in 98% of 52 sewage samples from widely diverse geographical areas (Bofill-Mas et al., 2000; Pina et al., 1998) and is excreted regularly in urine by healthy individuals.

Human adenoviruses (HAdV) are members of the genus Mastadenovirus in the Adenoviridae family, which comprises 51 serotypes classified into 6 species (A–F). They have double-stranded linear DNA and a non-enveloped icosahedral shell with fiber-like projections from each of the 12 vertices (Stewart et al., 1993). Human adenovirus infections occur worldwide, but many of them run their course asymptomatically. The most common HAdVs (Ad1, 2 and 5) infect 40–60% of children (Brandt et al., 1969). Some serotypes such as 40 and 41 are unique in being responsible for most cases of Ad-associated gastroenteritis in children. Others included in subspecies B are responsible for 5–10% of childhood respiratory diseases and conjunctivitis (Wold and Horwitz, 2007). In previous studies, methods for the molecular detection of HAdV in sewage, shellfish, river water and drinking water have been developed (Albinana-Gimenez et al., 2006; Cho et al., 2000; Formiga-Cruz et al., 2002; Lee et al., 2004; Pina et al., 1998; Puig et al., 1994; Van Heerden et al., 2005).

At a previous stage of this study, several concentration methods were compared and two methods based on adsorption-elution in glass wool were selected (Albinana-Gimenez et al., in press). At the present stage, three drinking-water treatment plants (DWTPs) were selected and several control points in each purification process were surveyed during a one-year period.

The aim of this study was to analyze the levels of HAdV and JCPyV by applying sensitive concentration methods in water and quantifying virus removal efficiency in several DWTPs, using quantitative PCR protocols. An overall goal is also to evaluate the applicability of HAdV and JCPyV quantified by qPCR as a tool for the control of the viral quality of source and treated water after a diversity of treatments.

2. Materials and methods

2.1. Sample collection

A total of 144 samples were collected over a period between January 2007 and March 2008 in three DWTPs (54 samples from plant 1, 42 samples from plant 2 and 48 samples from plant 3). The volume analyzed of raw river water samples from plants 1 and 3 was 1 L and the volume analyzed of sedimented water sample from plant 3 was 10 L. Small-volume samples (1 or 10 L) were collected, kept at 4 °C, filtered and processed within 24 h. Large-volume samples (400–2500 L) were filtered at the sampling point, the filters kept at 4 °C and processed within 24 h of collection.

2.2. Drinking-water treatment plants

DWTP 1 is situated in the Barcelona area, next to a river used as source water, and has a treatment capacity of 4.5 × 10^6 m^3/day. The raw water is breakpoint pre-chlorinated, and poly-electrolytes and aluminum sulphate are added to the water running into the sedimentation tanks for flocculation. After sedimentation, the water goes through sand filters. At this point there is an intake of ground water. Then the mixed water goes through ozonation and granular activated carbon (GAC) filters. Finally, the water is stored in tanks in which it is post-chlorinated. Samples were collected at the intake point (raw water), after sand filtration, at the intake of ground water, after ozonation, after GAC filtration and after post-chlorination (finished).

DWTP 2 has a treatment capacity of 1.8 × 10^6 m^3/day. The source water for this plant comes from three wells in the area. The plant has three parallel treatment lines. In the first line...
the water goes through filter cartridges, UV treatment and a final membrane filtration. In the other two lines the water goes through filter cartridges, UV treatment and reverse osmosis. The three lines converge in a tank where the water is post-chlorinated. Samples were collected at the ground water intake of both lines (raw), after filtration cartridges of both lines (filtered), after nanofiltration in line 1, after reverse osmosis in line 2 and after post-chlorination (finished).

DWTP 3 is situated in a rural farming area, next to a river used as source water and has a treatment capacity of \(1.4 \times 10^4\) m\(^3\)/day. The plant intake is situated about 100 m downstream from a wastewater treatment plant effluent. The raw water is breakpoint pre-chlorinated and polyelectrolytes are added to the water running into the sedimentation tanks for flocculation. After sedimentation, the water goes through GAC filters and is finally stored in tanks where it is post-chlorinated. Samples were collected at the intake (raw), after sedimentation, after GAC filtration and after post-chlorination (finished).

### 2.3. Bacteriological analysis

The bacteriology of the samples from the rivers (Ter and Llobregat) and from the DWTPs was analyzed by previously validated and accredited (ISO 17025) methods, some of them based on ISO Standards: for Heterotrophic Plate Count (aerobic bacteria) at 22 °C, Water Plate Count Agar (ISO) (Oxoid Inc., Basingstoke, UK); for Coliform bacteria and Escherichia coli, Colilert-18 Quanti-Trap (Idexx Inc. Westbrook, ME, USA); for Enterococci, Sklanetz & Barley Agar + Bile-esculin Agar (Oxoid); for Clostridium perfringens, TSC Agar (Oxoid) with the fluorogenic probe JEP3 described by Pal et al. (2006). HAdV genomes were quantified with 0.9 \(\mu\)M of the primers AdF and AdR and 0.225 \(\mu\)M of the fluorogenic probe JE3P described by Hernroth et al. (2002). Following activation of the uracil N-glycosilase contained in the core mix (2 min at 50 °C) and activation of the AmpliTaq Gold for 10 min at 95 °C, amplifications were carried out as described in previous studies, using two amplification rounds with 30 cycles each (Allard et al., 2001). Standard precautions were taken when performing the PCR assays: the PCR mix was prepared in a DNA-free environment, the samples were added at a separate location, and positive and negative controls were included in each assay.

### 2.4. Concentration of viruses from water and nucleic extraction

Two variations of the same method were used when processing different volumes. These methods were selected from previous studies (Albinana-Gimenez et al., in press) and are based on methods originally described by Vilaginès et al. (1993). Small-volume samples (1–10 L) were pre-acidified to pH 2.5 by adding HCl, and for detection limit was 2.5 \(\mu\)g/L. Large-volume samples (>15 L) were processed following the same protocol, but without pre-acidification.

As variable volumes of sample were concentrated and analyzed, the detection limits are different for each sample. For river water samples (1 L) the detection limit was approximately 2.5 \(\times\) 10^2 GC/L for 1000 L samples (treated water) the detection limit was 2.5 \(\times\) 10^-1 GC/L. Nucleic acids were extracted using Nucleospin RNA virus F (Macherey–Nagel). As samples from DWTP 3 had inhibition problems, a second serial extraction was performed with NucliSens magnetic extraction reagents (Biomerieux, The Netherlands).

### 2.5. Enzymatic amplification

(i) Nested-PCR. Nested-PCR was used for the typification of HAdV-positive samples. Ten-\(\mu\)L aliquots of the extracted nucleic acid and their respective 10-fold dilution were used in each test. Amplifications were carried out as described in previous studies, using two amplification rounds with 30 cycles each (Allard et al., 2001). Standard precautions were taken when performing the PCR assays: the PCR mix was prepared in a DNA-free environment, the samples were added at a separate location, and positive and negative controls were included in each assay.

(ii) Quantitative Real-Time PCR (qPCR). For the specific detection and quantification of JCPyV and HAdV genomes, 10 \(\mu\)L of the 10-fold and 100-fold dilutions of every DNA extraction were also assayed. These dilutions were made to avoid amplification inhibition due to this assay’s high sensitivity to inhibitors. Amplification was performed in a 25-\(\mu\)L reaction mixture with the PCR Master Mix (Applied Biosystems). The reaction contained 10 \(\mu\)L of a DNA sample or 10 \(\mu\)L of a quantified plasmid DNA, 1 \(\times\) TaqMan master mix, and the corresponding primers and TaqMan probes at their corresponding concentrations. JCPyV genomes were quantified with 0.5 \(\mu\)M of the primers JE3F and JE3R and 0.15 \(\mu\)M of the fluorogenic probe JE3P described by Pal et al. (2006). HAdV genomes were quantified with 0.9 \(\mu\)M of the primers AdF and AdR and 0.225 \(\mu\)M of the AdP1 probe described by Hernroth et al. (2002). Following activation of the uracil N-glycosilase contained in the core mix (2 min at 50 °C) and activation of the AmpliTaq Gold for 10 min at 95 °C, 40 cycles (15 s at 95 °C and 1 min at 60 °C) were performed with an MX3000P sequence detector system (Stratagene). All samples were run in quadruplicate, analyzing two replicates of the neat and –1 dilution for well water and treated water and two replicates of the –1 and –2 dilution in river water. Positive and negative controls were included. Oligonucleotide primers and probes for qPCR and nested-PCR are shown in Table 1.

The plasmid used as standard for quantification of HAdV was pAd41, containing the hexon region of HAdV41 in pBR322 kindly donated by Dr. Annika Allard of the University of Umeå (Sweden). The plasmid used as standard for quantification of JCPyV was JCPyV, containing the whole JCPyV genome strain Mad-1 in pBR322 kindly donated by Dr. Andrew Lewis Jr. of the Office of Vaccine Research and Review, CBER, FDA (USA). The amount of DNA was defined as the average of the quadruplicate data obtained. Inhibition controls were analyzed too: known quantities of viral genomes (10^3 GC/reaction) were added to reactions with sample, these controls were performed with all the samples.

### 2.6. Cell culture infection

Infection assays of HAdV found in finished water, GAC-filtered water samples (plants 1 and 3) and nanofiltered and reverse-osmosis water samples (plant 2) were performed on A549 cell line. A549 is an epithelial cell line from human lung carcinoma
and was propagated in Earl’s minimum essential medium (EMEM) supplemented with 1% glutamine, 50 μg of gentamicin per mL and 5% (growth medium) or 2% (maintenance medium) of heat-inactivated FBS (Foetal Bovine Serum). The assays were performed in duplicate and neat and 10-fold dilution of every sample’s concentrate was inoculated. Culture media were removed from the cell culture flasks. Two 85 cm² cell culture flasks were inoculated with 500 μl of the concentrate and three 25 cm² cell culture flasks were inoculated with 100 μl of the 10-fold dilution of the concentrate in PBS. A negative control 25 cm² flask was inoculated with PBS. washed twice and fresh media added. One of the 25 cm² flasks was frozen at –80 °C as T0 and the rest were incubated at 37 °C. The flasks were washed twice and fresh media added. One of the 25 cm² flasks was frozen at –80 °C as T0 and the rest were incubated at 37 °C for two weeks and then frozen at –80 °C.

2.7. Typification of human adenoviruses

Amplicons obtained from HAdV-positive treated water samples were purified using the QIAquick PCR purification kit, following the manufacturer’s instructions. Both strands of the purified DNA amplicons were sequenced with the ABI FRISM BigDye Terminator Cycle Sequencing Ready Reaction V 3.1 kit with AmpliTaq DNA polymerase FS (Applied Biosystems), following the manufacturer’s instructions. The results were checked using the ABI FRISM 3700 DNA analyzer (Applied Biosystems), and the sequences were compared with those present in GenBank and the European Molecular Biology Library by means of the basic BLAST program of the National Center for Biotechnology Information (available from URL: http://www.ncbi.nlm.nih.gov/BLAST/).

3. Results

3.1. Bacteriological analyses

3.1.1. Plant 1

Raw river water had significant concentrations of bacterial indicators with average values of 3.4 × 10² total coliforms cfu/100 ml, 2.1 × 10³ E. coli cfu/100 ml, 2.5 × 10³ C. perfringens cfu/100 ml, 4.4 × 10² Enterococci cfu/100 ml and 2.8 × 10³ Heterotrophic bacteria cfu/ml. The counts decreased drastically after sand filtration, with sporadic low counts found in the following treatments. However, decreasing Heterotrophic Plate Counts were observed during all the treatments.

3.1.2. Plant 2

Negative results and sporadic low counts (~10 cfu/100 ml) of all bacterial indicators were found throughout the process. In the case of Heterotrophic bacteria, 10⁻¹⁻² cfu/ml were found in all the steps of the process, showing no reduction.

3.1.3. Plant 3

Raw river water had higher concentrations of bacterial indicator than plant 1, with average values of 7.5 × 10⁴ total coliforms cfu/100 ml, 6.7 × 10³ E. coli cfu/100 ml, 5.2 × 10³ C. perfringens cfu/100 ml, 7.3 × 10³ Enterococci cfu/100 ml and 5.1 × 10³ Heterotrophic bacteria cfu/ml. One log reduction in the bacterial counts was seen after the sedimentation. GAC-filtered water had no bacterial indicators, except 1.5 × 10² Heterotrophic bacteria cfu/ml. No finished water sample had any positive standard bacterial parameter.

3.2. Quantification of viruses in three drinking-water treatment plants

The concentration of virus detected is expressed as genome copies (GC) per L, and the results are shown in Tables 2–4. All the river water samples from plant 1 were positive for HAdV in 1 L analyzed in this study, with a mean concentration of 1.24 × 10⁴ GC/L; and 44% were positive for JCPyV, with a mean concentration of 7.4 × 10² GC/L. The results of river water obtained in plant 3 are similar, with 83% of the samples positive for HAdV (mean concentration: 9.24 × 10³ GC/L) and for JCPyV (mean concentration: 1.3 × 10³ GC/L).

The raw water samples from plant 2 had lower levels of viral contamination, both for HAdV (mean concentration in both lines: 5.38 × 10⁴ GC/L) and for JCPyV (mean concentration in both lines: 2.15 × 10³ GC/L) as expected in ground water. Similar levels of viral contamination were found throughout the treatment and higher reduction was observed after the final chlorination. The values found in nanofiltered samples were 3.61 × 10³ HAdV GC/L and 1.01 × 10³ JCPyV GC/L, and in

Table 1 – Oligonucleotide primers for qPCR and nested-PCR amplification of JCPyV and HAdV.

<table>
<thead>
<tr>
<th>Virus (Prototype)</th>
<th>Name</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>JCPyV (Mad-1)</td>
<td>J3F</td>
<td>4317–4339</td>
<td>5’TATTTGCGCATGATGATAA-3’</td>
</tr>
<tr>
<td></td>
<td>J3R</td>
<td>4251–4277</td>
<td>5’GGAAGCTTTTGGCTTCTATCCT-3’</td>
</tr>
<tr>
<td></td>
<td>J3P</td>
<td>4282–4314</td>
<td>5’FAM-AGGATCCCCAAGACTCTAC-GTCAAA-BHQ1-3’</td>
</tr>
</tbody>
</table>

HAdV (HAdV2)

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAdF</td>
<td>18,868–18,887</td>
<td>5’TCTGTATCCATGCCATTC-3’</td>
</tr>
<tr>
<td>HAdR</td>
<td>18,919–18,938</td>
<td>5’TACGGGGCRAAYTGCACACAG-3’</td>
</tr>
<tr>
<td>HAdP1</td>
<td>18,890–18,917</td>
<td>5’FAM-CCGGGCTCAGGTACTCCG-3’</td>
</tr>
</tbody>
</table>

JCPyV (Mad-1)

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>JE3F</td>
<td>18,858–18,883</td>
<td>5’GGCCSCARTGKGWATACGGACATC-3’</td>
</tr>
<tr>
<td>Hex2deg</td>
<td>19,916–19,158</td>
<td>5’CAGCACGCTCGATGTCCA-3’</td>
</tr>
<tr>
<td>Nehex3deg</td>
<td>18,931–18,954</td>
<td>5’GCCGGYGCCAGCAACTACTTC-3’</td>
</tr>
<tr>
<td>Nehex4deg</td>
<td>19,077–19,102</td>
<td>5’CCYACRCCAGITWRRGCGMCYTTGTA-3’</td>
</tr>
</tbody>
</table>
reverse-osmosis treated samples were $1.24 \times 10^6$ HAdV GC/L and $5.00 \times 10^{-1}$ JCPyV GC/L, as it is shown near the detection limit. It is known that quantification in the higher Ct values is not as accurate as for higher concentrations of target sequences.

The reduction of the concentrations of HAdV and JCPyV quantified as genome copies during treatment in the DWTPs is calculated by the difference between the concentration in raw water and the concentration in finished water (Tables 2–4). Data from plant 1 indicate that the reduction in HAdV is more than 5.13 logs for finished water, and 5.13 logs between raw water and GAC-filtered water; for JCPyV, the reduction is >5 logs for finished water, and 5.13 logs between raw water and GAC-filtered water. According to the low viral numbers detected in the source water used in plant 2, the virus removal efficiency has been quantified as more than 2 logs, however it is possible that if more contaminated source water is treated, the plant could present higher removal efficiencies. One finished water sample was positive, with removal efficiency occasionally quantified as decreasing to 0.67 logs. The removal efficiency of JCPyV reaches >2 logs. Data from plant 3 show that the reduction of HAdV through the process reaches >5 logs, but occasionally (as the two positive finished water samples show) it decreases to 4.94 logs; for JCPyV, efficiency is >5 logs.

HAdV genomes were detected in one finished water sample from plant 2 and two finished water samples from plant 3 at low concentrations ($6.11 \times 10^6$ GC/L, $1.26 \times 10^6$ GC/L and $4 \times 10^{-2}$ GC/L, respectively). No finished water samples from DWTP 1 were found to be positive however, HAdV was found in one sample of the GAC-filtered water samples and in 7 GAC-filtered water samples from plant 3. JCPyV was not found in any of the finished water samples, but was found in one sample from GAC-filtered water in plant 1 and 5 samples from GAC-filtered water in plant 3.

As expected, results at the limit of detection presented low reproducibility between replicates and the higher dilutions in the more contaminated samples presented higher reproducibility with more than 90% of the quantifications within the same log.

### 3.3. Infection assays

The three positive finished water samples from plants 2 and 3, one GAC-filtered water sample from plant 1, three GAC-filtered water samples from plant 3, one nanofiltered water sample and one water sample after reverse osmosis from plant 2, were inoculated in A549 cell culture. No CPE was observed at 10 days post-infection and no DNA amplification was detected when PCR was performed in the culture supernatants.

### 3.4. Characterization of human adenoviruses

HAdV nucleic acids from the positive finished water sample concentrates were amplified by conventional nested-PCR and sequenced. All the sequences had 98% nucleotide homology with HAdV2 (prototype GenBank accession number J01917.1).

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**Table 2 – Human adenoviruses and JC polyomavirus in DWTP 1.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol. (L)</th>
<th>% Positive</th>
<th>Mean GC/L</th>
<th>Std. Dev.</th>
<th>Reduct.</th>
<th>% Positive</th>
<th>Mean GC/L</th>
<th>Std. Dev.</th>
<th>Reduct.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>1</td>
<td>100 (9/9)</td>
<td>$1.24 \times 10^6$</td>
<td>$1.60 \times 10^4$</td>
<td>–</td>
<td>44.4 (4/9)</td>
<td>$7.40 \times 10^2$</td>
<td>$1.10 \times 10^3$</td>
<td>–</td>
</tr>
<tr>
<td>Sand-filtered</td>
<td>426</td>
<td>55.6 (5/9)</td>
<td>$7.02 \times 10^2$</td>
<td>$1.56 \times 10^1$</td>
<td>3.24</td>
<td>22.2 (2/9)</td>
<td>$2.03 \times 10^{-2}$</td>
<td>$5.70 \times 10^{-2}$</td>
<td>4.56</td>
</tr>
<tr>
<td>Ground water</td>
<td>796</td>
<td>44.4 (4/9)</td>
<td>$2.05 \times 10^2$</td>
<td>$3.87 \times 10^{-1}$</td>
<td>–</td>
<td>44.4 (4/9)</td>
<td>$1.43 \times 10^1$</td>
<td>$3.82 \times 10^0$</td>
<td>–</td>
</tr>
<tr>
<td>Ozonated</td>
<td>508</td>
<td>44.4 (4/9)</td>
<td>$8.70 \times 10^2$</td>
<td>$1.58 \times 10^1$</td>
<td>3.15</td>
<td>44.4 (4/9)</td>
<td>$8.24 \times 10^0$</td>
<td>$1.57 \times 10^1$</td>
<td>1.95</td>
</tr>
<tr>
<td>GAC-filtered</td>
<td>655</td>
<td>11.1 (1/9)</td>
<td>$9.10 \times 10^{-2}$</td>
<td>$2.37 \times 10^{-1}$</td>
<td>5.13 (&gt;5.13)</td>
<td>11.1 (1/9)</td>
<td>$2.35 \times 10^{-1}$</td>
<td>$7.05 \times 10^{-1}$</td>
<td>3.51 (&gt;4)</td>
</tr>
<tr>
<td>Finished</td>
<td>980</td>
<td>0 (0/9)</td>
<td>ND</td>
<td>ND</td>
<td>&gt;5.13</td>
<td>0 (0/9)</td>
<td>ND</td>
<td>ND</td>
<td>&gt;5</td>
</tr>
</tbody>
</table>

ND: Not detected.

a Accumulative elimination efficiency expressed in log(10). In brackets: potential efficiency without the sporadic positive sample.

**Table 3 – Human adenoviruses and JC polyomavirus in DWTP 2.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol. (L)</th>
<th>% Positive</th>
<th>Mean GC/L</th>
<th>Std. Dev.</th>
<th>Reduct.</th>
<th>% Positive</th>
<th>Mean GC/L</th>
<th>Std. Dev.</th>
<th>Reduct.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw line 1</td>
<td>1261</td>
<td>66.7 (4/6)</td>
<td>$7.36 \times 10^0$</td>
<td>$1.73 \times 10^1$</td>
<td>–</td>
<td>50.0 (3/6)</td>
<td>$2.46 \times 10^0$</td>
<td>$3.76 \times 10^0$</td>
<td>–</td>
</tr>
<tr>
<td>Filtered line 1</td>
<td>1479</td>
<td>16.7 (1/6)</td>
<td>$1.20 \times 10^{-1}$</td>
<td>$2.93 \times 10^{-1}$</td>
<td>1.78</td>
<td>50.0 (3/6)</td>
<td>$2.81 \times 10^0$</td>
<td>$5.49 \times 10^0$</td>
<td>0</td>
</tr>
<tr>
<td>Nanofiltered line 1</td>
<td>1659</td>
<td>33.3 (2/6)</td>
<td>$3.61 \times 10^0$</td>
<td>$8.78 \times 10^0$</td>
<td>0.3</td>
<td>50.0 (3/6)</td>
<td>$1.01 \times 10^0$</td>
<td>$1.53 \times 10^0$</td>
<td>0.38</td>
</tr>
<tr>
<td>Raw line 2</td>
<td>1607</td>
<td>50.0 (3/6)</td>
<td>$3.45 \times 10^0$</td>
<td>$4.46 \times 10^0$</td>
<td>–</td>
<td>50.0 (3/6)</td>
<td>$1.84 \times 10^0$</td>
<td>$2.79 \times 10^0$</td>
<td>–</td>
</tr>
<tr>
<td>Filtered line 2</td>
<td>2100</td>
<td>66.7 (4/6)</td>
<td>$4.21 \times 10^0$</td>
<td>$4.64 \times 10^0$</td>
<td>0</td>
<td>66.7 (4/6)</td>
<td>$1.47 \times 10^0$</td>
<td>$1.85 \times 10^0$</td>
<td>0.09</td>
</tr>
<tr>
<td>Osmosis line 2</td>
<td>1845</td>
<td>50.0 (3/6)</td>
<td>$1.24 \times 10^0$</td>
<td>$2.85 \times 10^0$</td>
<td>0.44</td>
<td>16.7 (1/6)</td>
<td>$5.00 \times 10^{-1}$</td>
<td>$1.23 \times 10^0$</td>
<td>0.56</td>
</tr>
<tr>
<td>Finished</td>
<td>1285</td>
<td>16.7 (1/6)</td>
<td>$1.02 \times 10^0$</td>
<td>$2.49 \times 10^0$</td>
<td>0.67 (&gt;2)</td>
<td>0 (0/6)</td>
<td>ND</td>
<td>ND</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>

ND: Not detected.

a Accumulative elimination efficiency expressed in log(10). In brackets: potential efficiency without the sporadic positive sample.
4. Discussion

High viral loads are being shed in the environment and in rivers used as source water by DWTPs. In previous studies, levels of human viral contamination in sewage and wastewater treatment plants were analyzed (Albinana-Gimenez et al., 2006; Bofill-Mas et al., 2000, 2006; He and Jiang, 2005; Katayama et al., 2008; Pina et al., 1998). All these studies reported high concentrations of viruses in sewage. During the present study, in the geographical area studied here, the levels of HAdV found in urban sewage were $3.8 \times 10^4$ HAdV GC/L and $3.4 \times 10^7$ JCPyV GC/L (Bofill-Mas et al., 2006). The levels found in the effluent of a wastewater treatment plant with conventional secondary treatments were $8.08 \times 10^4$ HAdV GC/L (Bofill-Mas et al., 2006). Effluents of wastewater treatment plants are commonly discharged into rivers that are used as source water in DWTPs. This viral load could represent a risk of infections in the population if efficient drinking-water treatment is not applied and properly controlled before tap water distribution and consumption. HAdV have been described as stable in the environment and highly resistant to UV disinfection (Thompson et al., 2003). The stability of HAdV and JCPyV in sewage has been calculated by qPCR to be very high, showing $t_{50}$ of 60.9 and 63.9 and $t_{90}$ of 132.3 and 127.3 days for HAdV and JCPyV, respectively (Bofill-Mas et al., 2006).

In this study, HAdV and JCPyV were quantified by qPCR in several treatment steps of three DWTPs in North-eastern Spain. The concentration method tested in five replicates of 10 and 50 L showed mean values of 4.18% recovery for HAdV in raw water (2.02–6.91%), and 50.0% (9.51–17.57%) recovery in treated water; 4.36% (1.23–9.48%) recovery for JCPyV in river water (2.02–6.91%), and 1.21% (0.71–1.57%) recovery in treated water; 4.36% (1.23–9.48%) recovery for JCPyV in river water (2.02–6.91%), and 1.21% (0.71–1.57%) recovery in treated water; 4.36% (1.23–9.48%) recovery for JCPyV in river water (2.02–6.91%), and 1.21% (0.71–1.57%) recovery in treated water.

Table 4 – Human adenoviruses and JC polyomavirus in DWTP 3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol. (L)</th>
<th>HAdV</th>
<th>JCPyV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Positive</td>
<td>Mean GC/L</td>
</tr>
<tr>
<td>Raw</td>
<td>1</td>
<td>83.3 (10/12)</td>
<td>9.24 × 10^4</td>
</tr>
<tr>
<td>Sedimented</td>
<td>10</td>
<td>16.7 (2/12)</td>
<td>9.58 × 10^1</td>
</tr>
<tr>
<td>GAC-filtered</td>
<td>926</td>
<td>58.3 (7/12)</td>
<td>2.21 × 10^3</td>
</tr>
<tr>
<td>Finished</td>
<td>1099</td>
<td>16.7 (2/12)</td>
<td>1.07 × 10^2</td>
</tr>
</tbody>
</table>

ND: Not detected.
a Accumulative elimination efficiency expressed in log(10). In brackets: potential efficiency without the sporadic positive sample.

The ozone treatment applied seems to have little effect on the quantification of viral genome copies, although 98.8% elimination of Heterotrophic bacteria was observed (data not shown). Ozone is a very strong oxidizing agent and an effective alternative to chlorine for pathogen reduction in water, but has some disadvantages such as its short half-life, its need to be generated on-site, its corrosivity and its toxicity (AWWA, 1999). The treatment designed in plant 1, if correctly applied, guarantees 0.1–0.2 mg/L residual ozone in the ozonation chambers where water remains an average of 15–20 min, although the systems can suffer occasional shutdowns. Several studies report effective inactivation of indicator bacteria and viruses by ozone. Herbold et al. (1989) reported 93.2% reduction of E. coli in ozone treatment of 0.1 mg/L at 20 °C; 93.2% reduction of Hepatitis A virus (HAV) at 0.18 mg/L, and 98.95% reduction of Poliovirus 1 at 0.02 mg/L. The ozone concentration needed to inactivate different microorganisms is highly dependant on the microorganism itself. There may be differences in resistance even between two viruses of the same family. More recent studies by Thurston-Enriquez et al. (2005) report 76% elimination of HAV40 with 0.01 mg/L. These studies were performed in ozone-demand–free buffered water and the viruses were quantified by cell culture infection, whereas in the present study total viral DNA was quantified in samples consisting of a mix of ground water and sand–filtered water. It is possible that most of the viruses were inactivated during the ozonation treatment, but the particles and the DNA remained relatively intact. However, the tendency of these viruses to aggregate should not be forgotten: the aggregates could protect some viral particles from the action of disinfectant treatments. Even though ozone can also cause DNA damage (Ito et al., 2005), HAdV genome copies were found in wool filtration protocol used in this study. The qPCR protocol used in the present study detects mostly human adenoviruses but may also detect some strains of animal adenovirus (Bofill-Mas et al., 2006). The human origin of the viruses is confirmed by specific nested-PCR and also by the JCPyV qPCR which is specific for this human virus so the use of these two viruses gives confirmation of the human origin of the viral contamination.

Plant 1 showed >5.13 logs of HAdV removal between raw and finished water and 5.13 logs of virus removal between raw and GAC-filtered water. Only one of the GAC-filtered water samples was positive. For JCPyV, >5 logs of virus removal were observed between raw and finished water and 3.51 logs between raw and GAC-filtered water. The ozonation treatment applied seems to have little effect on the quantification of viral genome copies, although 98.8% elimination of Heterotrophic bacteria was observed (data not shown).
GAC-filtered water; the fact that these viruses were not capable of infecting cell culture supports the hypothesis that the viral genomes quantified may correspond to non-infectious viruses.

In the present case, the highest virus removal was found after sand filtration, but the most effective treatment would probably be the breakpoint chlorination performed at the intake of the plant. However, other problems arise when chlorine is added to highly contaminated water, such as production of trihalomethanes and other toxic compounds that would need to be also considered when a DWTP is designed.

Plant 2 had lower levels of virus contamination. Since it uses ground water, this source water is less likely to be fecally contaminated, although filtrations or contact with contaminated aquifers may be related to the presence of low levels of fecal contamination in the samples. The virus quantification results in this plant were quite unexpected, as the plant uses treatments such as membrane nanofiltration and reverse osmosis. Although, according to the literature, no viral particle or even DNA fragments can pass through these kinds of filters; as shown in Table 3, low virus loads after all the treatments in the plant. One possible explanation for the sporadic detection of viruses after these treatments is that the membranes or installations in the plant may have small leaks. Mi et al. (2004) studied the retention of MS2 bacteriophage through reverse-osmosis membranes. The virus rejection with intact membranes was >99.9995% but decreased to 99.95–99.9% when membranes with compromised integrity were used. There are also studies of virus removal by nanofiltration. Yokoyama et al. (2004) tested the virus removal of nanofiltration membranes (35 nm pore diameter) with human parvovirus B19, human encephalomyocarditis virus (EMC) and porcine parvovirus (PPV), all of them between 24 and 30 nm, under diverse conditions. They found that viruses aggregate in the presence of certain kinds of amino acids and thus can be removed by nanofiltration; but that, in the absence of amino acids, there was no removal. These viruses are smaller than HAdV (90 nm) and JCPyV (42–45 nm) used in the present study. The membranes used in plant 2 have a pore range of 10–30 nm, so viruses should not be able to pass through them. It is probable, as commented above for reverse osmosis, that the installation has microleaks and/or the membranes have imperfections or small fissures that could let through a small quantity of viral particles. The viral concentrations observed in plant 2 are at the limit of detection and the overall removal efficiency is very difficult to estimate accurately, since sporadic positive samples are identified throughout the treatment.

The virus concentrations in plant 3 were similar to those in plant 1. The high levels of viruses found in raw river water can be explained by there being a wastewater treatment plant effluent a few meters upstream and on the same bank. The HAdV removal efficiency calculated was higher than 5 logs, but two of the finished water samples were positive at low concentrations. Thus, in the worst case, removal efficiency decreases to 4.94 logs. No finished water samples were positive for JCPyV; the removal efficiency calculated is >5 logs. The most effective treatments in this plant were again the two chlorinations, one at the intake of the plant and the other after GAC filtration.

Three finished water samples were positive for HAdV in plants 2 and 3, but the viruses found did not produce infection in the cell culture assay. The performance of a treatment unit can affect the efficiency of downstream treatment units. The presence of suspended solids increases the resistance of most microbes to disinfection (LeChevallier and Au, 2004). Therefore, failure in the removal efficiency of turbidity or particles can decrease the inactivation efficiency of disinfection processes.

Three water treatment plants from the Paris area were also sampled and analyzed successfully with the method developed in this project, demonstrating that the method can easily be applied in other laboratories (data not shown).

Viruses were consistently found in samples from all the plants with absence or very low concentration of bacterial indicators, supporting previous studies on the lack of correlation between bacterial and viral indicators (Albinana-Gimenez et al., 2006; Formiga-Cruz et al., 2003; Gerba et al., 1979; Lipp et al., 2001; Tree et al., 2003). Concentrations of E. coli MPN and Enterococci PFU in 1 L are comparable to the concentrations of adenovirus GC, being adenoviruses GC in the same logarithm or higher. These data strongly support the applicability of human adenoviruses quantified by qPCR as a molecular index of faecal contamination in water, considering that the estimated quantities of viruses in the raw and treated water samples studied are equivalent or higher than the bacterial standards analyzed by the currently used standard methods.

Although a significant reduction of viral GC quantified by qPCR was observed after chlorination, human Adenovirus 2 was found in post-chlorinated water from plants 2 and 3. However, no infective HAdV particles were found in these samples or in GAC-filtered water, suggesting that the viruses could be inactivated during the treatment.

In previous studies of several DWTPs in Egypt, Ali et al. (2004) studied the presence of enteroviruses by plaque infectivity assay in BGM (Buffalo Green Monkey kidney cells), HAV, HEV and of Norovirus by RT-PCR. Viruses were detected at every step of the treatment, but none was detected in post-chlorinated water. Removal efficiencies of >1 log and >2 logs were observed for the infecting enteroviruses. Abbaspazadegan et al. (2008) performed studies in a pilot DWTP, including sedimentation and sand filtration evaluating bacteriophages as viral surrogates to study adenovirus and Feline Calicivirus removal. The removal efficiencies obtained for HAdV were 1.4 logs after sand filtration. In the present study higher removal efficiencies after sand filtration were observed, but included chlorination. Another important difference is that the efficiencies found by Ali et al. and Abbaspazadegan et al. were calculated using infectious viral particles in cell culture.

qPCR techniques are highly sensitive and produce quick quantitative data on the presence of viral genomes. Negative qPCR results are a strong indicator of efficient virus removal in DWTPs, but positive results in finished water where disinfectants have been used would require their infection capacity to be tested before the risk associated could be realistically evaluated. Simple methods based on adsorption-elution of viruses in glass wool were successfully used to survey the virological quality of the water at three DWTPs in
North-eastern Spain. Quantification of JCPyV and HAdV using qPCR proved useful for evaluating virus removal efficiency in DWTPs, identifying HACCP (Hazard Analysis Critical Control Points) and as a molecular index of the virological quality of source water, including identification of microbial fecal contamination of human origin.

5. Conclusions

- Concentrations of E. coli MPN and Enterococci PFU in 1 L source water are comparable to the concentrations of adenovirus GC. This data strongly supports the applicability of human adenoviruses quantified by qPCR as a molecular index of viral contamination in water.
- Even considering the high removal efficiency of most of the DWTPs, and the fact that qPCR values are highly reduced after final chlorination treatments, sporadic qPCR positive samples have been detected in finished water in some plants. The negative results in the infectivity assays performed suggest that these viruses may not be infectious.
- Quantification of JCPyV and HAdV using qPCR has shown to be useful for evaluating virus removal efficiency in water treatment plants, identification of HACCP and as an index of the virological quality of water including identification of the human origin of the faecal contamination.

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References


