An epidemiological study of enteric viruses in sewage with molecular characterization by RT-PCR and sequence analysis

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ABSTRACT

The aim of this study was to assess the presence and seasonal frequency of various enteric viruses in wastewater treatment. The detection of astrovirus, norovirus, enterovirus, hepatitis A virus (HAV) and rotavirus was carried out by molecular analyses in concentrated water samples collected over 18 months at the entrance and exit of an activated sludge sewage treatment plant. The reverse transcriptase-polymerase chain reaction (RT-PCR) results were confirmed by sequencing, and comparative phylogenetic analysis was performed on the isolated strains. Genomes of human astrovirus and human rotavirus were identified in 26/29 and 11/29 samples of raw sewage, respectively, and in 12/29 and 13/29 treated effluent samples, respectively. Some rotavirus sequences detected in environmental samples were very close to those of clinical strains. Noroviruses, enteroviruses and HAV were not detected during the study period. This could be related to the small sample volume, to the sensitivity of the detection methods or to local epidemiological situations. Frequent detection of viral RNA, whether infectious or not, in the exit effluent of sewage treatment indicates wide dispersion of enteric viruses in the environment. Consequently, viral contamination resulting from the use of these treated waters is a risk that needs to be addressed.

Key words | detection, genotyping, PCR, viruses, wastewater

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INTRODUCTION

A large variety of viruses are excreted in human faeces and hence are potential water pollutants. Astroviruses, noroviruses, and rotaviruses can cause gastroenteritis, enteroviruses are responsible for a wide range of infections, and hepatitis A virus (HAV) is the main cause of acute hepatitis worldwide. All these viruses have been implicated in waterborne outbreaks (Metcalf *et al.* 1995). Investigation of the presence of these enteric viruses in raw sewage and in effluents released by wastewater treatment plants is important. Except for some enteroviruses, most viruses of public health concern are not readily cultivable in cell lines and have to be detected by molecular techniques. Reverse transcriptase-polymerase chain reaction (RT-PCR) is a useful tool in the routine monitoring of viruses in water samples since it allows simultaneous detection of a number of viruses and is able to distinguish between virus genotypes. However, relevant indicators of viral pollution are still lacking. Routine monitoring of the circulation of human enteric viruses in wastewater samples could provide useful insights into the extent of viral pollution. Several studies have been performed on the viral contamination of wastewater but very few have systematically investigated a large panel of enteric viruses in raw sewage and treated effluents for an extended period. Some studies were conducted over a few weeks or months only (Schvoerer *et al.* 2000; Morace *et al.* 2002) and yet viral circulation is seasonal. Other studies lasted at least one year but involved only one or two virus families (Lodder *et al.* 1999; Baggi & Peduzzi 2000; Pinto *et al.* 2001; Taylor *et al.* 2001; Nadan *et al.* 2003; Komninou *et al.* 2004; van Zyl *et al.* 2004). It would be interesting therefore to gather data on the

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simultaneous circulation of the main medically important enteric viruses. In addition, data on nucleotide sequences can provide accurate information on circulating viruses.

The aim of our study was to determine during an 18 month period the incidence of viral contamination in the raw sewage and treated effluent of a large sewage treatment plant using molecular techniques and to characterize by partial sequencing the viral strains detected. For a complete viral study of the sewage samples, the presence of astroviruses, noroviruses, enteroviruses, HAV and rotaviruses was determined.

METHODS

Cell culture infection

Poliovirus was used to assess the recovery rate of the aluminium sulphate concentration method. The titre of poliovirus was determined by plaque assay in BGM cells as previously described (Arraj *et al.* 2005).

Sewage sample collection and preparation (concentration)

The study was performed on raw sewage and final effluent samples from an activated sludge wastewater treatment plant receiving the effluent of an urban area comprising 250,000 inhabitants. Aluminium sulphate was added to 1 litre sewage samples at a final concentration of 0.002% and pH was adjusted to 5.4. The solution was homogenized by magnetic stirring for 5 min and stored at room temperature for 2 hours to allow viral adsorption on the aluminium sulphate and sedimentation. After discarding the upper phase, the solution was centrifuged for 30 min at 6,000 rpm, and the pellet was suspended in 1 ml of an elution buffer (pH 4.7) containing 1M citric acid. The concentrate was then vortexed vigorously for 5 min with one volume of 3% beef extract/Freon (1/1) solution. The suspension was allowed to stand for 5 min at room temperature, sonicated for 2 min and centrifuged at 6,000 × g for 15 min. The upper aqueous phase was recovered and used for analysis or kept frozen at −80°C until use.

RNA extraction

Total RNA was extracted from 1 ml aliquots of the concentrated sewage samples with NucleoSpin RNA spin columns (Macherey Nagel, Hoerdt, France).

Primers

Published sets of oligonucleotide primers were used for rotaviruses (Gouvea *et al.* 1990), human astroviruses (Belliot *et al.* 1997), enteroviruses (Zoll *et al.* 1992) and HAV (Cromeans *et al.* 1987). For human noroviruses, three set of primers located in the conserved RNA polymerase region were used: the primers designed by Ando *et al.* (1995) yield a 123 bp product, those designed by Green *et al.* (1995) yield a 113 bp product, and those designed by Jiang *et al.* (1999) yield a 319 bp product.

Reverse transcriptase-polymerase chain reaction

Reverse transcription and PCR were performed in one single step using the QIAGEN OneStep RT-PCR Kit (QIAGEN, Hilden, Germany). All the RNA extracts were analysed without dilution and after a 10^{-1} dilution in sterile distilled water to overcome inhibitory effects of the extracts on RT-PCR reactions. A volume of 5 μl of the RNA extract or its 10^{-1} dilution was added to a 45 μl mix. Reverse transcription was performed for 30 min at 50°C followed by a Taq polymerase activation step for 15 min at 95°C. The PCR conditions on the thermocycler (Perkin Elmer, 2400) were as follows: 40 amplification cycles with denaturation at 94°C for 30 s, annealing at 40°C for 60 s for one set of norovirus primers (Green *et al.* 1995), annealing at 50°C for 30 s for HastV, 60 s for enterovirus, and 80 s for two sets of norovirus primer (Ando *et al.* 1995; Jiang *et al.* 1999), annealing at 55°C for 60 s for HAV and rotavirus, and elongation for 1 min at 72°C for all viruses. The final elongation step lasted 10 min for all viruses. Analysis of RT-PCR products was by 2% agarose gel electrophoresis stained with ethidium bromide and visualized by short-wave UV trans-illumination.

Sensitivity of the viral detection in the sewage samples by the one-step RT-PCR was tested in both inlet and outlet wastewater concentrate samples. Concentrates obtained

by the method described above were seeded with serial dilutions of a viral suspension with known viral titres. For norovirus, a suspension was made by mixing 100 μl of the stool obtained from a child with diarrhoea due to norovirus (genogroup II) in 900 μl sterile distilled water. The suspension was treated with 1 volume of Freon® and was clarified by centrifugation at 7,000 × g for 5 min. Serial dilutions of this clarified suspension was used to seed the wastewater concentrates. RNA was then extracted and amplified with one step RT-PCR. Sterile distilled water was used as a control.

Sequencing and analysis of PCR products

RT-PCR positive samples were sequenced using the same primer pairs with dichlororhodamine-labelled dideoxynucleotides (Perkin Elmer). Multiple sequence alignments of detected sequences and reference sequences from GenBank were created by use of CLUSTALW. Five additional sequences corresponding to clinical stool specimens positive for rotavirus that had been sampled from children hospitalized in the university hospital of the city served by the wastewater treatment plant were included in the rotavirus multiple sequence alignments. Phylogenetic trees were constructed from the nucleic acid sequence alignments by using the UPGMA method with MEGA version 2.1 software.

RESULTS AND DISCUSSION

In this study, RT-PCR assays followed by sequencing of the amplified products were applied for the routine detection of HAV, noroviruses, astroviruses, rotaviruses and enteroviruses over an 18 month period. Adenoviruses which are also important enteric pathogens potentially found in polluted water were not included in this study. Our results show that, during this period, astroviruses and rotaviruses were commonly found in wastewater while the other viruses were not detected.

The recovery rate of the aluminium sulphate flocculation step was determined using cell culture with poliovirus as a model virus. Four experiments were performed on four different 1 litre wastewater samples. These samples were seeded with a poliovirus amount ranging from 3.6×10^7 to 1×10^8 plaque forming units (PFU). The total amount of poliovirus recovered under a volume of 5 ml ranged from 1.5×10^7 to 3×10^7 PFU. The mean recovery rate calculated on these four experiments was $51 \pm 21\%$. We did not determine the recovery rate using low viral titres which may be more likely found in the environment particularly in treated wastewater. However, we found a recovery rate close to that obtained by others with an aluminium precipitation technique (Gerba *et al.* 1978; Hucko 1989). Gerba *et al.* (1978), using filtration followed by aluminium sulphate precipitation, were able to concentrate poliovirus from large volumes of sewage with an average efficiency of 50%. The aluminium sulphate procedure used by Hucko (1989) to concentrate poliovirus from experimentally contaminated cow milk yielded 14 to 58% of the inoculated amount with an average of 27%. Concentration of viruses from wastewater using aluminium sulphate is easy, inexpensive and rapid.

The detection thresholds of the RNA extraction and RT-PCR steps were estimated for astrovirus, HAV, rotavirus and poliovirus in raw wastewater and treated water concentrates. Viral stocks with known titres were serially diluted (tenfold dilutions) in raw wastewater concentrates, treated water concentrates, and sterile distilled water as a control. All the dilutions were subjected to specific RT-PCR (Table 1). The experiments were made once for each virus in raw wastewater and treated water concentrates and twice in sterile distilled water.

For HAV, poliovirus and rotavirus, RT-PCR detection was more sensitive in sterile distilled water than in water concentrates. In water concentrates, detection tends to be more sensitive in treated water than in raw wastewater. The sensitivity of our protocol for the detection of rotavirus in raw sewage was similar to that reported in a previous study by Gajardo *et al.* (1995) where sensitivity was 2×10^4 most probable number of cytopathogenic units (MPNCU) ml^{-1} with RT-PCR. The sensitivity reported in a recent study for the detection of HAV in sewage by RT-PCR ranged between 10^1 and 10^2 50% Tissue Culture Infective Dose ($TCID_{50}$) ml^{-1} (Morace *et al.* 2002), while the sensitivity for HAV in our study was 10^3 PFU ml^{-1}. Detection of astrovirus was as sensitive in raw wastewater concentrates as in treated water concentrates and sterile distilled water.

Table 1 | Detection thresholds of the RT-PCR in water concentrates and sterile distilled water seeded with astrovirus, HAV, poliovirus and rotavirus. The values correspond to the lowest dilutions resulting in positivity of the RT-PCR

Virus (viral stock titres)	Sterile distilled water	Raw sewage concentrates	Treated effluent concentrates
Astrovirus (1×10^7 PFU ml^{-1})	10^1 PFU ml^{-1}	10^1 PFU ml^{-1}	10^1 PFU ml^{-1}
HAV (1.8×10^7 PFU ml^{-1})	10^2 PFU ml^{-1}	10^3 PFU ml^{-1}	10^3 PFU ml^{-1}
Poliovirus (3×10^7 PFU ml^{-1})	10^1 PFU ml^{-1}	10^4 PFU ml^{-1}	10^1 PFU ml^{-1}
Rotavirus (2×10^7 FFU ml^{-1})	10^1 FFU ml^{-1}	10^4 FFU ml^{-1}	10^2 FFU ml^{-1}

FFU: focus-forming unit.

For norovirus, the stool suspension was serially diluted with fivefold dilutions. RT-PCR detection was obtained up to a 1/500,000 dilution in sterile distilled water and in treated water concentrates and up to a 1/50,000 dilution in raw wastewater concentrates.

A total of 58 water samples from the activated sludge treatment plant were analysed from June 2002 to December 2003. These samples were drawn periodically from the plant about every two weeks and they corresponded to 29 raw entrance samples and 29 final effluent samples. Entrance and final effluent samples were taken on the same day. The RT-PCR results for the detection of viruses in these water samples are summarised in Table 2.

Astroviruses were detected by RT-PCR in 26 of 29 raw effluent concentrates. Of note, only five of these concentrates were positive when undiluted RNA was used for RT-PCR whereas 26 of 29 were positive with 10^{-1} diluted RNA. Astroviruses were detected in 12 concentrates of final effluents and the use of 10^{-1} diluted RNA for RT-PCR did not yield additional positive samples. Rotaviruses were demonstrated by RT-PCR in 11 concentrates of raw effluents. As observed for astrovirus, only one sample was positive with undiluted RNA and ten additional positive samples were detected using 10^{-1} diluted RNA. Thirteen concentrates of final effluents were positive for rotaviruses and the use of 10^{-1} diluted RNA for RT-PCR did not yield additional positive samples.

One of the main disadvantages of RT-PCR is the sensitivity of the enzymes to the presence of inhibitors that can be concentrated with the virus during the concentration process. To overcome inhibitory effects of the water concentrates on RT-PCR reactions, the purification of the nucleic acids with silica columns is a recommended method to eliminate inhibitory compounds (Hale *et al.* 1996). The RNA extraction and purification kit used in our study is based on this type of column and has been shown to efficiently remove inhibitors (Baggi & Peduzzi 2000). In addition, we analysed all the RNA extracts both undiluted and after a 10^{-1} dilution in sterile distilled water to dilute potential inhibitors.

No noroviruses, enteroviruses or HAV were detected in the sewage samples irrespective of whether undiluted RNA or 10^{-1} diluted RNA was used for RT-PCR. Noroviruses and enteroviruses have a potential active circulation in the environment (Lodder *et al.* 1999; Schvoerer *et al.* 2000). Because of the known genetic variability of the noroviruses, all the wastewater samples have been analysed with three different sets of previously published norovirus primers (Ando *et al.* 1995; Green *et al.* 1995; Jiang *et al.* 1999). Therefore, it is unlikely that the absence of detection of noroviruses in our study was due to primers unsuitable for the circulating strains. It may be that the aluminium sulphate concentration is not suitable for norovirus, that our method is not sensitive enough for noroviruses or that the circulation of noroviruses was low during the study period. Concerning enteroviruses, we can assume that the aluminium sulphate concentration is suitable since its recovery rate was evaluated using poliovirus. In addition, the primers we used have been shown to be pan-enterovirus primers (Zoll *et al.* 1992). Of note, the clinical virology laboratory of our main city hospital which monitors infections due to enteroviruses (Bailly *et al.* 2002) reported that enterovirus isolations from clinical samples, especially Coxsackie virus, were remarkably rare during the study period. This suggests that enterovirus circulation was not active at the time.

Astroviruses and rotaviruses were present throughout the year. However, there were more final effluent samples positive for astroviruses during the winter than the summer. For rotaviruses, a seasonal peak was obvious in winter.

Table 2 | RT-PCR results for the detection of astroviruses and rotaviruses in water samples

	Astrovirus		Rotavirus	
Sampling date (month/day/year)	**Raw sewage**	**Treated effluent**	**Raw sewage**	**Treated effluent**
06/24/02	+	+	−	−
07/15/02	+	−	+	−
07/29/02	+	−	−	−
08/19/02	+	−	−	−
08/26/02	−	−	+	−
09/16/02	+	+	−	−
09/30/02	+	−	−	−
10/21/02	+	−	−	−
10/28/02	+	−	−	−
11/25/02	+	−	−	−
12/16/02	+	−	−	−
12/30/02	+	+	−	+
01/13/03	+	+	−	+
01/27/03	+	+	+	+
02/10/03	+	+	+	+
02/24/03	+	+	+	+
03/10/03	+	−	+	+
03/31/03	+	−	+	+
04/28/03	+	−	+	+
05/05/03	+	+	+	+
05/19/03	+	−	−	−
06/16/03	+	+	+	−
07/07/03	+	−	−	+
07/28/03	+	−	−	+
08/25/03	+	−	+	−
09/29/03	−	−	−	−
10/27/03	+	+	−	+
11/10/03	+	+	−	−
12/01/03	−	+	−	+

+, detected; −, not detected.

All the astrovirus and rotavirus RT-PCR products detected in final effluents were sequenced. In raw sewage, 9 of 26 and 4 of 11 RT-PCR products were sequenced for astrovirus and rotavirus, respectively. A total of 21 of 38 astrovirus RT-PCR products and 17 of 24 rotavirus RT-PCR products were sequenced. The 21 astrovirus sequences were compared with published human and animal astrovirus sequences (Figure 1).

No animal astrovirus sequences were found in the samples. The astrovirus sequence analysis showed the diversity of the astrovirus strains detected in wastewater in this study. Of note, most of the strains we detected seem to have closer genetic relationships with astrovirus type 3 than astrovirus type 1. We confirmed that, as observed recently by Le Cann *et al.* (2004), the frequency of astrovirus detection was high both in raw sewage and in treated effluents and that astroviruses were detected throughout the year. Our study provides further information about the circulating genotypes. With quantitative

RT-PCR, Le Cann *et al.* (2004) estimated an average concentration in treated effluents of 1.01×10^5 astrovirus genomes per litre corresponding to 670 infectious viruses. In our study, this concentration should be at least 2×10^4 infectious astroviruses per litre since the sensitivity threshold of our non-quantitative RT-PCR was estimated at 10^1 virus per ml of concentrate and the mean recovery rate of the concentration method was 50%. This high prevalence of astroviruses in sewage was also suggested by the findings of Chapron *et al.* (2000), who, using RT-PCR, detected astroviruses in 15 of 16 sewage sludges. Nevertheless, in the study by Chapron *et al.* and in a separate study by Pinto *et al.* (2001), sewage samples had to be incubated on Caco-2 cells prior to RT-PCR amplification.

The 17 rotavirus sequences were compared with published human and animal rotavirus sequences and with five rotavirus clinical strains isolated from children hospitalized in the main university hospital of the city which was served by the wastewater treatment plant (Figure 2).

All samples were classified as type G1 rotavirus. The phylogenetic analysis of different animal rotavirus VP7 sequences resulted in a clearly distinct cluster that did not match any of our samples, evidence of the ability of our protocol to study epidemiological relationships between water contamination and strains involved in human infections. Our results indicate that the rotaviruses detected in environmental and clinical samples are closely related. Given the RT-PCR sensitivity threshold that we determined for rotavirus in

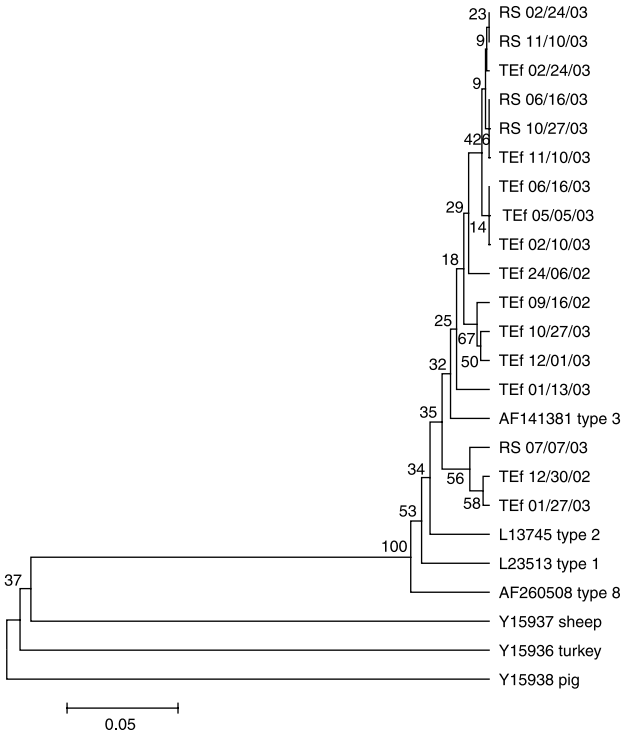

Figure 1 | Genetic clustering of the astrovirus sequences in the ORF1 region (289 bp) identified from the wastewater treatment plant and from human and animal prototype strains. Raw sewage (RS) and treated effluent (TEf) are indicated with the sampling date. GenBank accession numbers of the prototype strains were as follows: human astrovirus standard strains L23513, L13745, AF141381, AF260508; animal astrovirus standard strains Y15936 (avian astrovirus), Y15937 (ovine astrovirus), Y15938 (porcine astrovirus). The MEGA version 2.1 program was used to generate the dendrogram with the UPGMA method. Bootstrap values of the internal nodes are indicated. Branch length is drawn to the indicated scale (number of nucleotide substitutions per site).

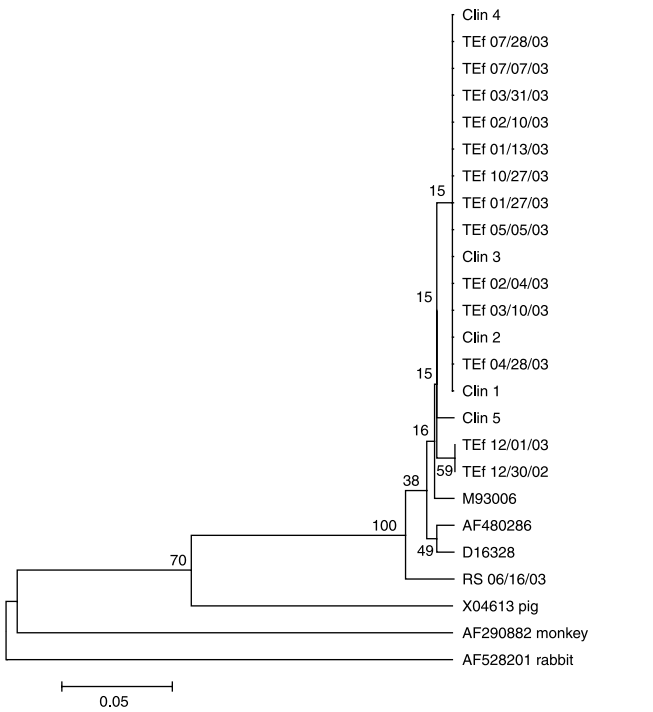

Figure 2 | Genetic clustering of the rotavirus sequences in the VP7 region (392 bp) identified from the wastewater treatment plant and from human and animal prototype strains. Raw sewage (RS) and treated effluent (TEf) are indicated with the sampling date. GenBank accession numbers of the prototype strains were as follows: human group A rotavirus standard strains AB081799, M93006, D16325, AF480286, D16328; animal standard strains AF528201 (rabbit rotavirus), XO4613 (porcine rotavirus), AF290882 (simian rotavirus SA11). Five sequences from rotavirus clinical strains were also included (Clin − 1 to − 5). The MEGA version 2.1 program was used to generate the dendrogram with the UPGMA method. Bootstrap values of the internal nodes are indicated. Branch length is drawn to the indicated scale (number of nucleotide substitutions per site).

treated wastewater concentrates (10^2 virus ml^{-1}) and the mean recovery rate of the concentration method (50%), the viral load in the analysed samples should be equal to or greater than 2×10^5 virus l^{-1}. This suggests that faecally polluted water could be a reservoir for human infection.

CONCLUSION

We have assessed the presence and seasonal frequency of astrovirus, norovirus, enterovirus, HAV and rotavirus in wastewater treatment. In 1-litre samples collected regularly over 18 months at the entrance and exit of the activated sludge sewage treatment plant of Clermont-Ferrand (France) we have detected by RT-PCR and confirmed by sequencing numerous astroviruses and rotaviruses strains. Comparative phylogenetic analysis performed on the isolated strains showed that some clinical and environmental rotavirus strains were identical. We believe that the type of astrovirus and rotavirus that we found is a reflection of their clinical epidemiology. Frequent detection of astrovirus and rotavirus RNA in the exit effluent of sewage treatment indicates widespread dispersion of enteric viruses in the environment. It is not known whether these viral RNAs correspond to infectious viruses, but the problem of the risk of viral contamination resulting from use of the treated waters must be addressed.

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