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Research Paper

Detection of enteric viruses in activated sludge by feasible concentration methods

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Abstract

Human enteric viruses are responsible to cause several diseases, including gastroenteritis and hepatitis, and can be present in high amounts in sewage sludge. This study compared virus recovery efficiency of two feasible concentration methods used for detecting human adenovirus (HAdV), rotavirus species A (RV-A), norovirus genogroup II (NoV GII) and hepatitis A virus (HAV) in sewage sludge from an activated sludge process. Twelve sewage sludge samples were collected bi-monthly from January to July, 2011. Ultracentrifugation was compared with a simplified protocol based on beef extract elution for recovering enteric viruses. Viruses were quantified by quantitative real-time PCR assays and virus recovery efficiency and limits of detection were determined. Methods showed mean recovery rates lower than 7.5%, presenting critical limits of detection (higher than 10^2 - 10^3 genome copies - GC L^{-1} for all viruses analyzed). Nevertheless, HAdV were detected in 90% of the analyzed sewage sludge samples (range: 1.8×10^4 to 1.1×10^5 GC L^{-1}), followed by RV-A and NoV (both in 50%) and HAV (8%). Results suggesting that activated sludge is contaminated with high viral loads and HAdV are widely disseminated in these samples. The low virus recovery rates achieved, especially for HAV, indicate that other feasible concentration methods could be developed to improve virus recovery efficiency in these environmental matrices.

Key words: enteric viruses, sewage sludge, activated sludge process, virus concentration methods.

Introduction

Raw sewage sludge is an ideal system for assessing viral diversity. Recently, 234 known viruses were identified in these environmental matrices, including humans, plant, animals, algal viruses, as well as bacteriophages (Cantalupo *et al.*, 2011).

Different species of pathogenic human enteric viruses may be present in raw sludge (Sano *et al.*, 2003; Schlindwein *et al.*, 2010; Cantalupo *et al.*, 2011; Jebri *et al.*, 2012), including members of the *Picornaviridae* family (hepatitis A virus - HAV), *Caliciviridae* (human noroviruses - NoV GI, GII and GIV) and *Reoviridae* (rotavirus species A - RV-A) (ICTV, 2011). HAV and NoV are the primary human viral pathogens of concern responsible to cause hepatitis and gastroenteritis in children and adults worldwide, but RV-A seems to be widely disseminated in wastewaters in Brazil (Fumian *et al.*, 2011; Prado *et al.*, 2011) and are the main responsible to cause acute gastroenteritis in children lower than 5 years old in developing countries (Linhares *et al.*, 2011).

Human adenoviruses (HAdV) species A-G (family: *Adenoviridae*, genus: *Mastadenovirus*) (ICTV, 2011) are too recognized as important pathogens related to several diseases, including gastroenteritis, respiratory diseases, conjunctivitis, among others (Okoh *et al.*, 2010). These viruses are largely disseminated in several environmental matrices (Bofill-Mas *et al.*, 2006; Barrella *et al.*, 2009; Okoh *et al.*, 2010; Schlindwein *et al.*, 2010; Prado *et al.*, 2011) and have been considered as suitable index to evaluate water microbiological contamination (Bofill-Mas *et al.*, 2006; Okoh *et al.*, 2010; Schlindwein *et al.*, 2010).

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However, many issues remain to be elucidated, such as attachment properties of viruses onto sludge flocs and variable removal rates for different viruses in WWTPs (Silva *et al.*, 2007; Wen *et al.*, 2009; Okoh *et al.*, 2010; Simmons and Xagoraraki, 2011; Jebri *et al.*, 2012). Moreover, there is a scarcity of information about enteric virus detection in sewage sludge, mainly for some viral groups, including rotavirus (RV) and norovirus (NoV) (Sidhu and Toze, 2009). The knowledge about viral contamination levels in sewage sludge is important to evaluate virus attachment properties onto sludge flocs and the capacity of the subsequent stabilization or disinfection processes in virus removal (Silva *et al.*, 2007; Sidhu and Toze, 2009; Wen *et al.*, 2009).

Molecular biology methods, notably PCR and their subsequently development by quantitative real-time PCR (qPCR) presents several advantages compared with other methods and can be incorporated into regular monitoring program to assess virus levels in wastewater effluents, mainly in developing countries (Girones *et al.*, 2010; Okoh *et al.*, 2010). However, natural inhibitors in environmental samples affect PCR performance and false negative results can be obtained, mainly for detecting enteric viruses in sewage sludge (Sano *et al.*, 2003; Guzmán *et al.*, 2007; Rock *et al.*, 2010; Jebri *et al.*, 2012).

Simplified concentration methods have been proposed for recovering viruses from sewage sludge (Sano *et al.*, 2003; Guzmán *et al.*, 2007), but detection sensitivity for different viral groups has not yet been established. Therefore, the aim of this study was to evaluate efficiency of two virus concentration methods used for detecting HAdV, RV-A, norovirus genogroup II (NoV GII) and HAV in sewage sludge from an urban wastewater treatment plant (WWTP) that uses activated sludge process, located in Rio de Janeiro city, Brazil.

For this purpose an ultracentrifugation method commonly used for concentrating viruses from wastewater samples (Heim *et al.*, 2003; Bofill-Mas *et al.*, 2006; Calgua *et al.*, 2012; Jebri *et al.*, 2012) was compared with a second virus concentration method based on beef extract elution (Guzmán *et al.*, 2007), which is also recommended by a Resolution of the National Council of the Environment - CONAMA (Conama, 375/2006). This Resolution provides guidelines for monitoring enteric viruses in sewage sludge.

Quantitative PCR (qPCR) was used for viruses detection. The primers and probes used in TaqMan qPCR assays target more conservative regions of the virus genome and have been considered suitable for detecting enteric viruses in environmental and clinical samples (Heim *et al.*, 2003; Villar *et al.*, 2006; Fumian *et al.*, 2010, 2011; Prado *et al.*, 2011, 2012). Virus recovery rates and detection limits of the assays were also determined to avoid false negative results.

Materials and Methods

Sampling collection at WWTP

The urban WWTP analyzed in this study is located in the metropolitan area of Rio de Janeiro city, Brazil. The plant operates by a secondary treatment (conventional activated sludge process) with a mean inflow of 1600 L s^{-1}. The treatment process begins with a preliminary treatment and primary sedimentation at a hydraulic retention time (HRT) of 2 h. There are 4 aeration tanks placed in parallel that have the capacity to treat 625 L s^{-1} of effluent at an HRT of 4 h. Secondary sedimentation is performed in secondary settling tanks at an HRT of 4 h. The total HRT for the system is approximately of 12 h. Twelve primary sewage sludge samples were collected bi-monthly (15 days intervals) from January to July, 2011. Sewage sludge samples (100 mL) were collected in sterile plastic bottles, kept on ice and transported to the laboratory for immediate analysis.

Virus concentration methods

Ultracentrifugation-based method initially described by Pina *et al.* (1998) for detecting enteric viruses from wastewaters was used for concentrate enteric viruses in sewage sludge. Minor modifications were performed. Briefly, 25 mL of sewage sludge was suspended in 10 mL of 25 mM glycine buffer (pH 9.5). After incubation in ice for 30 min, the solution was neutralized by the addition of 10 mL of 2 x phosphate-buffered saline (PBS, pH 7.2). The mixture was centrifuged (12,000 *g* for 15 min at 4 °C) and the supernatant was submitted to an ultracentrifugation (Beckman ultracentrifuge) at 100,000 x g for 1 h at 4 °C. Pellet was resuspended in 1.0 mL of 1 x PBS pH 7.2.

A second method based on elution with beef extract was performed as described by Guzmán *et al.* (2007). Briefly, a beef extract solution (10%, pH 7.2, 10:1 (v/v) or (w/v) (LP029B, Oxoid Ltd. Basingstoke, Hants., England), was added in 25 mL of sewage sludge sample. The sample was stirred by magnetic stirring for 20 min at room temperature. After, the sample was centrifuged at 4,000 *g* for 30 min at 4 °C (Thermo Scientific, Sorvall ST40 Centrifuge). The supernatant was recovered and filtered through a low protein binding 0.22 μm pore size membrane filters (Millipore) for decontamination.

Viruses and sludge spike experiments

RVA G1P[8] (GenBank accession no. GU831596), NoV GII/4 strain (GenBank accession no. DQ997040) isolated from acute gastroenteritis outbreaks in Brazil were used in spiked experiments. HAdV serotype 5 propagated in cell culture (Hep-2) and HAV strain (HAF-203) in Rhesus kidney cell cultures (FRhK-4) were used to perform all experiments (Villar *et al.*, 2006).

For spiked experiments, sludge samples were autoclaved at 121 °C for 30 min for decontamination. Viruses were spiked in 25 mL of the sludge sample and ad-

sorbed onto sludge flocs by adjusting the pH to 3.5 ± 0.1 with HCl (1 M), as described by Sano *et al.* (2003) and stirring with a magnetic stirrer for 30 min. The sludge samples were centrifuged (10,000 *g*, for 15 min at 4 °C) and pellet generated in each experiment were analyzed through concentration methods and Real-Time PCR assay for determining virus recovery yields. Procedures were performed in triplicate and repeated at different sampling dates. In all experiments, negative controls (decontaminated sewage sludge samples without viruses seeded) were also included.

Detection limits of methods were tested diluting initial spiked viral titers to the samples. Viral titers varying from 10^4 to 10^1 viral particles were inoculated in all experiments performed in triplicate.

Viral genomic extraction and reverse transcription (RT) reaction

Nucleic acids were extracted from 140 μL of the eluate to obtain a final volume of 60 μL, using the QIAamp Viral RNA (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

cDNA synthesis was carried out by RT using a random primer (PdN_6; $50A_{260}$ units; Amersham Biosciences, Chalfont St Giles, Buckinghamshire, UK) for RV, NoV, HAV 2 μL of dimethyl sulfoxide (Sigma, St. Louis, MO) and 10 μL of RNA were mixed briefly, heated at 97 °C for 7 min, and chilled in ice for 4 min. The components of the mixture and their final concentrations for a 50-μL RT reaction were carried out as follows: 2.5 mM each deoxynucleoside triphosphate (GIBCO BRL, Life Technologies, Inc., Grand Island, NY), 1.5 mM $MgCl_2$, 200U of Superscript III reverse transcriptase (Invitrogen), and 1 μL of PdN_6. The RT reaction mixture was incubated in a thermal cycler (PTC-100 Programmable Thermal Controller; MJ Research, Inc., Watertown, MA) at 25 °C for 5 min, 50 °C for 60 min and 70 °C for 20 min.

Virus quantification by Real-Time PCR

Real-time PCR protocols including sequence of primers and probes, region of amplification of the genome and references can be found in Table 1. For all viruses, a standard curve (SC; 10^8 to 10^1 copies per reaction) was generated using a tenfold serial dilution of pCR2.1 vectors (Invitrogen, USA) containing the target region. The qPCR reaction was performed in the final volume of 25 μL by using 12.5 μL of the Universal PCR Master Mix (Applied Biosystems, CA, USA) and 5 μL of the cDNA on the following incubation conditions: 50 °C for 2 min to activate UNG, initial denaturation at 95 °C for 10 min, and then 40-45 cycles of 95 °C for 15 s and 50-60 °C for 1 min. Amplification data were collected and analyzed using Applied Biosystems 7500 Software® v2.0 (Applied Biosystems, Foster City, CA). All reactions were performed in duplicate. Positivity was considered when samples signals crossed the threshold line, presenting a characteristic sigmoid curve. The number of viral particles was determined by adjusting the values according to the volumes used for each step of the procedure (extraction, cDNA syntesis and qPCR reaction). The values (genome copies - GC) found in each methodology were reported in ml of concentrated samples.

Results

The results of virus recovery rates obtained by ultracentrifugation and beef extract methods used to concentrate sewage sludge samples are shown on Table 2. According to results it was possible to observe that both methods present virus recovery efficiency lower than 7.5%. HAdV was better recovered by the two concentration methods and initial spiked viral titers of NoV and HAV were not detected

Table 1 - Viruses analyzed, PCRs assays, primers' sequences, genome region and references.

Viruses and qPCR assay	Primers and probe	Sequences 5' to 3'	Genome region	References
RV-A RT-qPCR	NSP3 F	ACCATCTWCACRTRACCCTCTATGAG [a]	NSP3	Zeng *et al*., 2008
	NSP3 R	GGTCACATAACGCCCCTATAGC		
	NSP3 probe	VIC-AGTTAAAAGCTAACACTGTCAAA		
HAdV qPCR	AQ1 F	GCCACGGTGGGGTTTCTAAACTT	Hexon	Heim *et al*., 2003
	AQ2 R	GCCCCAGTGGTCTTACATGCACATC		
	Probe	FAM-TGCACCAGACCCGGGCTCAGGTACTCCGA-TAMRA		
NoV RT-qPCR	COG2F	CARGARBCNATGTTYAGRTGGATGAG[a]	ORF1-ORF2 junction region	Kageyama *et al*., 2003
	COG2R	TCGACGCCATCTTCATTCACA		
	RING2-probe	FAM-TGGGAGGGCGATCGCAATCT-TAMRA		
HAV RT-qPCR	Forward primer	CTGCAGGTTCAGGGTTCTTAAATC	5' non-coding region (NC)	Villar *et al*., 2006
	Reverse primer	GAGAGCCCTGGAAGAAAGAAGA		
	Probe	FAM-ACTCATTTTTCACGCTTTCTG		

[a]IUB code: W = A/T, R = A/G, B = C/G/T, Y = C/T, N = A/C/G/T.

Table 2 - Virus recovery efficiency (%) obtained by two concentration methods and qPCR results (GC mL^{-1}) in primary sludge samples.

Concentration method*	HAdV	RV-A	NoV	HAV
Method 1				
Virus titers spiked	4.2×10^6	7.9×10^6	1.0×10^5	6.6×10^5
Recovery	3.1×10^5 (± SD 3.0×10^5)	1.0×10^4 (± SD 8.6×10^3)	8.3×10^2 (± SD 1.4×10^2)	4.3×10^4 (± SD 3.2×10^4)
Recovery efficiency (%)	7.3	0.1	0.8	6.5
Method 2				
Virus titers spiked	4.2×10^6	7.0×10^6	1.5×10^4	6.0×10^5
Recovery	1.8×10^5 (± SD 1.4×10^5)	3.0×10^1 (± SD 5.2×10^1)	0	0
Recovery efficiency (%)	4.2	0.0004	0	0

*Methods were run in triplicate, SD= standard deviation. Method 1 = ultracentrifugation, Method 2 = beef extract.

by method 2. RV-A was also very poorly recovered by this method.

Detection limits of the assays were tested diluting initial viral titers spiked into sludge samples. RV-A, NoV and HAV were not detected by method 2 when viral concentrations of 10^3 GC mL^{-1} were seeded in sludge samples, but HAdV were detected (mean genome load recovery: $1.3 \times 10^2 \pm$ SD 2.1×10^2 GC mL^{-1}). By using ultracentrifugation based-method, initial viral genome load of HAdV (4×10^3 GC mL^{-1}), RV-A (2.6×10^3 GC mL^{-1}) and NoV (4×10^3 GC mL^{-1}) were detected at mean recovery rates as following: $1.2 \times 10^3 \pm$ SD 2×10^3 GC mL^{-1} for HAdV, $8 \times 10 \pm$ SD 1.3×10^2 GC mL^{-1} for RV-A and $3.3 \times 10^2 \pm$ SD 5.7×10^2 for NoV, but HAV was negative when 10^3 GC mL^{-1} was spiked. In concentrations of 10^2 - 10^1 GC mL^{-1} all analysis showed negative results for viruses detection in both methods evaluated.

Table 3 shows the results for enteric viruses detected in naturally contaminated sewage sludge samples using two concentration methods. Method 2 (beef extract elution based method) was better for detecting HAdV, RV-A and NoV when compared with method 1. HAV was only detected in one sample by the ultracentrifugation method (Table 3). The total frequency of detection, independently of used methods, demonstrated that HAdV was the most detected (91%) (range: 1.8×10^4 to 1.1×10^5 GC L^{-1}) , followed by RV-A (range: 8×10^3 to 8×10^5 GC L^{-1}), NoV (range: 1.6×10^4 to 4.9×10^5 GC L^{-1}) (both 50%) and HAV (8%) (8.6×10^6 GC L^{-1}).

Discussion

Surveys on environmental virology need more focus on relevant issues addressed to ensure a reliable viral detec-

Table 3 - Viral genome loads (GC L^{-1}) in sewage sludge obtained by concentration methods following detection using qPCR.

Sampling date*	Viral groups							
	HAdV		RV-A		NoV		HAV	
Methods	1	2	1	2	1	2	1	2
January	2×10^4	7.4×10^4	0	0	0	0	0	0
February	0	9.4×10^4	0	0	0	0	8.6×10^6	0
	0	1.8×10^4	0	0	0	0	0	0
March	0	1.1×10^5	0	0	0	0	0	0
	0	4.9×10^4	0	0	0	2.4×10^5	0	0
April	3.1×10^4	3.6×10^4	0	0	0	0	0	0
	0	2.5×10^4	0	8×10^5	0	4.6×10^5	0	0
May	0	1.1×10^5	0	8×10^3	2.4×10^4	4×10^5	0	0
June	0	0	2×10^4	0	0	0	0	0
	0	8.7×10^4	0	2.4×10^5	1.6×10^4	5.4×10^4	0	0
July	0	5.3×10^4	0	4×10^5	4.9×10^5	1.2×10^5	0	0
	2×10^4	7.4×10^4	0	1×10^5	5×10^4	2.4×10^4	0	0
Frequency of detection	25%	91%	8%	41%	33%	50%	8%	0

Method 1 = ultracentrifugation, Method 2 = beef extract. * Samples were collected twice per month (except in Jan and May) with a 15 days intervals.

tion, avoiding underestimation of the viruses presence in several environmental matrices. For this purpose, it is necessary the establishment of a suitable indicator to predict viral contamination levels in the environment. Second, virus recovery rates and limits of detection achieved by specific methods should be determined.

In this study, an ultracentrifugation based-method was used for recovering enteric viruses from sludge samples because it has been proven to present advantages over other concentration methods, with a mean recovery rate of 47% for RV-A in sewage (Fumian *et al.*, 2010). However, very lower mean recovery rates were found for detecting enteric viruses in sewage sludge, demonstrating that these wastes contain higher inhibitor levels that can affect PCR performance.

Inhibitors are humic and fulvic acids, fats, proteins, organic and inorganic compounds, including polyphenols and heavy metals that form complexes with nucleic acids and inhibit amplification enzymes (Sano *et al.*, 2003; Sidhu and Toze, 2009; Rock *et al.*, 2010).

Simplified method based on beef extract elution that was previously tested for recovering coliphages from sewage sludge (Guzmán *et al.*, 2007) was better for detecting HAdV, RV-A and NoV GII from naturally contaminated sewage sludge when compared with ultracentrifugation.

Rock *et al.* (2010) has reported that beef extract and glycine buffer can concentrate different inhibitor compounds responsible by causing different results when using qPCR assay. Nevertheless, it is difficult to determine which characteristic could affect qPCR efficiency, since ultracentrifugation was used in this study as a final strategy to concentrate viruses. Pellet generated can contain viruses and other substances, such as suspended solids of the final eluate, which could affect the procedure of nucleic acid extraction. Other studies have demonstrated that ultracentrifugation is not the better method used to recovering enteric viruses from water and wastewater samples (Albinana-Gimenez *et al.*, 2009; Calgua *et al.*, 2012). This method need acquisition of an ultracentrifuge and can be more expensive when compared with other methods (Calgua *et al.*, 2012).

Limits of detection demonstrated that only relative high viral loads can be detected using elution with beef extract, but it seems to be applicable for sludge samples since these residues can contain high concentrations of pathogenic viruses, mainly HAdV.

HAdV have been proposed as a suitable index for the indication of viral contaminants of human origin since they were reported to occur worldwide throughout the year and are widely disseminated in several environments, being more resistant to sewage treatment processes, as suggested elsewhere (Bofill-Mas *et al.*, 2006; Okoh *et al.*, 2010; Schlindwein *et al.*, 2010). In this study HAdV was the most detected virus in sewage sludge samples analyzed, similarly to another study reported in Brazil (Schilindwein *et al.*, 2010) and seems that there isn't seasonal variation for their occurrence in environmental matrices, as verified previously (Barrella *et al.*, 2009; Schilindwein *et al.*, 2010).

Different seasonal patterns could be observed for other viruses. HAV was only detected in February, similarly to a recent study on HAV occurrence during the warmer and rainy seasons in wastewaters from Rio de Janeiro, Brazil (Prado *et al.*, 2012).

NoV GII was predominantly detected in the colder months, suggesting a higher burden and circulation of these viruses during this period, which corroborates previous results about peaks of NoV occurrence in sewage samples in Brazil (Victoria *et al.*, 2010). RV-A was also detected in the colder months, although seasonal variation has been not verified in wastewaters from Rio de Janeiro (Fumian *et al.*, 2011).

RV-A was poorly detected in sewage sludge samples compared with AdV present in these samples, although recent studies have shown a large dissemination of RV-A ($\geq$ 90% of detection) in wastewaters from Rio de Janeiro (Fumian *et al.*, 2011; Prado *et al.*, 2011). RV-A could be detected in higher frequencies in sewage sludge because are charged particles recognized to adsorb onto sludge flocs (Sidhu and Toze, 2009). Nevertheless, some authors have described that RV could poorly adsorb on solid fractions contained in sewage sludge (Arraj *et al.*, 2005). Viral adsorption mechanisms in sewage sludge could vary according to distinct viruses types (Arraj *et al.*, 2005; Silva *et al.*, 2007) and these issues need further investigation.

The low frequency of RV-A found in these residues could also be related to limits of detection obtained by virus concentration methods and qPCR assays. Method based on beef extract elution seems to be not suitable for detecting RV in sewage sludge (Arraj *et al.*, 2005).

The lower frequency of HAV detection in comparison with other enteric viruses in sewage sludge is in accordance with another study carried out in Brazil (Schilindwein *et al.*, 2010). The real presence of HAV in these wastes could also be underestimated based on limits of detection of virus concentration methods, as recently reported (Jebri *et al.*, 2012). Therefore, other virus concentration methods could be developed to improve virus recovery efficiency from sewage sludge.

Results also suggest that activated sludge samples are contaminated with higher viral genome loads than the observed. Based on data provided by previous studies in this WWTP (Fumian *et al.*, 2011; Prado *et al.*, 2012), sewage sludge samples can contain viral concentrations at least 1 to 2 log units more elevated than the raw sewage ones. Contamination levels of primary sewage sludge indicate that an adequate stabilization process should be employed to treat these wastes in activated sludge process.

It is also emphasized that the knowledge about viral contamination levels in sewage sludge and treated effluent of the current WWTPs could contribute to implement strat-

egies or necessary alternatives to improve the performance of conventional sewage treatment processes and to ensure a better effluent and solid wastes quality in the near future.

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