Comparative study of two extraction methods for enteric virus recovery from sewage sludge by molecular methods

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The aim of this study was to compare two nucleic acid extraction methods for the recovery of enteric viruses from activated sludge. Test samples were inoculated with human adenovirus (AdV), hepatitis A virus (HAV), poliovirus (PV) and rotavirus (RV) and were then processed by an adsorption-elution-precipitation method. Two extraction methods were used: an organic solvent-based method and a silica method. The organic-based method was able to recoup 20% of the AdV, 90% of the RV and 100% of both the PV and HAV from seeded samples. The silica method was able to recoup 1.8% of the AdV and 90% of the RV. These results indicate that the organic-based method is more suitable for detecting viruses in sewage sludge.

Key words: sewage sludge - virus detection - RT-nested-PCR

Sludges derived from wastewater treatment are foul-smelling, biologically unstable substances. They contain numerous pathogenic microorganisms, mostly of faecal origin.

Wastewater treatment processes are likely to reduce the number of pathogens in the water prior to its return to the environment. The high incidence of pathogenic viruses in sewage sludge is due to the preferential adsorption of viruses to sludge solids. Depending upon the method used to treat domestic sewage, between 50-99.99% of the viruses can be inactivated. Therefore, infectious viruses potentially remain after treatment. With enteric viruses, a low infectious dose can be enough to cause illness (Moe 1991).

Studies that compared the persistence of enteric viruses indicated that enteroviruses such as poliovirus (PV) are not reliable indicators of other human enteric viruses of major health significance such as rotavirus (RV), astrovirus, hepatitis A virus (HAV) (Abad et al. 1994) and adenovirus (AdV). For this reason, we selected four enteric viruses (AdV, HAV, PV and RV) for use in a model of virus recovery in sewage sludge.

Nucleic acids can be extracted from contaminated environmental samples by many different protocols. These include organic-based extraction by phenol (in the case of DNA viruses) or Trizol® (in the case of RNA viruses), silica methods and commercial kits. These methods aim to extract and purify nucleic acids by removing cell debris and inhibitors. This extraction step is crucial to environmental virology because the main challenge in molecular detection techniques [such as polymerase chain reaction (PCR)] is inhibition by substances such as humic acids, polysaccharides and other chemicals (Kingsley & Richards 2001). Non-kit-based methods are preferred for routine laboratory procedures all over the world because they are less expensive than kit-based protocols. The reagent Trizol LS is a mono-phasic solution of phenol and guanidine isothiocyanate that is preferred for total RNA isolation from cells and tissues because it has the ability to lyse cells and inactivate nucleases (Boom et al. 1990). When this method is used, total RNA segregates into the aqueous phase, while DNA and proteins remain in the phenol phase and interface. For this reason, the traditional phenol/chloroform protocol is more suitable for DNA isolation. Silica methods are rapid, easy to use and efficient at removing inhibitors (Jiang et al. 2001).

In this study, we compared organic-based and silica nucleic acid extraction methods on the basis of their ability to isolate viruses seeded into sewage sludge samples. The efficiency of viral elution was evaluated by PCR, reverse transcription (RT) PCR and RT-nested-PCR. The use of samples contaminated with known amounts of the viruses facilitated direct comparisons between the different extraction methods.

MATERIALS AND METHODS

HAV-cytophatic strain HM 175 and human AdV (genogroup C, serotype 5) (AdV5) were propagated in a continuous line of foetal FRHk-4 cells (rhesus kidney-derived cells) and Hep-2 cells (human larynx carcinoma), respectively. Poliovirus type 2 (PV2) and Simian RV SA11 (group A, serotype G3) were propagated in VERO cells (an established line of African green monkey kidney fibroblasts) and MA104 cells (a continuous line of foetal rhesus kidney cells), respectively. Cells were cultured in Eagle's minimal medium (MEM-Sigma), supplemented with foetal bovine serum (Gibco-BRL), streptomycin (100 µg/mL), penicillin G (100 U/mL) and amphotericin (0.025 µg/mL) (Gibco-BRL). For determination of viral

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titres, a previously described indirect immunofluorescence assay was used for RV and HAV, with modifications (Barardi et al. 1999), and a lysis plaque assay was the method of choice for AdV5 and PV2 titre determination (Kingchington et al. 1995). The titre of AdV5 was 7.0 x 10^6 plaque forming units (PFU)/mL, HAV was at 4.5 x 10^5 focus forming units (FFU)/mL, PV2 was at 4.7 x 10^5 PFU/mL and RV was at 6.0 x 10^6 FFU/mL.

Activated sludge samples were obtained from the wastewater treatment plant in Florianópolis, state of Santa Catarina, located in the South of Brazil. The samples were autoclaved at 120°C for 30 min and seeded with serial 10-fold dilutions of AdV5, HAV, PV2 or RV either before the elution step or after the decontamination step (as a positive control). Each experiment was performed in quadruplicate. The virus detection strategy is outlined in Fig. 1.

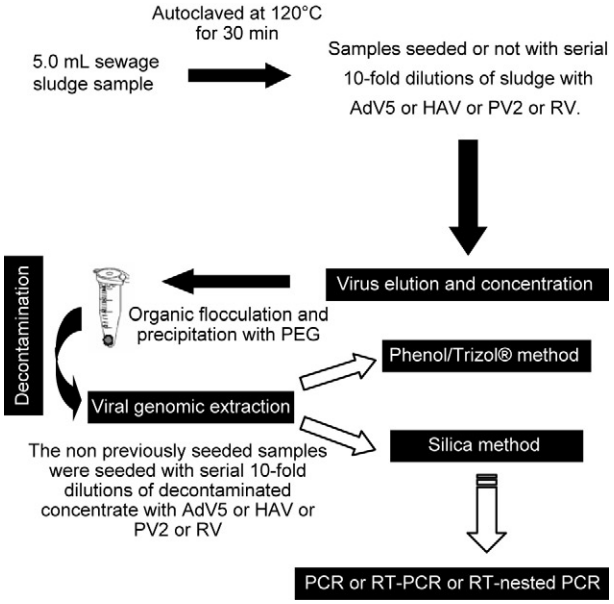

Fig. 1: schematic diagram describing the strategy for the detection of enteric viruses in sewage sludge samples. AdV: human adenovirus; HAV: hepatitis A virus; PV: poliovirus; RV: rotavirus.

The technique of virus elution used for the extraction of viruses from sludge was described by the Environmental Protection Agency (EPA 1992) with brief modifications. In this technique, a 0.05 M $AlCl_3$ solution was added (1%, vol/vol) to a 5.0 mL sludge sample and the pH was adjusted to 3.5 with 5M HCl. The mixture was then stirred at 500 rpm for 30 min. After centrifugation at 2,500 *g* for 15 min at 4°C, the pellet was suspended in 35 mL of buffered (pH 7) 10% beef extract (LP029B; Oxoid). Next, the mixture was again stirred at 500 rpm for 30 min and centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant was collected and the pH was adjusted to 7.2 when required. For virus concentration, PEG 6000 precipitation was employed as described by Lewis and Metcalf (1988). The pellet, suspended in 5.0 mL of 0.1 M phosphate buffer (pH 7.2), constituted the concentrate. The decontamination step was as described by Mignotte et al. (1999). In order to evaluate the efficiency of the extraction methods, AdV5 and RV were used as models of DNA and RNA genomes, respectively. The more sensitive method was then selected to extract the nucleic acid from the other viruses (HAV and PV2).

For DNA/RNA extraction, 500 µL samples of processed sewage sludge were treated with proteinase K at a final concentration of 400 µg/mL in a digestion buffer composed of Tris-HCl (pH 7.5), sodium dodecyl sulphate and ethylenediamine tetra-acetic acid (final concentrations of 10mM, 0.5% and 5mM, respectively) for 60 min at 56°C. The sewage sludge samples were then treated with cetyltrimethylammonium bromide and NaCl at final concentrations of 1.3% and 0.6 M, respectively, and incubated at 56ºC for 30 min. Next, DNA was extracted from all AdV5-seeded samples using the phenol:chloroform:isoamyl alcohol method, according to Sambrook and Russell (2001). The nucleic acids were suspended in 50 µL of sterile MilliQ water.

RNA was purified from the HAV, RV and PV2-seeded samples with Trizol® LS (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The nucleic acids were suspended in 50 µL of sterile diethylpyrocarbonate-treated water. Purified nucleic acids were stored at -80°C for the RT-PCR assays.

Extraction of nucleic acids with silica was performed as described by Boom et al. (1990), with modifications. The nucleic acids were recovered in 45 µL and stored at -80°C until PCR analysis. AdV DNA was detected in sewage sludge samples by using the oligonucleotide primer pair hexAA 1885 and hexAA 1913, which amplifies a 300 bp fragment from the AdV *hexon* gene (Allard et al. 1992). HAV RNA was detected in sewage sludge samples by RT nested-PCR using the oligonucleotide primer pair F6 (+) and F7 (-), which amplifies a 392 bp fragment suitable for detecting all HAV genotypes (universal primers). The internal primers were F8 (+) and F9 (-), which amplify a 247 bp fragment (De Paula et al. 2004). PV2 RNA was detected in sewage sludge samples by RT-PCR with the oligonucleotide primer pair Polio-R and Polio-L, which amplifies a 394 bp fragment from the 5' non-coding region (Atmar et al. 1993). RV RNA was detected in sewage sludge samples by RT-PCR with the oligonucleotide primer pair VP6-F (+) and VP6-R (-), which amplifies a 379 bp fragment of the *VP6* gene (Iturriza-Gómara et al. 2002). Conventional RT-PCR was performed using random and specific primers for reverse transcription and genome amplification. Negative and positive controls were included in all experiments. The sensitivity of the RT-nested-PCR procedure was determined by performing assays with 10-fold serial dilutions of the virus stocks in sludge samples before the elution step or after the decontamination step (as a positive control). The viral detection limit was considered to be the highest viral dilution that yielded a positive result. The recovery efficiencies of the various viruses from the

sewage sludge samples were calculated with the following equation and Fisher's exact test was applied for the comparison of two proportions:

$$\text{virus recovery efficiency (\%)} = \frac{\text{seeded sample after elution and decontamination processes}}{\text{seeded sample before virus elution}} \times 100$$

RESULTS AND DISCUSSION

As shown in Figs 2 and 3, the detection limits for the viruses seeded before and after the elution step using the organic-based method were, respectively: 0.63 PFU and 0.126 PFU (AdV5), 24 FFU and 21.6 FFU (RV), 0.0072 FFU (HAV) and 18.8 PFU (PV) (the last 2 viruses gave the same results both before and after elution). The detection limits of the silica method were: 560 PFU and 10.08 PFU (AdV5), 192 FFU and 172.8 FFU (RV) (Fig. 2). The results from the tests of the different viral extraction methods using sewage sludge samples artificially contaminated with AdV5, RV, PV or HAV are shown in Table. When the detection limits were compared with the positive controls (100% recovery), the organic-based method detected 20% of the AdV5, 90% of the RV and 100% of both the HAV and the PV2. The silica method detected 1.8% of the AdV5 and 90% of the RV. Our results showed that the sensitivity of the RT-nested-PCR was different depending on the nucleic acid extraction technique used. However, the efficiency of both methods with regard to the recovery of RV was the same (90%). Therefore, it is likely that the organic solvents associated with the chloroform clarification steps of the organic-based method removed inhibitory substances normally present in sewage-derived sludge and thereby increased the limit of sensitivity. The efficiencies of the different methods differed significantly ($p = 0.0286$). Thus, the organic-based method was preferable to the silica extraction method for detecting AdV and RV.

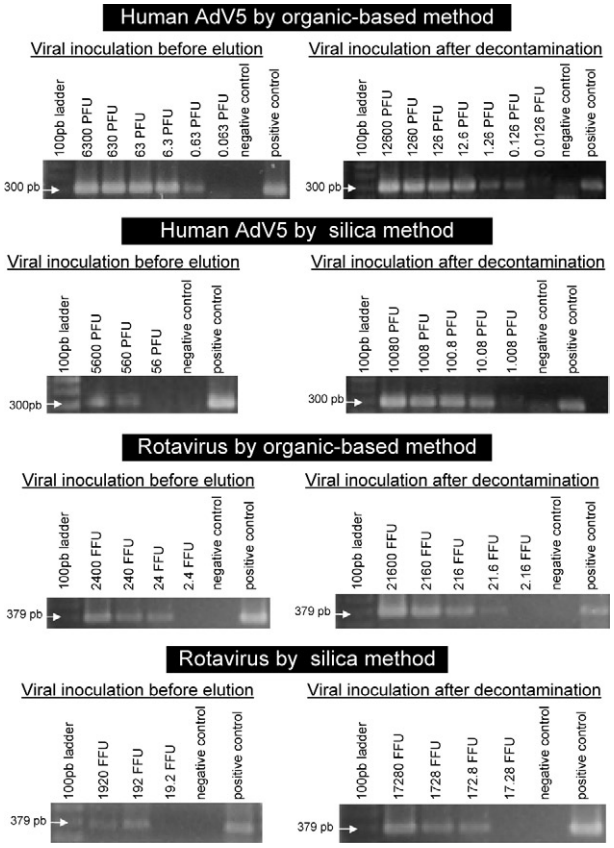

Fig. 2: agarose gel electrophoresis showing sensitivity limits of PCR and RT-PCR for the human human adenovirus (AdV5) and rotavirus artificially seeded in sewage sludge samples. FFU: focus forming units; PFU: plaque forming units.

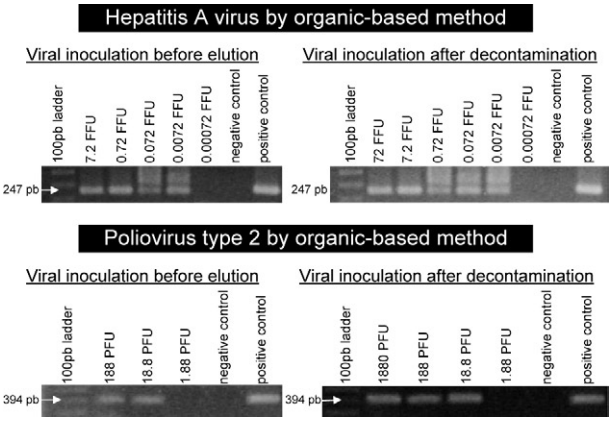

Fig. 3: agarose gel electrophoresis showing sensitivity limits of RT-nested-PCR or RT-PCR for the hepatitis A virus and poliovirus 2 artificially seeded in sewage sludge samples. FFU: focus forming units; PFU: plaque forming units.

The organic-based method gave a better yield of RNA viruses (90% recovery of RV and 100% recovery of both PV2 and HAV) than of the DNA virus (20% recovery of AdV5). This could be due to the nature of the virus genomes (DNA for AdV and RNA for HAV, PV2 and RV) and/or to the strong aggregation of AdV into solids that make it difficult to isolate from environmental samples. However, this would have to be confirmed by further studies involving other types of environmental samples because the contaminants and PCR inhibitors that they contain can vary dramatically from sample to sample. The molecular detection of AdV, HAV and enteric viruses by PCR can provide reliable data about the presence of these viruses in the environment, thus overcoming the technical limitations of isolating of these viruses in cell culture (Pina et al. 1998). To improve the sensitivity of the detection methods, molecular methods could be used in conjunction with other protocols. For example, quantitative PCR could be coupled with viral viability assays.

Several lines of evidence support the hypothesis that PCR-based AdV detection is a reliable index for human viral contamination. AdV and HAV are more stable in various environments than enterovirus and they are more resistant to UV irradiation and other treatments used by water purification plants (Meng & Gerba 1996). The detection of viruses in the environment by PCR, however, has important limitations: the viability of the detected viruses is unknown, the rules governing laboratory personnel and materials must be very strict and stringent

TABLE

Detection limits of human adenovirus (AdV5), hepatitis A virus (HAV), poliovirus (PV2) and rotavirus (RV) viral particles from sewage sludge with two different methods of acid nucleic extraction: organic-based and silica

Sample	Sewage sludge			
Virus and method	Seeded sample before virus elution	Seeded sample after elution and decontamination processes	Recovery %	Number of positive samples/ number of sample analyzed
AdV5 (PFU)				
Phenol	0.63	0.126	20	4/4
Silica	560	10.08	1.8	4/4
RV(FFU)				
Trizol	24	21.6	90	4/4
Silica	192	172.8	90	4/4
PV2 (PFU)				
Trizol	18.8	18.8	100	4/4
HAV (FFU)				
Trizol	0.0072	0.0072	100	4/4

FFU: focus forming units; PFU: plaque forming units.

quality controls must be applied in order to avoid false-negative and false-positive results. On the other hand, it is also clear that this approach has advantages of specificity, sensitivity and speed relative to the isolation of viruses in cell culture (Pina et al. 1998).

In conclusion, organic-based extraction protocols are a suitable, sensitive and economical method for detecting enteric viruses in sewage sludge samples when commercial kits are not available. Additional research should increase the sensitivity of these methods and further facilitate the detection of viruses in environmental samples.

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