NEW METHODS FOR THE CONCENTRATION OF VIRUSES FROM URBAN SEWAGE USING QUANTITATIVE PCR

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ABSTRACT

Viruses are among the most important pathogens present in water contaminated with feces or urine and represent a serious risk to human health. Four procedures for concentrating viruses from sewage have been compared in this work, three of which were developed in the present study. Viruses were quantified using PCR techniques. According to statistical analysis and the sensitivity to detect human adenoviruses (HAdV), JC polyomaviruses (JCPyV) and noroviruses genogroup II (NoV GGII), (i) a new procedure (elution and skimmed-milk flocculation procedure (ESMP)) based on the elution of the viruses with glycine-alkaline buffer followed by organic flocculation with skimmed-milk was found to be the most efficient method when compared to (ii) ultrafiltration and glycine-alkaline elution, (iii) a lyophilization-based method and (iv) ultracentrifugation and glycine-alkaline elution. Through the analysis of replicate sewage samples, ESMP showed reproducible results with a coefficient of variation (CV) of 16% for HAdV, 12% for JCPyV and 17% for NoV GGII. Using spiked samples, the viral recoveries were estimated at 30%–95% for HAdV, 55%–90% for JCPyV and 45%–50% for NoV GGII. ESMP was validated in a field study using twelve 24-h composite sewage samples collected in an urban sewage treatment plant in the North of Spain that reported 100% positive samples with mean values of HAdV, JCPyV and NoV GGII similar to those from other studies. Although all of the methods compared in this work yield consistently high values of virus detection and recovery in urban sewage, some require expensive laboratory equipment. ESMP is an effective low-cost procedure which allows a large number of samples to be processed simultaneously and is easily standardizable for its performance in a routine laboratory involved in water monitoring. Moreover, in the present study, a CV was applied and

proposed as a parameter to evaluate and compare the methods for detecting viruses in sewage samples.

Keywords: sewage, virus concentration method, human adenovirus, JC polyomavirus, norovirus

1. Introduction

Raw sewage is the most important source of pathogens that enter the environment, especially viruses that show a high stability in environmental conditions. Although raw sewage from urban areas, hospitals and slaughterhouses is usually treated before being released into the environment, several studies have documented the presence of pathogenic viruses in treated water (Gantzer *et al*., 1998; Pusch *et al*., 2005; van den Berg *et al*., 2005; Bofill-Mas *et al*., 2006; Fumian *et al*., 2010). Untreated and treated sewage may represent a source of environmental contamination.

A recently published metagenomic study of viruses present in urban sewage reported the presence of nearly 600,000 new virus-related sequences; 43,381 associated with known viruses and 596,146 that may be new viruses unrelated to previously identified ones (Cantalupo *et al*., 2011). New viruses, such as the picornavirus Klassevirus and the Asfarvirus-like virus, have recently been also reported in urban sewage (Hotlz *et al*., 2009; Loh *et al*., 2009). Moreover, studies of urban sewage have provided valuable information on the prevalence of many viral infections and the dissemination of new viruses in diverse populations; Bofill *et al*. (2010) described the presence of new polyomaviruses such as Merkel cell, KI and WU; Rodriguez-Manzano *et al*. (2010) analyzed the evolution in the circulation of the hepatitis A and E viruses in the population of Eastern Spain; and Prado *et al*. (2011) detected different enteric viruses in effluent water from two hospitals. All of these data suggest that raw sewage represents a useful matrix to study viruses excreted by human and animal populations.

Classical enteric human viruses, such as adenoviruses, rotaviruses, noroviruses and enteroviruses, and viruses excreted by urine such as the BK and JC polyomaviruses, have been widely detected in sewage from different geographical areas (Bofill-Mas *et al*., 2000; Miagostovich *et al*., 2008; Fumian *et al*., 2010; Victoria *et al*., 2010). Interestingly, various studies have reported that the levels of classical bacterial indicators (*E. coli* and enterococci) do not always correlate with viruses, particularly when bacterial indicator concentrations are low (Brownell *et al*., 2007; Colford *et al*., 2007; Calgua *et al*., 2008; Wyn-Jones *et al*., 2011). Improved indicators will be useful and human adenoviruses (HAdV) and JC polyomaviruses (JCPyV) have been proposed as viral indicators of human fecal contamination in the environment and have played an important role in recent studies on water quality (Puig *et al.*, 1994; Bofill-Mas *et al*., 2000; Albinana-Gimenez *et al*., 2006; McQuaig *et al*., 2006, 2009; Miagostovich *et al*., 2008; Tong and Lu, 2011; Wyn-Jones *et al*., 2011), showing high stability in the environmental conditions and to disinfection treatments commonly applied to sewage and drinking water (Bofill *et al*., 2006; Ogorzaly *et al*., 2010; Wong and Xagoraraki, 2011). According to previous studies, HAdV are almost always present in sewage samples from different geographical areas and show a mean concentration of 10^3 and 10^2 genomic copies (GC)/mL for HAdV and JCPyV, respectively (Bofill-Mas *et al*., 2006; Fong *et al*. 2009; Rodriguez-Manzano *et al.*, 2012).

HAdV is grouped in 53 serotypes, which have been widely reported to cause a broad range of clinical manifestations including respiratory tract infection, acute conjunctivitis, cystitis, gastroenteritis and systemic infections. JCPyV is a human virus in the *Polyomaviridae* family that triggers latent and chronic infections that

persist indefinitely in individuals and causes healthy individuals to regularly excrete viral particles in their urine (Shah, 1995). JCPyV is commonly associated with progressive multifocal leukoencephalopathy (PML) in immunocompromised individuals and has attracted new attention due to its reactivation in a small percentage of patients with multiple sclerosis and other autoimmune diseases treated with immunomodulators (Berger and Major, 1999; Yousry *et al*., 2006). The noroviruses are a major cause of sporadic outbreaks of infectious gastroenteritis, which occasionally requires hospitalization (Glass *et al*., 2009). Outbreaks commonly occur in closed populations such as childcare centers and cruise ships (Khan and Bass, 2010), with older children and adults being infected more frequently than infants (Glass *et al*., 2009). Based on the phylogenetic analysis of the viral capsid (VP1) gene, NoV is classified into five genogroups, which are further subdivided into genotypes. Genogroups I (GGI), II (GGII) and IV (GGIV) infect humans (Glass *et al*., 2009; Koo *et al*., 2010). Despite this diversity, only a few strains, primarily those of genogroup II, genotype 4 (GGII.4), have been responsible for the majority of recent cases and outbreaks (Barreira *et al*., 2010; Ferreira *et al*., 2010; Bull and White, 2011; Prado *et al*., 2011).

Methods based on ultracentrifugation and glycine-alkaline elution, have been described by Pina *et al*. (1998) and have been widely used in this laboratory (Pina *et al*., 1998; Bofill-Mas *et al.*, 2000; Clemente-Casares *et al*., 2003, 2009; Rodriguez-Manzano *et al.*, 2010). In order to define concentration methods with high level of cost-efficiency and applicability, new protocols have been developed and evaluated in this study for quantifying viruses present in sewage. DNA viruses such as HAdV and

JCPyV, and RNA virus such as NoV have been selected as representative viruses for the study.

2. Material and methods

2.1. Sewage samples

Four sets of sewage samples were used in this study. Each sample was harvested in a sterile 1,000-mL polyethylene container and kept at 4 °C for less than 24 h until the virus particles were concentrated:

(**i**) Comparison of methods: five samples of 200 mL raw urban sewage were collected between November and December 2010 at the entrance of a sewage treatment plant located in Barcelona (Catalonia, Spain) that receives sewage from a human population of about 1.8 million inhabitants. Each sample was vortexed for 1 minute and divided into four aliquots (n=20) and each set of aliquots (n=5) was processed using one specific concentration method based on flocculation, ultrafiltration, lyophilization and ultracentrifugation.

(**ii**) Repeatability assay for the elution and skimmed-milk flocculation procedure: one sample of raw urban sewage was collected at the entrance of a sewage treatment plant located in Barcelona (Catalonia, Spain) and divided into ten 50-mL aliquots.

(**iii**) Recovery assay for the elution and skimmed-milk flocculation procedure: one sample of raw urban sewage was collected at the entrance to a sewage treatment plant located in Barcelona (Catalonia, Spain) and divided into ten 50-mL aliquots. The aliquots were processed using two different assays for estimating virus recovery.

(**iv**) Field study: twelve 24-h composite 50-mL samples were collected between September and December 2010 at the entrance of a sewage treatment plant in Vitoria (Basque Country, Spain) that receives sewage from a human population of about 240,000 inhabitants.

2.2. Virus-concentration methodology

According to the virus-concentration method applied and the limitations caused by the volume capacity of the filters and rotors the used sample volumes ranged from 42 to 50 mL depending on the method used. Considering that NA from 100–140 μL of viral concentrate have been extracted and resuspended in a final volume of 100 μL of elution buffer, and 10 μL of which have been finally analysed by qPCR, the volume of sewage sample analysed by each assay was 1.4 mL for the elution and skimmed-milk flocculation procedure (ESMP) and lyophilization-based method (LP), and 4.2 mL and 4.5 mL for ultracentrifugation- based method (UC) and ultrafiltration-based method (UF), respectively. Viral concentrates obtained by applying the different procedures were dissolved with the same phosphate buffer at pH 7.5 (1:2, v/v of Na_2HPO_4 0.2 M and NaH_2PO_4 0.2 M). When necessary, the final viral concentrates were stored at −80 °C.

2.2.1 Elution and skimmed-milk flocculation procedure

The sewage sample (50 mL) was transferred to a 500-mL centrifuge pot and the viruses present were eluted using 100 mL of glycine buffer 0.25 N, pH 9.5 (1:2 v/v). The sample was stirred rapidly for 30 min on ice and centrifuged at 8,000 x*g* for a

178 further 30 min at 4 °C. The supernatant (150 mL) was transferred to a new centrifuge
179 pot, the pH was adjusted to 3.5 with HCl 1N, and 1.5 mL of pre-flocculated skimmed-
180 milk solution (final concentration of skimmed-milk 0.01% (w/v)) was added. The pre-
181 flocculated skimmed-milk solution (1% (w/v)) was prepared in advance according to
182 Calgua *et al*. (2008) by dissolving 1 g of skimmed-milk powder (Difco, Detroit, MI,
183 USA) in 100 mL artificial seawater and carefully adjusting the pH to 3.5 with HCl 1
184 N. The sample was then stirred for 8 h to allow the viruses to be adsorbed into the
185 skimmed-milk flocs at room temperature (RT). Then flocs were sedimented by
186 centrifugation at 8,000 x*g* for 30 min at 4 °C. The supernatants were carefully
187 removed without disturbing the sediment and the pellet was dissolved in 500µL of
188 phosphate buffer (pH 7.5).
189
190 2.2.2. Ultrafiltration-based method
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192 Millipore Ultrafree-15 Centrifugal Filters 100,000-MW cutoff (Millipore, Milford,
193 MA, USA) were washed with 10 mL of bi-distilled sterile water (four filters per
194 sample), centrifuged at 2,000 x*g* for 10 min and the filtered water was discarded. A
195 45-mL sample of sewage was transferred to three pre-washed filters (15 mL each),
196 centrifuged at 2,000 x*g* for 1 h at RT and the filtered volume was discarded. The
197 viruses were eluted from each filter by using 4 mL of glycine buffer 0.25 N and pH
198 9.5. The eluted viruses (approximately 12 mL) were transferred to a sterile 50-mL
199 tube, incubated for 30 min at 4 ºC (vortexed every 10 min) and centrifuged at 3,000
200 x*g* for 30 min at 4 ºC. The supernatant was then recovered and transferred to a pre-
201 washed filter. The filter was centrifuged at 2,000 x*g* for 1 h at RT and subsequently

mixed by vortex and centrifuged at 2,000 x*g* for 2 min at RT. Finally, the volume retained by the filter was collected in 100 µL.

2.2.3. Lyophilization-based method

A 50-mL sample of sewage was frozen to -80 ºC and lyophilized for 24 to 36 h. The lyophilized sample (powder) was then dissolved in 500 µL of phosphate buffer (pH 7.5).

2.2.4. Ultracentrifugation-based method

This procedure had been described previously and applied to several studies (Pina *et al*., 1998; Clemente-Casares *et al*., 2003, 2009; Bofill-Mas *et al.*, 2000; Rodriguez-Manzano *et al*., 2010). Briefly, 42 mL of sewage were ultracentrifuged at 100,000 x*g* for 1 h at 4 °C to pellet all the viral particles together with any suspended material. The viruses present in the pellet were eluted by mixing it with 4 mL of 0.25 N glycine buffer (pH 9.5) on ice for 30 min, and after the addition of 4 mL of phosphate buffer, the suspended solids were separated by centrifugation at 3,000 x*g* for 20 min. Finally, the viruses were concentrated by ultracentrifugation at 100,000 x*g* for 1 h at 4 °C and resuspended in 100 µL of phosphate buffer (pH 7.5).

2.3. Extraction of nucleic acids from viral concentrates

The extraction of nucleic acids (NA) was performed with QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) and the automated system QIACube (Qiagen,

227 Valencia, CA, USA), both according to manufacturer's instructions. NA extracts were
228 stored at 4 °C and assayed on the same day using quantitative PCR (qPCR) or
229 quantitative reverse transcription-PCR (qRT-PCR).
230
231 2.4. Viral enzymatic amplification and quantitation by qPCR
232
233 Quantitative PCR was performed in a 25-µL final volume containing 1X Master Mix
234 (Applied Biosystems, TaqMan® Environmental Master Mix 2.0, Foster City, CA,
235 USA). The reaction contained 10 µL of a NA extraction or 10 µL of a quantified DNA
236 and the corresponding primers and TaqMan probes. HAdV genomes were quantified
237 with 0.9 µM of the AdF and AdR primers and 0.225 µM of the AdP1 probe as
238 described by Hernroth *et al*. (2002). JCPyV genomes were quantified with 0.5 µM of
239 the JE3F and JE3R primers and 0.15 µM of the JE3P fluorogenic probe as described
240 in Pal *et al*. (2006). AmpliTaq Gold was activated for 10 min at 95 °C followed by 40
241 cycles (15 s at 95 °C and 1 min at 60 °C) using an MX3000P sequence detector
242 system (Stratagene, La Jolla, CA, USA). The qPCR method used for the quantitation
243 of HAdV was tested in previous studies where the qPCR successfully detected human
244 adenoviruses from all species, including 40 and 41 (data not shown). This assay also
245 showed a higher sensitivity in the quantitation of human viruses from urban sewage
246 compared to other previously described assays (Bofill-Mas *et al*., 2006). HAdV and
247 JCPyV qPCR demonstrated high specificity and their sensitivity was estimated as 1–
248 10 genome copies.
249
250 Quantitative reverse transcription-PCR was also performed in a 25-µL reaction
251 mixture containing 1X of QuantiTect™ Probe RT-PCR kit (Qiagen, Valencia, CA,

252 USA). The reaction contained 5 μL of NA extraction or 5 μL of a quantified plasmid
253 cDNA, 1 μM of the JJV2F and COG2R primers and 0.1 μM of the RING2-TP probe,
254 as described by Jothikumar *et al*. (2006). Following retrotranscription (30 min at 50
255 °C) and activation of the HotStarTaq (15 min at 95 °C), 45 cycles (10 s at 95 °C, 20 s
256 at 55 °C, 15 s at 72 °C) were performed using an MX3000P sequence detection
257 system (Stratagene, La Jolla, CA, USA). The qPCR assay used for noroviruses has
258 been shown to be specific and to present a sensitivity of <10 genome copies per
259 reaction (Jothikumar *et al*., 2006).
260
261 For the detection and quantitation of specific viral genomes, 10 μL (for HAdV and
262 JCPyV) and 5 μL (for NoV GGII) of neat and 10-fold dilution of every DNA/RNA
263 extraction were tested; these dilutions were designed to detect and reduce
264 amplification inhibition caused by the potential presence of inhibitory substances that
265 may interfere with the qPCR. All samples were run in quadruplicate (two replicates
266 per dilution), and positive and negative controls were included. Known quantities of
267 target DNA were added to a parallel amplification reaction containing qPCR mix and
268 the plasmid. In every assay, the amplification plots of samples and standard dilutions
269 were compared. A sample was considered positive if it produced correct amplification
270 curves and the quantitation data was within the detection limit. The amount of DNA
271 was defined as the average of the duplicate data obtained.
272
273 2.5. qPCR standards
274
275 For the generation of standards to use in the real-time qPCR assays, three plasmid
276 constructions were employed. The plasmid pJCPyV, which contained the whole

277 JCPyV genome strain Mad-1 in pBR322, was kindly donated by Andrew M. Lewis at
278 the Office of Vaccine Research and Review, CBER/FDA, MD, USA. The plasmid
279 pAd41, containing the hexon region of HAdV 41 in pBR322, was kindly donated by
280 Dr. Annika Allard of the University of Umeå, Sweden. For NoV II, the plasmid
281 pNoV, containing the ORF1–ORF2 junction in pTrueBlue, was kindly donated by Dr.
282 Vinjé and Dr. Jothikumar at the CDC (Atlanta, GA, USA). *Escherichia coli* JM109
283 cells (Promega, Madison, WI, USA) were transformed with the plasmid (pJCPyV,
284 pAdV41 or pNoV). The plasmids were purified from bacteria using the QIAGEN
285 Plasmid Midi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's
286 instructions and the DNA was quantified with a GeneQuant pro (Amersham
287 Biosciences, Piscataway, NJ, USA). To reduce the possibility of DNA contamination,
288 the plasmids were linearized with EcoRI (pJCPyV and pNoV) or NruI (pAd41)
289 (Promega, Madison, WI, USA), and then purified and quantified again. Suspensions
290 containing 10^{-2}–10^{7} viral DNA molecules per 10 μL were made in TE buffer and used
291 as standard dilutions, then aliquoted and stored at −80 °C until use.
292
293 2.6. Statistical analysis
294
295 The data obtained from virus quantification was analyzed using the non-parametric
296 Friedman test for relative efficiency analysis and ranks were allotted by assigning the
297 higher value to the better yield method (Friedman *et al*., 1937). A *P-value* of <0.05
298 was considered significant. The statistical analysis was performed using R software
299 version 2.14.1 (Verzani, 2004; R, 2008).

3. Results

3.1. Comparison of methods for concentrating viruses from raw sewage

The samples/aliquots evaluated (*n*=20) by the four different viral concentration methods were positive for all viruses tested and showed high concentration values (Figure 1). For HAdV, the different methods differed significantly (Friedman test, *P-Value* = 0.033) and the associated multiple comparison test showed that UC was significantly different from all the other methods tested in terms of the amount of HAdV detected, being of HAdV significantly lower. However, the assigned ranks showed that the higher values were obtained with ESMP (rank = 3.60), followed by LP (rank = 2.60), UF (rank = 2.60) and UC (rank = 1.20). For JCPyV, there were no statistically significant differences (Friedman test, *P-Value* = 0.948), although ESMP showed the higher rank value (rank = 2.80), followed by UC (rank = 2.40), LP (rank = 2.40) and UF (rank = 2.40). For NoV GGII detection, there were no significant differences (Friedman test, *P-Value* = 0.077) and ESMP (rank = 3.60) showed the higher ranking values, followed by UC (rank = 2.80), UF (rank = 1.80) and LP (rank = 1.80) (Table 1).

3.2. Repeatability assay for ESMP validation

To evaluate ESMP efficiency and ensure the repeatability of the procedure, 10 aliquots of raw urban sewage were evaluated for HAdV, JCPyV and NoV GGII as a model for DNA and RNA viruses. The percentage of coefficient of variation (CV) ([standard deviation/mean] × 100) was used as an estimator of intra-laboratory

variability. The results obtained are shown in Figure 2. The estimated CV was 15.9% for HAdV (mean: 6.89 x 10^2; Min: 5.27 x 10^2; Max: 8.64 x 10^2; SD: 1.09 x 10^2 GC/mL) and 12.2% for JCPyV (mean: 1.39 x 10^3; Min: 1.12 x 10^3; Max: 1.59 x 10^3; SD: 1.71 x 10^2 GC/mL), whereas it was 17.4% for NoV GGII (mean: 3.17 x 10^3; Min: 2.45 x 10^3; Max: 3.81 x 10^3; SD: 5.52 x 10^2 GC/mL).

3.3. Recovery assay for ESMP

The recovery of ESMP for HAdV, JCPyV and NoV GGII was evaluated using two different assays from one raw-sewage sample collected in Barcelona, Spain.

(**i**) By analyzing the losses in the viral concentration of four aliquots (50 mL) through the quantitation of viruses present in the pellet from the first centrifugation (A) and the supernatant from the last centrifugation (B), depending on the total viruses quantified in the final concentrate (C) and according to the following equation: Recovery (%) = [C/(A+B+C)] x 100. The viruses present in the A portion were resuspended in 7 mL of glycine buffer (0.25 M, pH 9.5) and then ultracentrifuged, whereas the viruses from the B portion were directly ultracentrifuged. The viruses from both pelleted portions were eluted in 100 µL of phosphate buffer and the NA directly extracted for the qPCR assay.

(**ii**) Six aliquots of 50 mL each were concentrated by ESMP and the titer was determined among spiked samples with known amount of viruses (*n*=4). Non-spiked samples (*n*=2) were used to determined endogenous viruses.

For HAdV, JCPyV and NoV GGII, the estimated recoveries were around 30%–95%, 55%–90% and 45%–90%, respectively.

3.4. Field study

In winter 2010 (September to December), 50 mL of twelve 24-h composite samples collected at the entrance of a sewage treatment plant located in Vitoria (Basque Country, Spain) were analyzed by applying the ESMP. The NA from viral concentrates were extracted and analyzed using qPCR and qRT-PCR. As expected, all samples were positive for HAdV, JCPyV and NoV GGII, with values (GC/mL) ranging from 4.56×10^2 to 3.41×10^3 (SD: 8.47×10^2), from 1.59×10^2 to 1.61×10^3 (SD: 4.07×10^2) and from 1.41×10^0 to 7.98×10^1 (SD: 2.27×10^1), respectively.

4. Discussion

There is a need for cost-effective easily standardizable virus concentration methods design to be used in routine laboratories. In the present study, a previously described method that has been successfully applied in many studies (UC) was compared and evaluated using three proposed new protocols. Moreover, the repeatability of the results and the virus recovery of the most sensitive method, i.e. the ESMP protocol, were further evaluated.

The first step of the ESMP is to elute the viruses (50 mL of sewage) from the organic matter using an alkaline-glycine buffer, then the viruses present in the supernatant are concentrated using organic flocculation under acidic conditions and the addition of a skimmed-milk solution, a process based on previous methods to concentrate viruses from sea, river and ground water with a viral recovery of about 50% for HAdV, JCPyV, NoV and rotaviruses (Calgua *et al*., 2008; Bofill *et al*., 2011). Although no significant statistical differences were observed in accordance with Friedman's ranking analysis, this method gave a better yield of HAdV (DNA virus), JCPyV (DNA virus) and NoV GGII (RNA virus) in natural samples when compared to three alternative methods: (i) Ultracentrifugation and glycine-alkaline elution (Pina *et al*., 1998), which has been described as an efficient method that allows 42 mL of sewage to be concentrated to 100 µL of PBS, but may be limited due to the need for a high-cost ultracentrifugation device which most routine laboratories involved in water-quality analysis do not have access to. (ii) Ultrafiltration and alkaline-glycine elution, an alternative method also described in this study. This showed the same ratio of concentration as the ultracentrifugation method and although it does not require

special equipment, the cost of the filters required for one sample is from 25-50 times the cost of applying the ESMP and the potential problems associated with the clogging of high organic matter could limit this method. (iii) The lyophilization-based method is a one-step procedure also described in the present study as a new method for the concentration of viruses in sewage. This allows 50 mL of sewage to be concentrated in 500 µL of phosphate buffer with high virus-recovery rates. One advantage of this method is that none of the samples is lost during the concentration process. However, the time required for the method (24–36h) and the high cost of a lyophilization device would make this method probably unfeasible for most laboratories. On the other hand, the time needed to concentrate viral particles using each proposed protocol would depend on the number of samples as well as on the acquaintance with the methodology (handling). Nevertheless, considering as an example that only two sewage samples have been concentrated and the maximum number of samples depends on centrifuge/ultracentrifuge rotor type (mainly for UF and UC), the estimated time to complete the protocols ranges from 4 h for UC and UF, 10 h for ESMP to 24–36 h for LP. When the number of samples is higher, the estimated spent time to complete the UC and UF protocols will increase significantly. Although there are more procedures described for the concentration and detection of viruses from sewage, most of them use membranes and/or filters that require pre-treatments. The use of a pre-filter before virus concentration using membranes or filters to avoid clogging may be associated with the loss of viruses in the organic matter that may be retained in the pre-filter. This represents a significant limitation for the detection of viruses usually present in low concentrations. The detection of specific pathogens that may be expected to be in very low concentrations may require

412 the analysis of larger sample volumes and in these cases, for example for the analysis
413 of HEV in sewage, it may be considered to use UC if available.
414
415 The reproducibility of concentration methods for the quantification of viruses in water
416 has been defined as a significant limitation in current protocols (Girones *et al*., 2010).
417 The CV of the ESMP, the most sensitive method, was also evaluated by using the
418 values of the viruses detected in natural samples together with the virus-recovery test
419 using a spiked sample. The percentage of CV (in the present study: CV = standard
420 deviation [SD]/mean-viruses recovered [MVR] × 100) is the ratio between SD and
421 MVR and, as expected, when a method is developed successfully, this value is lower.
422 There is a lack of information in the literature about the CV applied to methods for
423 virus concentration from water samples. ESMP showed mean recovery values of
424 about 50% for HAdV, JCPyV and NoV GGII, and CVs (n=10) ranging between 12%
425 and 17%. Nupen *et al.* (1970) compared two procedures for concentrating viruses
426 from sewage and two methods (cell culture-based methods) to detect the virus-
427 recovery rate; the results showed CV values from 40% to 80%, depending on the
428 detection method selected. Lambertini *et al*. (2008) showed CVs ranging between
429 21% and 91% with trials of more than three samples for polioviruses, HAdV and NoV
430 detection in different ground and tap water samples by using the glass-wool based
431 procedure as a virus-concentration method and qPCR as a method for detection and
432 quantification. Moreover, different CVs were reported for poliovirus detection (24–
433 81%) compared to Vilaginès *et al*. (2003) (CV 8–40%). In addition, the molecular
434 protocols for detecting and quantifying viruses using qPCR TaqMan® in the present
435 study have been applied previously with different environmental samples such as
436 sewage, seawater and river water (Albinana-Gimenez *et al*., 2009; Bofill-Mas *et al*.,

2010; Calgua *et al*., 2011; Wyn-Jones *et al*., 2011). The variability in quantifying RNA viruses is expected to be higher than DNA viruses, since an additional RT-PCR step is required. Each qPCR assay included a standard curve (each point per triplicate) that showed significantly lower variability with a correlation close to 1, meaning that the molecular assays for virus detection did not introduce any variation to the results of the processes. Furthermore, the NA-extraction protocol had previously been compared with other kits and in-house procedures (data not show), and the most sensitive one, which represents a good approach for avoiding potential inhibitors that may hamper molecular detection, was selected. According to the data available in the literature, most of the studies based in comparing methods for viral recovery from water estimated the virus-recovery rate, but sometimes used different means. Due to the importance of the repeatability of the results and the fact that variable data from the viruses detected can also give high recovery rates, the inclusion of the CV showing acceptable ratios that should not be more than 0.5 for these types of methods, as previously mentioned (Hill *et al*., 1971), provides a very significant information related to the applicability of the assay.

Finally, the ESMP was validated in a field study to detect HAdV, JCPyV and NoV GGII in sewage samples. The values detected are in concordance with previous data detected in Spanish samples (Bofill-Mas *et al*., 2006; Rodriguez-Manzano *et al.*, 2012). Regarding the quantitative detection assay, each sample and virus were evaluated by quadruplicate; duplicate for the undiluted NA and duplicate for the 10-fold dilution, which led to quantitative data with robust values and an absence of the inhibition effect on the qPCR.

Therefore, as demonstrated in the present study, the methods described recover satisfactorily DNA and RNA viruses and the criteria used for the selection of the proposed method are based on the efficiency, low cost and lack of requirements for expensive equipments.

5. Conclusions

5.1. A total of four alternative procedures for concentrating RNA or DNA viruses from sewage samples are reported: three new procedures (ESMP, LP and UF) and one previously reported (UC) with high efficiency values in virus recovery and quantification by qPCR.

5.2. ESMP has shown to be the most cost-efficient procedure and shows about 50% viral recovery (DNA and RNA viruses). Considering the CV values (12%–17%), this procedure produces reproducible results. Moreover, the procedure can be applied to large-scale virus-detection programs since it is effective, inexpensive and easily standardizable for its application in a routine laboratory involved in water quality.

5.3 The molecular qPCR protocols applied are useful tools for the rapid detection and quantification of viruses from sewage samples.

5.4. In the present study, the CV measurement is proposed as an essential parameter in the evaluation and comparison of methods that have been developed to detect viruses in water samples. In addition, $CV \times 100 \leq 50\%$ is the proposed limit to indicate the

reproducibility and efficiency of a method designed for concentrating and detecting viruses in water samples.

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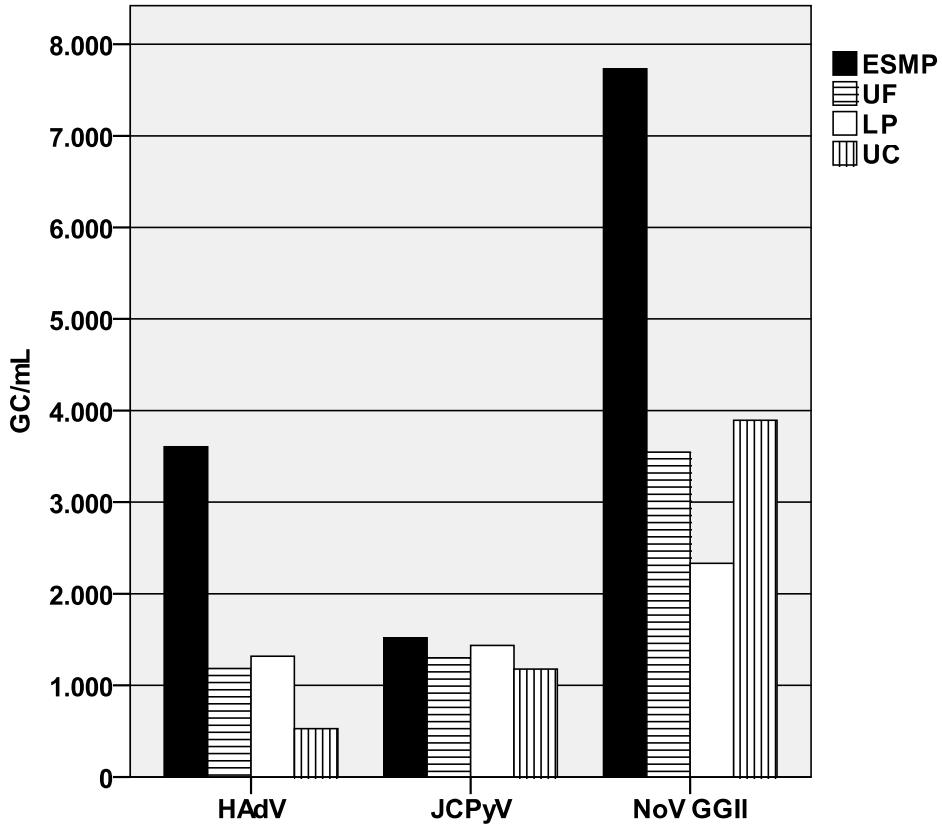

Fig. 1. Evaluation of four different methods for virus concentration from raw sewage. **UC:** ultracentrifugation method; **UF:** ultrafiltration method; **ESMP:** elution skimmed-milk procedure; **LP:** lyophilization method; **GC/mL:** genomic copies/milliliter; **HAdV:** human adenovirus; **JCPyV:** JC polyomavirus; **NoV GGII:** norovirus genogroup II. Bars show means of quantitation.

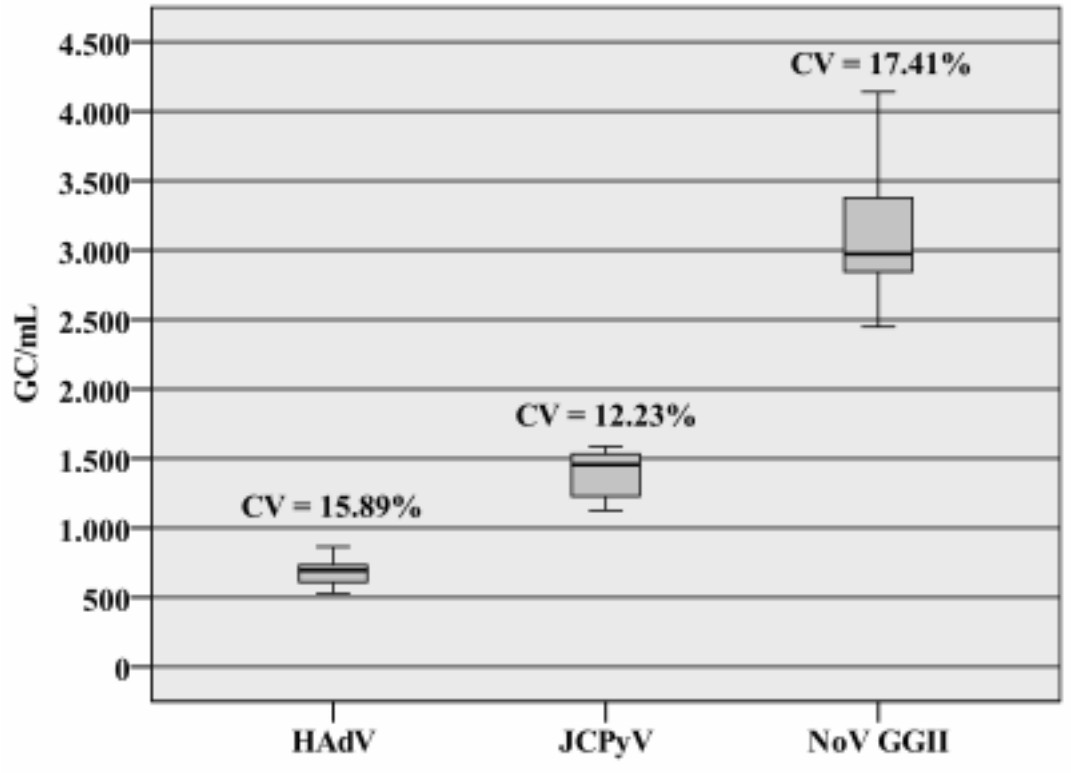

Fig. 2. Repeatability assay for ESMP evaluated with HAdV (DNA), JCPyV (DNA) and NoV GGII (RNA). **HAdV:** human adenovirus; **JCPyV:** JC polyomavirus; **NoV GGII:** norovirus genogroup II; **CV:** coefficient of variation percentage; **GC/mL:** genomic copies/milliliter. The box plots show the first (bottom of box) and third (top of box) quartiles (equivalent to the 25th and 75th percentiles), the median (the horizontal line in the box) and the range (excluding outliers and extreme scores).

Table 1.
Quantitation of viruses in raw-sewage samples by four different concentration methods.

Virus	Method	qPCR (GC/mL)					
		Mean	**SD**	**Minimum**	**Maximum**	**Rank[a]**	**P-Value**
HAdV	ESMP	3.6×10^3	1.8×10^3	1.9×10^3	6.4×10^3	3.60	0.033
	UF	1.2×10^3	7.2×10^2	3.9×10^2	1.9×10^3	2.60	
	LP	1.3×10^3	1.2×10^3	4.4×10^2	2.9×10^3	2.60	
	UC	5.3×10^2	3.2×10^2	2.3×10^2	1.0×10^3	1.20	
JCPyV	ESMP	1.5×10^3	5.7×10^2	9.9×10^2	2.4×10^3	2.80	0.948
	UF	1.3×10^3	7.9×10^2	4.7×10^2	2.4×10^3	2.40	
	LP	1.4×10^3	4.5×10^2	7.8×10^2	1.9×10^3	2.40	
	UC	1.2×10^3	6.6×10^2	4.2×10^2	2.1×10^3	2.40	
NoV GGII	ESMP	7.7×10^3	3.2×10^3	2.7×10^3	1.2×10^4	3.60	0.077
	UF	3.5×10^3	4.2×10^3	1.1×10^3	1.1×10^4	1.80	
	LP	2.3×10^3	1.3×10^3	5.4×10^2	4.0×10^3	1.80	
	UC	3.9×10^3	2.0×10^3	2.3×10^3	7.3×10^3	2.80	

UC: ultracentrifugation method; **UF:** ultrafiltration method; **ESMP:** elution skimmed-milk procedure; **LP:** lyophilization method; **HAdV:** human adenovirus; **JCPyV:** JC polyomavirus; **NoV GGII:** norovirus genogroup II; **GC/mL:** genomic copies/milliliter; **SD:** standard deviation.
[a]Friedman's statistical ranks were assigned using the better yield method.