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Evaluation of concentration efficiency of the *Pseudomonas aeruginosa* phage PP7 in various water matrixes by different methods

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Abstract Enteric viruses monitoring in surface waters requires the concentration of viruses before detection assays. The aim of this study was to evaluate different methods in terms of recovery efficiencies of bacteriophage PP7 of *Pseudomonas aeruginosa*, measured by real-time PCR, using it as a viral control process in water analysis. Different nucleic acid extraction methods (silica–guanidinium thiocyanate, a commercial kit (Qiagen Viral RNA Kit) and phenol–chloroform with alcohol precipitation) exhibited very low recovery efficiencies (0.08–4.18 %), being the most efficient the commercial kit used for subsequent experiments. To evaluate the efficiency of three concentration methods, PBS (as model for clean water) and water samples from rivers were seeded to reach high (HC, 10^6 pfu ml^{-1}) and low concentrations (LC, 10^4 pfu ml^{-1}) of PP7. Tangential ultrafiltration proved to be more efficient (50.36±12.91, 17.21±9.22 and 12.58±2.35 %

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for HC in PBS and two river samples, respectively) than adsorption–elution with negatively charged membranes (1.00±1.34, 2.79±2.62 and 0.05±0.08 % for HC in PBS and two river samples, respectively) and polyethylene glycol precipitation (15.95±7.43, 4.01±1.12 and 3.91±0.54 %, for HC in PBS and two river samples, respectively), being 3.2–50.4 times more efficient than the others for PBS and 2.7–252 times for river samples. Efficiencies also depended on the initial virus concentration and aqueous matrixes composition. In consequence, the incorporation of an internal standard like PP7 along the process is useful as a control of the water concentration procedure, the nucleic acid extraction, the presence of inhibitors and the variability of the recovery among replicas, and for the calculation of the sample limit of detection. Thus, the use of a process control, as presented here, is crucial for the accurate quantification of viral contamination.

Keywords Virus concentration · Surface water · PP7 · Absorption/elution · Polyethylene glycol · Ultrafiltration · qRT-PCR

Introduction

Gastroenteritis is one of the most common causes of morbidity and mortality among children under the age of five in the developing world, with diarrhoea and vomiting being the prominent symptoms. The World Health Organization (WHO) estimates that about 1.5 million deaths per year from diarrhoeal diseases, mainly in children, are attributable to environmental factors such as contaminated drinking water, poor sanitation and poor hygiene (Prüss-Üstün and Corvalán 2006). This WHO report observes that a large portion of the total burden of diarrhoeal disease is caused by fecal–oral pathogens from both human and animal sources. The list of pathogens can be very extensive and includes bacteria, viruses and parasites.

The microbiological quality of water was traditionally assessed only by the determination of indicator bacteria. Use of this specific methodology may be legally required. However, as bacterial counts do not correlate with the presence of viruses or parasites (Schroeder et al. 2002; Pusch et al. 2005), these other pathogens also need to be specifically monitored (Ferguson et al. 1996). Among the potential viral contaminants are rotavirus, adenovirus, enterovirus, norovirus, hepatovirus and astroviruses. The detection of viruses has traditionally been done by culture methods, requiring long times to obtain results and being restricted to the viruses that can grow in tissue culture. In recent years molecular methods have made great progress, in particular the polymerase chain reaction (PCR) that allows the specific and fast detection of a target viral DNA sequence. Additional advantages have come from the development of real-time PCR (qPCR), which allows quantification using standards. Numerous studies are currently being conducted in order to set up proper methodology for virus detection and quantification in water samples, including the best methods to increase target virus recovery efficiency.

Many of the enteric viruses excreted in human feces are present in wastewater. Even after the usual wastewater treatment processes (Skraber et al. 2004; Blatchley et al. 2007; Petrinca et al. 2009), these viruses may be discharged to surface waters. A few viral particles may produce disease, even though the concentration may be below their detection limit (Straub and Chandler 2003). Thus, it is usually necessary to concentrate the viruses from a large volume of water before detection assays are applied, and the selection of the method to achieve this becomes a crucial step in water analysis. There are different approaches available for concentration of viruses in water, based on different properties of virus particles, and each technique may also have numerous variations.

Adsorption/elution methods are based on viral particle properties such as polarity and hydrophobicity that allow viruses to adsorb to a wide variety of charged matrixes. Virus adsorption to microporous filters is promoted by electrostatic interactions between the viruses and the filters, while the addition of salts can also strengthen hydrophobic interactions in a pH-dependent manner (Farrah 1982; Lukasik et al. 2000). As viruses are reversibly adsorbed, they can be eluted in small volumes of solutions, thereby concentrating them during this process.

Precipitation with polyethylene glycol (PEG) may also be used to concentrate viruses from water samples. Precipitation by PEG is due primarily to excluded volume effects. Proteins are sterically excluded from regions of the solvent occupied by the inert synthetic polymers and are thus concentrated until their solubility limit is exceeded and precipitation occurs (Atha and Ingham1981).

Ultrafiltration (UF) is a separation method based on size. The selection of the proper pore size allows for the simultaneous removal and concentration of larger

molecules, particles or even microorganisms like viruses, bacteria and parasites from large volumes of water samples (Morales-Morales et al. 2003).

The aim of the present study was to evaluate different existing methods for the concentration of the bacteriophage PP7 of *Pseudomonas aeruginosa* used as a viral control process in water analysis.

Materials and methods

The general strategy of this work comprised experiments to determine the viral recovery efficiency of different concentration methods of viruses from water. PP7 (ATCC 15692-B2), an RNA bacteriophage of *P. aeruginosa*, was used as a virus surrogate for all of the experiments.

To enable comparisons among three different concentration methods, PP7 was inoculated in PBS (as a model of clean water, shared by all the laboratories). In addition, two water samples from local rivers (upstream and downstream a wastewater discharge) were also inoculated. Each concentration method was applied to these three water matrixes in the laboratories of the Consortium. Aliquots of the inoculated samples and the concentrates were shipped to Salta laboratory for evaluation of the viral recovery efficiency. With this purpose, the RNA from the samples were extracted by the method previously evaluated as the most efficient and quantified by TaqMan RT-PCR.

Consortium

The consortium performing these studies consists of five partner laboratories from three different regions in Argentina. From Buenos Aires City (denominated as BA), the participants were Universidad Nacional de Buenos Aires, Instituto Nacional de Enfermedades Infecciosas INEI-ANLIS Dr. Carlos G. Malbrán, and Prefectura Naval Argentina. From Córdoba Province (denominated as CO), the laboratory was Universidad Nacional de Córdoba. Finally, from Salta Province (denominated as SA), the participant laboratory was Universidad Nacional de Salta.

Efficiency of nucleic acid extraction methods

The nucleic acid extraction efficiency was evaluated for three different methods (EM). Only PBS samples, as a model of clean water (best-case scenario), spiked with high (10^{10} pfu ml^{-1}) and low (10^{6} pfu ml^{-1}) concentrations of PP7 were used. To calculate the efficiency of each process, as described in the next sections, the RNA was quantified by qRT-PCR (Rajal et al. 2007).

EM-BA

The method, previously described by Boom et al. (1990), consisted of nucleic acid purification with silica particles in the presence of the chaotropic agent guanidinium thiocyanate.

EM-SA

Qiagen Viral RNA Kit (Qiagen, Valencia, CA) was used according to the manufacturer's directions.

EM-CO

Viral nucleic acids were extracted by the phenol–chloroform method followed by alcohol precipitation according to standard procedures (Perry et al. 1972).

The efficiency of each extraction method was calculated as the ratio of the number of plaque forming units (pfu) in the qPCR reaction mixture (5 μl of sample after the reverse transcription) determined experimentally (N^{e}) and the theoretical number (N^{t}), according to

$$E_{ex}(\%) = \frac{N^{e}}{N^{t}} \times 100. \quad (1)$$

The efficiencies of the reverse transcription and the qPCR reactions were verified to be 100 %. The number of plaque forming units in the qPCR reaction mixture was determined from the standard curve as

$$N^{e} = 10^{(36.82 - Ct^{e}/3.25)} \quad (2)$$

while the theoretical number was calculated assuming 100 % extraction efficiency

$$N^{t} = \frac{C_{v}}{1000} \times \frac{V_{i,ex}}{V_{f,ex}} \times \frac{V_{i,rt}}{V_{f,rt}} \times 5 \quad (3)$$

where C_v (pfu milliliter^{-1}) is the concentration of genomic copies in the samples analyzed. The variables V_i and V_f (both in microliters) are the initial and final

volumes at the extraction (*ex*) and reverse transcription (*rt*) steps.

The extraction method found as the most efficient in terms of recovery of nucleic acids was selected to be used in all the locations to continue the experiments.

Two-tube TaqMan RT-PCR (qRT-PCR)

This procedure consists of two stages: reverse transcription to produce complementary DNA (cDNA) and amplification–detection with TaqMan PCR.

Production of cDNA

Five microliters of nucleic acids extracted was added to 15 μl of the reaction mixture using a commercial kit (Invitrogen Superscript III). Final concentrations were 1X RT buffer, 500 μmol l^{-1} dNTPs, 5 mmol l^{-1} $MgCl_2$, 2 U μl^{-1} RNaseOUT, 10 U μl^{-1} SuperScript III and 2.5 ng μl^{-1} of random hexamers. cDNA was synthesized by incubating the mixture at 50 °C for 50 min, followed by an incubation step at 85 °C for 5 min to inactivate the RT enzyme.

TaqMan PCR

Each 25 μl of PCR reaction mixture contained 12.5 μl of commercially available 2X TaqMan® Universal PCR Master Mix (Applied Biosystems) with 1000 nmol l^{-1} each of forward (GTTATGAACCAATGTGGCCGT TAT, PP7R-247f) and reverse (CGGGATGCCTCT GAAAAA AG, PP7R-320r) primers, 300 nmol l^{-1} probe (6-FAMTCGGTGGTCAACGAGGAACTG GAAC-TAMRA, PP7R-274p), 5 μl of the cDNA sample, and water to complete the volume (Rajal et al. 2007). The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI PRISM 5700 Sequence Detection System, Applied Biosystems). Standard amplification conditions were used: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The Ct values were calculated using baseline values of 6–15 and a threshold of 0.05.

Water samples

Surface water can usually contain different inorganic and organic compounds as well as many target and non-target organisms that can interfere with the detection of viruses. A phosphate buffer solution (PBS) was used as a basic clean water matrix for the three concentration methods. In addition to PBS, water samples from the rivers that will be studied in the future for viruses at each location were collected upstream (US, as a model of real "clean" water matrix) and downstream (DS, as a model of real "dirty" water matrix) of a wastewater discharge. Basic physicochemical (temperature, pH, turbidity, conductivity and dissolved oxygen) and microbiological (total and fecal coliforms) characterization was carried out.

All the samples (PBS, US, DS) were seeded to reach high and low concentrations of PP7, and concentrated using any of the methods described in the next sections.

The use of PBS as a water matrix had two objectives. The first one was to compare the efficiency of the different concentration methodologies in the best-case scenario (clean water, no inhibition). The second goal was to use it as a reference at each location to evaluate the effect of the aqueous matrix on the virus concentration process, when compared to real water samples.

Simulation

A simulation of the concentration processes was performed assigning efficiencies to every step involved in order to ensure that at least the viral detection limit could be reached to allow for virus quantification. Thus, at least 5 pfu should be in the qPCR reaction mixture, which means 5.7×10^5 pfu ml^{-1} in the concentrated water, assuming that the efficiency of nucleic acid extraction is 10 % (Rajal et al. 2007).

The simulation was carried out for high and low seed levels corresponding to high (HC) and low (LC) initial concentrations of viruses in the environment to evaluate the effect of the initial virus concentration on their recovery. Samples were spiked with a known amount of PP7 bacteriophage to reach the established HC (10^6 pfu ml^{-1}) and LC (10^4 pfu ml^{-1}).

Evaluation of the concentration method

Three concentration methods (CM) were analyzed at the different locations.

CM-BA

The viral particles present in the samples were concentrated by the adsorption–elution method on negatively charged membranes, with the insertion of an

acid rinse step for the removal of cations, as described previously (Katayama et al. 2002), with minor modifications. Briefly, 30 ml of $MgCl_2$ 2.5 mol l^{-1} was added to 10 l of sample to a final concentration of 7.5 mmol l^{-1}, and the pH was adjusted to 5 with 37 % HCl. The sample was then filtered through glass fiber prefilters (MSI, G15WP14225) and a negatively charged membrane (Millipore, HAWP14250). After filtration, prefilters were removed, and the membrane was rinsed with 350 ml of 0.5 mmol l^{-1} H_2SO_4 (pH 3.0). The viral concentrate was obtained by elution with 15 ml of 1 mmol l^{-1} NaOH. The eluate was further concentrated using an Amicon Ultra® Millipore filter (50 kDa) to a final volume of 3 ml.

CM-CO

Concentration of specimens was performed using the PEG precipitation method previously described by Lewis and Metcalf (1988) and Greening et al. (2002), modified by Huang et al. (2005). The 1.5-l water samples were concentrated 100-fold to 15 ml by high-speed centrifugation (two centrifugation steps, each of 10,700*g* for 20–25 min), elution (two steps at room temperature for 1 h) and PEG precipitation (10 % PEG 6000, overnight at 4 °C). The concentrated samples were further treated with chloroform in order to obtain a clear sample for cell-culture virus isolation.

CM-SA

Twenty liters of water samples was filtered through two stainless-steel sieves (74 and 37 μm) to remove solids. Then it was pumped through the ultrafiltration unit with a 50,000-MW membrane cut-off (Microza AHP 1010, Pall Life Sciences, East Hills, NY), until the volume was reduced to about 30 ml. Two elution steps with 0.05 mol l^{-1} glycine/NaOH pH 7.0 and 0.1 % Tween 80 were performed to increase the PP7 recovery. The final concentrated sample, 50–70 ml, consisted of the mixture of the eluate from the ultrafiltration unit plus the final retentate.

The final concentrated samples from BA, CO and SA were analyzed in Salta. The nucleic acids were extracted using a Qiagen Viral RNA Kit (Qiagen, Valencia, Spain), found as the most effective extraction method, according to the manufacturer's directions, and the RNA was used for detection by qRT-PCR.

The percentage of recovery (R) for viruses in each case was calculated as the ratio of the number of plaque forming units in the concentrate (C) and the feed solution (F), according to

$$R\,(\%) = \frac{\frac{V^F_{\mathrm{i,ex}}}{V^F_{\mathrm{f,ex}}} \times E^F_{\mathrm{ex}} \times \frac{V^F_{\mathrm{i,rt}}}{V^F_{\mathrm{f,rt}}} \times E^F_{\mathrm{rt}} \times \frac{1}{\mathrm{DF}^F} \times \left(1 + E^F_{\mathrm{am}}\right)^{\mathrm{Ct}^F}}{\frac{V^F}{V^C} \times \frac{V^C_{\mathrm{i,ex}}}{V^C_{\mathrm{f,ex}}} \times E^C_{\mathrm{ex}} \times \frac{V^C_{\mathrm{i,rt}}}{V^C_{\mathrm{f,rt}}} \times E^C_{\mathrm{rt}} \times \frac{1}{\mathrm{DF}^C} \times \left(1 + E^C_{\mathrm{am}}\right)^{\mathrm{Ct}^C}} \times 100 \tag{4}$$

where V (milliliters) is volume and E is efficiency. The subscripts are i for initial, f for final, ex for extraction, rt for reverse transcription and am for amplification. The Ct values correspond to reactions without inhibition. Some samples contain inhibitors that affect the efficiency of amplification; the dilution factor (DF) is the dilution needed to overcome inhibition. As an example, if it is necessary to make a 1/10 dilution to be within the linear region in the graph of Ct versus Log(Concentration), then DF=10.

The general equation (Eq. 4) can be simplified if the following assumptions are made: $E^F_{\mathrm{rt}} = E^C_{\mathrm{rt}}$, $E^F_{\mathrm{rt}} = E^C_{\mathrm{rt}}$, and $E^F_{\mathrm{am}} = E^C_{\mathrm{am}} = 1$, the last verified experimentally.

Calculation of sample limit of detection (S_{LOD})

The limit of detection is the minimum viral concentration that is needed in the original sample in order to be detectable following a certain procedure. This concentration depends on the characteristics of the sample that affect the recovery and on the variables involved in the extraction, reverse transcription and limit of detection for real-time PCR. The S_{LOD} (in pfu milliliter^{-1}) for each sample was calculated according to Rajal et al. (2007):

$$S_{\mathrm{LOD}} = \frac{A_{\mathrm{LOD}}}{\frac{V^F}{V^C} \times \frac{R}{100} \times \frac{V^C_{\mathrm{i,rt}}}{V^C_{\mathrm{f,rt}}} \times E^C_{\mathrm{rt}} \times \frac{V^C_{\mathrm{i,ex}}}{V^C_{\mathrm{f,ex}}} \times E^C_{\mathrm{ex}} \times \frac{1}{\mathrm{DF}^C}} \times 1000 \tag{5}$$

where A_{LOD} (5 pfu μl^{-1}) is the assay limit of detection for this system by real-time PCR.

Statistical analysis

Recoveries results were analyzed by one-way ANOVA with Tukey–Kramer multiple comparisons test as a post-test, implemented with GraphPad InStat v3.01 software.

Results

The water matrixes used for the evaluation of the different concentration methods were physicochemically and microbiologically characterized (Table 1) in order to evaluate the possible effects of their composition on the recovery of viruses.

The simulation performed (Table 2) allowed us to plan the concentration experiments to ensure that the detection limit was reached at each condition. This permitted us to quantify the viral concentration and the recovery of each concentration method. With the assumptions of the efficiencies involved in the processes (global recovery variable obtained after assuming efficiencies for every step of each concentration method, 10 % for nucleic acid extraction, 100 % for the reverse transcription and for the amplification) and with the volume reduction factor corresponding to each procedure, the sample detection limits were calculated.

The application of each of the three RNA isolation procedures showed very low recovery efficiencies, between 0.08 and 4.18 % (Table 3), which increased for higher target concentrations. The EM-SA method (Qiagen kit), found the most efficient, was used to quantify PP7 to evaluate the concentration methods.

The ability to recover viruses (measured as viral RNA) from spiked samples using the three water concentration protocols is presented in Table 4. A high variability of the recovery was observed for all the replicas, giving standard deviations that are of the same magnitude as the measurement, especially for the CM-BA. However, differences among the efficiencies of the three methods are clearly observed. The higher recovery of the CM-SA (with HC and LC of PP7) compared with the other two methods was statistically confirmed.

Discussion

Concerning the different RNA isolation procedures (using PBS as a model of clean water sample), very low recovery efficiencies were observed. They ranged between 0.08 and 4.18 % (Table 3), which increased for higher target concentrations. The EM-SA method was 3.7 and 31.3 times more efficient than the EM-CO and EM-BA, respectively, for the lower target concentration, while it was 2.7 and 19.3 times more efficient than EM-CO and EM-BA, respectively, for the higher PP7 concentration. The EM-CO was 8.4 and 7.1 times more efficient than the EM-BA method for low and high concentrations, respectively. However, it is important to note that even though the efficiency of the EM-CO is lower than that for EM-SA, for the high target concentration the number of viral particles that is actually in the qPCR reaction is higher due to the volume reduction factor (= 5) inherent to the method when the elution is performed (100 μl to 20 μl, Table 3).

Table 1 Characterization of the different water matrixes: sterile phosphate saline buffer (PBS), upstream (UP) and downstream (DS) from a wastewater treatment plant at each location: De la Plata River (BA), Suquía River (CO), Arenales River (SA)

Parameter	PBS	BA		CO		SA	
		US	DS	UP	DS	UP	DS
Temperature (°C)	17.9 (0.7)	19.4 (0.22)	20 (0.22)	ND	ND	23.9 (0.8)	24.5 (1.1)
pH	7.07 (0.09)	6.88 (0.16)	7.19 (0.16)	7.36 (0.06)	7.45 (0.19)	7.83 (0.18)	7.68 (0.37)
Turbidity (NTU)	0	47	36	ND	ND	6 (2)	598 (46)
Conductivity ($mS\ cm^{-1}$)	1.78 (0.112)	0.204 (0.046)	0.418 (0.046)	ND	ND	0.146 (0.004)	0.177 (0.024)
Dissolved oxygen ($mg\ l^{-1}$)	7.74 (0.06)	7.32 (0.6)	3.68 (0.6)	7.63 (0.12)	3.57 (0.25)	9.27 (0.45)	7.77 (0.11)
Total coliforms (in 100 ml)	0	460	2.4×10^{6}	2.0×10^{4} (5.0×10^{3})	1.3×10^{5} (1.5×10^{4})	4.6×10^{3} (1.3×10^{3})	2.1×10^{4} (4.0×10^{3})
Fecal coliforms (in 100 ml)	0	150	2.4×10^{6}	1.9×10^{2} (4.0×10^{1})	7.0×10^{3} (2.0×10^{3})	4.6×10^{3} (1.3×10^{3})	2.1×10^{4} (4.6×10^{3})

The averages of three replicate samples are presented, with the standard deviation in parenthesis

ND not determined

Table 2 Values obtained from initial simulation of the process including the concentration methods (CM), the nucleic acid extraction (NA), and the reverse transcription (RT) and real-time PCR (qPCR) reactions

	BA	CO	SA
Lowest global recovery efficiency assumed for CM (%)[a]	38.9	21.3	51.0
Initial C_v—HC seeded (pfu ml^{-1})	1.0×10^{6}	1.0×10^{6}	1.0×10^{6}
Initial C_v—LC seeded (pfu ml^{-1})	1.0×10^{4}	1.0×10^{4}	1.0×10^{4}
Sample volume (l)	10	1.5	20
Volume reduction factor (sample volume/final volume)	3333	100	286
NA efficiency assumed (%)	10	10	10
C_v after NA extraction—from HC seeded (pfu ml^{-1})	8.6×10^{7}	3.7×10^{6}	2.6×10^{7}
C_v after NA extraction—from LC seeded (pfu ml^{-1})	8.6×10^{5}	3.7×10^{4}	2.6×10^{7}
RT efficiency assumed (%)[b]	100	100	100
C_v in RT reaction mixture—from HC (pfu ml^{-1})	2.1×10^{7}	9.3×10^{5}	6.4×10^{6}
C_v in RT reaction mixture—from LC (pfu ml^{-1})	2.1×10^{5}	9.3×10^{3}	6.4×10^{4}
qPCR efficiency assumed (%)[b]	100	100	100
C_v in qPCR mixture—from HC (pfu ml^{-1})	4.3×10^{6}	1.9×10^{5}	1.3×10^{6}
C_v in qPCR mixture—from LC (pfu ml^{-1})	4.3×10^{4}	1.9×10^{3}	1.3×10^{4}
Sample detection limit (pfu ml^{-1})	17.6	1072.1	156.8

C_v: PP7 concentration

[a]Obtained after assuming efficiencies for every step of each concentration method

[b]Assumed as 100 % according to the value obtained experimentally from slope of the standard curved for PP7

The efficiencies of extraction were evaluated using PBS as the sample medium, which probably represents the best-case scenario. For real environmental samples, which contain other chemicals and non-target nucleic acids that may interfere in the process, the methods are expected to have even lower recoveries, or there could be changes in the relative efficiencies among the methods, due to different abilities to eliminate the inhibitors.

With reference to the ability to recover PP7 viruses (measured as viral RNA) from spiked samples, ultrafiltration, CM-SA (with HC and LC of PP7), was the most efficient among the three procedures (Table 4).

Numerous studies have shown that ultrafiltration (UF) is a reliable and consistent method for sample concentration when applied to natural waters (Paul et al. 1991; Winona et al. 2001) or to concentrate different animal viruses from infected cells during the production of viruses (Weiss 1980; Subramanian et al. 2005). In our work ultrafiltration (CM-SA) was between 3.2 (compared to CM-CO, HC) and 50.4 (against CM-BA, HC) times more efficient than the other methods for PBS matrix (model of clean water, best scenario), 4.3–6.2 times more efficient than the other methods for US and 2.7 (compared to CM-CO, LC) to 252 (against CM-BA, HC) times better for DS.

Virus recovery rate depends upon the level of contamination of the water source, since virus recovery decreased in complex matrixes (river samples, US and DS) compared with simpler matrixes (PBS) for the three concentration methods. The recoveries in most

Table 3 Efficiencies (E_{ex}) of each extraction method (EM) used for the study

C_v (pfu ml^{-1})	EM	$V_{i,ex}$ (µl)	$V_{f,ex}$ (µl)	$N_{i,ex}$ (pfu)	N^t (pfu)	Ct^t	Ct^e (SD)	N^e (pfu)	E_{ex} (%)
10^6	EM-SA	140	80	1.4×10^{5}	2188	25.96	31.14 (0.25)	56	2.55
10^6	EM-CO	100	20	1.0×10^{5}	6250	24.48	31.51 (0.22)	43	0.69
10^6	EM-BA	100	60	1.0×10^{5}	2083	26.02	36.07 (0.47)	2	0.08
10^{10}	EM-SA	140	80	1.4×10^{9}	2.2×10^{7}	12.96	17.44 (0.16)	9.1×10^{5}	4.18
10^{10}	EM-CO	100	20	1.0×10^{9}	6.3×10^{7}	11.48	17.38 (0.15)	9.6×10^{5}	1.53
10^{10}	EM-BA	100	60	1.0×10^{9}	2.1×10^{7}	13.03	21.69 (0.06)	4.5×10^{4}	0.22

PBS was used in all locations as a model of clean water sample, representing the best-case scenario

C_v viral concentration, V volume, N number of viral particles, Ct threshold cycle. Sub-indexes: i initial, f final, ex extraction. Supra-indexes: t theoretical, e experimental

Table 4 Recoveries of the concentration methods (CM) studied for different water matrixes: phosphate saline buffer (PBS), upstream (US) and downstream (DS) from a wastewater treatment plant at each location

Procedure	Sample	HC (10^6 pfu ml^{-1})		LC (10^4 pfu ml^{-1})	
		Range	Average ± SD	Range	Average ± SD
CM-BA	PBS	0.11–2.75	1.00±1.34[a]	0.49–7.37	2.79±3.96[b]
	US	0.40–5.74	2.79±2.62	ND	ND
	DS	0.00002–0.14	0.05±0.08	ND	ND
CM-CO	PBS	10.56–24.42	15.95±7.43[c,e,f]	2.16–3.86	2.94±0.86[d]
	US	2.98–5.21	4.01±1.12[e]	2.54–7.07	4.78±2.27
	DS	3.31–4.35	3.91±0.54[f]	1.35–6.11	2.97±2.72
CM-SA	PBS	42.76–65.26	50.36±12.91[a,c,g,h]	24.54–33.68	28.56±4.67[b,d]
	US	7.95–26.39	17.21±9.22[g]	20.87–24.03	22.45±2.23
	DS	9.87–14.05	12.58±2.35[h]	4.18–10.58	7.87±3.31

All the samples were spiked with PP7 to reach a high concentration (HC) and a low concentration (LC). The average and standard deviation (SD) were calculated from three replicate samples

Superscript letters indicate the pair of compared results that were considered statistically different

ND not detected

[a] CM-SA vs CM-BA, $p<0.01$

[b] CM-SA vs CM-BA, $p<0.01$

[c] CM-SA vs CM-CO, $p<0.05$

[d] CM-SA vs CM-CO, $p<0.01$

[e] CM-CO-PBS vs CM-CO-US, $p<0.05$

[f] CM-CO-PBS vs CM-CO-DS, $p<0.05$

[g] CM-SA-PBS vs CM-SA-US, $p<0.05$

[h] CM-SA-PBS vs CM-SA-DS, $p<0.01$

of the cases were higher for PBS, followed by US and finally DS. The US water samples were of better quality than the DS samples. However, these differences were statistically significant only when the PBS matrixes were compared with both river matrixes.

The studied rivers clearly deteriorate along their path, increasing the pH in BA and CO, the turbidity in SA and the conductivity in BA and SA, decreasing the dissolved oxygen in all the cases, but most dramatically in BA and CO, and increasing the bacterial concentration in all the cases (see total and fecal coliform counts in Table 1), but especially in BA. The increase of particles and chemical species in the water was accompanied by decreased virus recovery. When filters are used to separate by size, like in the last step of the CM-BA and in CM-SA, fouling affects the membranes. That effect is partially reduced in SA by performing an elution step to resuspend materials that could have remained attached to the membranes and to the line.

For the ultrafiltration method, virus loss was expected to be small, since the virus particles are much larger than the selected pore size of the filter; however, the results obtained here show that the losses are significant and that there is also a great variability between replicate samples. Unlike other filtration systems, recovery of viruses using tangential filtration is less affected by complex chemical constituents found in natural water, as shown in other studies where viral recovery has been determined by conventional plaque assay methods, qualitative PCR and qPCR (Morales-Morales et al. 2003; Oshima 2001; Winona et al. 2001; Rajal et al. 2007). The recoveries obtained in this work are comparable to those obtained from Albinana-Gimenez et al. (2009) for the JC Polyomavirus using UF, and much higher than the ones obtained for adenovirus.

PEG precipitation has been shown to be a rapid, technically simple and relatively effective method for concentration of virus in eluates from water. The

method is flexible and inexpensive and can be used without extensive pH treatment or use of multiple reagents. Polyethylene glycol is a nontoxic water-soluble synthetic polymer that is widely used by biochemists, pharmacologists and virologists (Atha and Ingham 1981; Polson et al. 1964; Lewis and Metcalf 1988; Muller et al. 2009; Sheih et al. 1997). PEG–protein interaction is relatively insensitive to changes in solution conditions including pH, temperature and ionic strength up to 0.15 mol l^{-1}. This observation suggests that attractive or repulsive forces between PEG and protein are relatively unimportant in the precipitation mechanisms (Atha and Ingham 1981). Nevertheless, the protein charge has been emphasized as a secondary mechanism of protein precipitation by PEG treatment (Lee and Lee 1981). In this way, more highly charged or hydrophobic proteins will be more easily precipitated than those of lesser charge. This conclusion suggests that each particular interaction between PEG and virus needs to be evaluated.

On the other hand, a double chloroform organic treatment of the PEG concentrated samples results in a significant loss of PP7 phage (about 20 % of the spiked amount, data not shown). High rates of virus detection in cell cultures have been reported after chloroform treatment of PEG concentrates (Muller et al. 2009). In this way, the efficacy of PP7 recovery by the PEG method revealed the recovery rate that can be reached for viable virus.

For protocols using charged membranes, the limiting steps in viral recovery are considered to be adsorption and desorption onto the membrane. Soluble organic matter in water may interfere with their effectiveness by competing with viruses for adsorption sites, by forming complexes with virus particles thereby altering their ability to adsorb and by accumulating so extensively on filter surfaces that they interfere with virus elution (Sobsey and Hickey 1985). Thus, the presence of organic compounds in the water matrix of environmental samples can constitute a possible cause for the reduced recovery of PP7. Since the properties of viral particles vary among viruses from different families (e.g., polarity, surface charge density, hydrophobicity), the interactions of distinct viruses with charged membranes in the presence of suspended solids and soluble organics could be dissimilar, differentially influencing the rate of recovery of each different virus (Sobsey and Glass 1984). So, different behaviors could be expected for other viruses different from PP7. Méndez et al. (2004) reported higher recoveries than in this work for the concentration of different bacteriophages although the variability was larger. For human enteric viruses, the recovery broadly varies depending on the virus investigated, on the source of the environmental water and on the $MgCl_2$ concentration (Victoria et al. 2009).

Concentration steps led not only to the enrichment of viruses but also of impurities. Inhibitory effects on qRT-PCR were seen in all the environmental samples. A dilution approach was used to adequately account for the inhibition of PP7 quantification by qRT-PCR, but dilution can also negatively influence the detection of enteric viruses since they are commonly present at low concentrations in environmental samples. The quality of the nucleic acid extraction procedure is very important as a step in which qRT-PCR reaction inhibitors may be eliminated.

The assumption of global recovery when the simulation was performed (Table 2) was only accurate for PBS in SA and quite approximate (21.3 assumed versus 15.9 experimental) for PBS in CO, but totally wrong for BA. The low extraction efficiencies and recoveries obtained experimentally increased the detection limit of the samples (Table 5). The experiments performed in BA with LC are presented as non-detected (ND) since the concentration of the spiked feed did not reach the assay limit of detection as assumed. In spite of the low recovery for BA, it should be noted that in some cases the actual number of viral particles in the PCR reaction for the concentrated sample was higher than for SA and CO. This is due to the high volume reduction factor inherent to the concentration method that compensates for the losses during the concentration and the nucleic acid extraction. These variables, and also the inhibition factor, are sample-dependent and were considered for the calculation of the S_{LOD} (Eq. 5) for the spiked surrogate PP7 (Rajal et al. 2007) showing lower values for BA in some cases (Table 5) in spite of the low recovery.

These observations point up the risks involved in extrapolating data from investigations with any single water type to others, since virus recovery efficiencies are certainly influenced by water quality. In consequence, the incorporation of an internal standard like PP7 in all the processes is useful as a control of the water concentration procedure, the nucleic acid extraction and the presence of inhibitors. It is additionally important due to the high variability of the recovery observed among replicas.

Table 5 Theoretically (by simulation) and experimentally determined sample limits of detection (S_{LOD}) values

	S_{LOD} (pfu ml^{-1})		
	BA	CO	SA
Theoretical	17.6	1072.1	156.8
Experimental			
For HC			
PBS	2692.4	5626.2	622.6
US	965.02	22378.6	1824.2
DS	53848.3	22950.9	2490.2
For LC			
PBS	965.02	30523.2	1097.1
US	ND	18773.7	1394.5
DS	ND	30214.9	3971.8

The calculations took into account the volume reduction factor, the efficiency for each concentration method and the efficiency of extraction for the EM-SA used in all the experiments

ND not determined (the spiked amount was insufficient to reach the assay detection limit)

Since human enteric viruses in the aquatic environment constitute a risk for human health, reliable, sensitive and practical methods for concentrating and detecting them in water systems are needed.

While molecular detection offers a high level of sensitivity, the viral infectivity is often unknown. However, recent studies suggest that molecular techniques such as quantitative RT-PCR are suitable for viral detection in environmental waters, since a strong correlation between virus infectivity and the amount of viral genome detected was observed (Espinosa et al. 2008). The PEG concentration method coupled with chloroform extraction steps offers a tool to recover both the viral genome and virus particles feasible for culture isolation.

Finally, the use of viral surrogates to assess the performance of different procedures is necessary. Some authors suggest that the best idea is to use those that are as related as possible to the target virus. Such were the cases of mengovirus as surrogate for hepatitis A virus (HAV) studied in clinical and food samples (Costafreda et al. 2006), feline calicivirus for the detection of HAV in food and water samples (Mattison et al. 2009), and murine norovirus (MNV) for human norovirus (NV) to study inactivation in water (Bae and Schwab 2008), among others. Indeed, it is expected that a surrogate that is as closest as possible to the target virus will be the best approximation to the reality. However, the selection of such a model is not easy when it comes to evaluating procedures that will be applied in samples to be studied for different target viruses. In that situation (which is the current case, where the purpose of all the partner laboratories was to detect a variety of viruses, some of them RNA and others DNA, in water), it is not possible to use so many different surrogates, and thus it is practical to introduce a virus as a control process for all the procedures. On the other hand, the use of bacteriophages as surrogates (also for mammalian viruses) is much extended in environmental and medical virology (Mesquita et al. 2010; Rajal et al. 2007; Lute et al. 2004; Oshima. 2001) for several reasons: i) they are innocuous, ii) they can be cultivated to high titres ($> 10^8$/ml) increasing the sensitivity, iii) infectivity results can be obtained faster (in hours against days for the other viruses), iv) phage assays do not require specialized facilities (Aranha-Creado and Brandwein 1999) and finally v) it is not expected to find selected bacteriophages in the environmental waters. *Escherichia coli* K12 phage MS2 is probably the most studied phage including assessment of concentration by filtration procedures (Rhodes et al. 2011), evaluation of the inactivation using different chemicals (D'Souza and Su 2010), electrical discharges (Lee et al. 2011), UV treatment (Park et al. 2011), survival (Horm and D'Souza 2011; Bae and Schwab 2008) and fate and transport (Syngouna and Chrysikopoulos 2010) in the environment. In this work we used the bacteriophage PP7 as a viral control process for the future concentration, extraction and quantitative detection of a variety of enteric viruses. It is most likely that PP7 will have a similar behavior to MS2 since they are both from the genus *Levivirus*, non-enveloped, with RNA genome, and they have the same size (25 nm) and icosahedral shape, reasons for which they were both used as models for polio virus (Aranha-Creado and Brandwein 1999).

In summary, several methods for the extraction of RNA were compared in this work using PBS as a model of clean water sample and PP7 in different concentrations as a virus model. The EM-SA method, which is a commercial kit based on the use of a chaotropic agent and packed silica columns, was found to be the most efficient method although the recovery of virus RNA was extremely low. Also, three viral concentration methods from water samples were

compared. Hollow fiber ultrafiltration was found to be more efficient than adsorption/elution to charged membranes or PEG precipitation for the recovery of phage PP7. The protocols reported here constitute a good approach to the use of an internal standard to validate the entire process as a prerequisite to quantification of human viruses. PP7 recovery efficiencies were calculated by including concentration, extraction and PCR inhibition variables and by using an average correction factor.

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