Journal of Virological Methods 187 (2013) 215–221

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

# New methods for the concentration of viruses from urban sewage using quantitative PCR

Byron Calgua[a], Jesus Rodriguez-Manzano[a], Ayalkibet Hundesa[a], Esther Suñen[b], Miquel Calvo[c], Sílvia Bofill-Mas[a], Rosina Girones[a,*]

[a] Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 643, Barcelona 08028, Spain
[b] Department of Immunology, Microbiology and Parasitology, Faculty of Pharmacy, University of the Basque Country, Paseo de la Universidad 7, Vitoria-Gasteiz 01006, Spain
[c] Department of Statistics, Faculty of Biology, University of Barcelona, Av. Diagonal 643, Barcelona 08028, Spain

*Article history:*
Received 21 March 2012
Received in revised form 18 October 2012
Accepted 24 October 2012
Available online xxx

*Keywords:*
Sewage
Virus concentration method
Human adenovirus
JC polyomavirus
Norovirus

A B S T R A C T

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 observed in 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 working 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.



## 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 Asfarvirus-like virus and the picornavirus Klassevirus, have also been reported in urban sewage (Holtz et al., 2009; Loh et al., 2009). In addition, 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-Mas et al. (2010a,b) 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

* Corresponding author. Tel.: +34 93 402 1483; fax: +34 93 403 9047.
*E-mail address:* rgirones@ub.edu (R. Girones).

0166-0934/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2012.10.012

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-Mas et al., 2006; Ogorzaly et al., 2010; Wong and Xagoraraki, 2011). According to previous studies, HAdV and JCPyV 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, respectively (Bofill-Mas et al., 2006; Fong et al., 2009; Rodriguez-Manzano et al., 2012).

HAdV is grouped in 53 types, 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 and cause 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 occur commonly 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 subdivided further 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. Materials and methods

### 2.1. Sewage samples

Four sets of sewage samples were used in this study. Each sample was harvested in a sterile 1000-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 min 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 to 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 analyzed by qPCR, the volume of sewage sample analyzed 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 8000 × *g* for a further 30 min at 4 °C. The supernatant (150 mL) was transferred to a new centrifuge pot, the pH was adjusted to 3.5 with HCl 1 N, and 1.5 mL of pre-flocculated skimmed-milk solution (final concentration of skimmed-milk 0.01%, w/v) was added. The pre-flocculated skimmed-milk solution (1%, w/v) was prepared in advance according to Calgua et al. (2008) by dissolving 1 g of skimmed-milk powder (Difco, Detroit, MI, USA) in 100 mL artificial seawater and carefully adjusting the pH to 3.5 with HCl 1 N. The sample was then stirred for 8 h to allow the viruses to be adsorbed into the skimmed-milk flocs at room temperature (RT). Then flocs were sedimented by centrifugation at 8000 × *g* for 30 min at 4 °C. The supernatants were carefully removed without disturbing the sediment and the pellet was dissolved in 500 μL of phosphate buffer (pH 7.5).