Journal of Virological Methods xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Extraction and RT-qPCR detection of enteroviruses from solid environmental matrixes: Method decision tree for different sample types and viral concentrations

Julia Martín-Díaz[a,b,*], Francisco Lucena[a,b]

[a] Department of Genetics, Microbiology and Statistics, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain
[b] The Water Research Institute, University of Barcelona, C/Montalegre 6, 08001 Barcelona, Spain

ARTICLE INFO

Keywords:
Enteric viruses
Sediment
Sewage sludge
Soil
Quantitative PCR

ABSTRACT

Quantitative RT-PCR methods (RT-qPCR) are becoming increasingly desirable for the detection of enteric viruses in solid environmental matrixes such as sediments, soils and sewage sludge. However, effective methodologies that allow the extraction of high quality RNA ready for molecular quantification continue to be evaluated. In the present study, four different methods for enterovirus extraction from solid environmental matrixes were compared in terms of viral recovery and inhibitor removal. Three indirect methods based on glycine elution and concentration by ultracentrifugation were tested. The main differences between indirect methods were the sample to glycine buffer ratio, and the ultracentrifugation protocol applied. One commercial direct method was also tested. The indirect methods produced better results than the direct method. The ultracentrifugation led to viral losses in samples with high titers; however, as the virus concentration reduced, the ultracentrifugation became increasingly important for viral recovery. Two commercial RNA extraction kits were also evaluated and it was selected the most effective in removing RT-qPCR inhibitors. The results obtained allowed the development of a method decision tree with three versions that are suitable for different samples and viral concentrations.

1. Introduction

Enteric viruses are released into the environment through fecal material, and once there, they can pose a risk to human and animal health. *Adenovirus*, *Astrovirus*, *Norovirus*, *Enterovirus*, *Hepatovirus* and *Rotavirus* are some of the principal genera of concern (World Health Organization, 2011). The members of the genus *Enterovirus* (family *Picornaviridae*, order *Picornavirales*) are the cause of important diseases and symptoms such as meningitis and encephalitis in children; pneumonia; hand, foot and mouth disease; paralysis; hemorrhagic conjunctivitis; and myocarditis (US Environmental Protection Agency, 2012; World Health Organization, 2011).

Sewage sludge contains large amounts of fecal pathogens, including bacteria, viruses and parasites (Lepeuple et al., 2004; Sidhu and Toze, 2009). One of the preferred disposal options for this residue is land application as organic fertilizer (Kelessidis and Stasinakis, 2012), and therefore adequate hygienization treatments are required to prevent infectious pathogens spreading into the environment. In this context, some regulations require the control of enteroviruses as reference pathogens (Milieu Ltd. et al., 2010; US Environmental Protection Agency, 2003). River, coastal and estuarine sediments are also known to be important reservoirs of fecal pathogens (De Brauwere et al., 2014; Hassard et al., 2016) and enteroviruses have been found in different sediment types (Hassard et al., 2016; LaBelle et al., 1980; Lewis et al., 1985). In addition, soils may also be contaminated with fecal pathogens due to a variety of causes (Bradford et al., 2013; Santamaría and Toranzos, 2003).

Traditionally, enteroviruses have been detected using methods based on cell cultures, whereby samples are placed in contact with cultures of the Buffalo Green Monkey kidney (BGM) cell line (US Environmental Protection Agency, 2003). Although the method performs well, BGM cells are not specific for the genus *Enterovirus*, they are subjected to cytotoxicity effects and the confirmation of results often requires a long time (US Environmental Protection Agency, 2003). All these issues, together with the small numbers usually detected in certain types of environmental samples, have increased the desirability of molecular detection methods and some water regulations already include them (Hassard et al., 2016; Martín-Díaz et al., 2016; Sidhu and Toze, 2009; US Environmental Protection Agency, 2012; Wyn-Jones and Sellwood, 2001). The molecular detection of enteric viruses in solid or semisolid matrixes follows very similar protocols in samples as different as food, sediments, sewage sludge and clinical samples (Knight

* Corresponding author at: Department of Genetics, Microbiology and Statistics, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain.
E-mail address: juliamadi@hotmail.com (J. Martín-Díaz).

http://dx.doi.org/10.1016/j.jviromet.2017.10.004
Received 29 June 2017; Received in revised form 3 October 2017; Accepted 5 October 2017

et al., 2013; Miura et al., 2011; Monpoeho et al., 2001; Stals et al., 2012). Direct methods consist in the extraction of nucleic acids directly from the sample. The general scheme for indirect methods consists in the elution of viral particles (preceded or not by acid adsorption), followed by a concentration step and then finally the extraction of nucleic acids (Miura et al., 2011; Stals et al., 2012). During acid adsorption, an acid buffer is used to promote the association between viral and solid particles; after this, the sample is centrifuged and the supernatant is discarded. In the elution step, viral particles are detached from solids by the combination of an alkaline or neutral buffer and mechanical forces. Magnetic stirring, shaking, vortexing and homogenization with stomacher are frequently used methods, and the selection of one of them often depends on the facilities available or the sample characteristics (Goyal and Aboubakr, 2016; Guzmán et al., 2007). In addition, the buffer is frequently supplemented with proteins or amino acids such as glycine, beef extract or soya protein that exchange for viruses adsorbed to the solid matrix, thereby favoring viral elution. The proportion of sample and elution buffer is another factor to consider: larger quantities of elution buffer are expected to be more effective than smaller ones, but they will produce higher dilution of viral particles. For the concentration step, the most commonly used methods are polyethyleneglycol (PEG) precipitation, organic flocculation, ultracentrifugation and ultrafiltration. The main difficulties affecting extraction and molecular detection of enteric viruses in solid samples are: (i) low or very low recovery rates, which have been described as usually less than 10% (Hassard et al., 2016; Miura et al., 2011); (ii) the high number of steps in the protocol, which leads to higher risk of viral losses; (iii) the fact that they do not distinguish between infectious and non-infectious viral particles; (iv) the great variety of inhibitory substances present in environmental matrixes (Schrader et al., 2012); and (v) the lack of standardization in molecular methods which hinders accuracy, repeatability and interpretation of results (Bustin et al., 2009).

The present study aimed at developing a simple and versatile method for the extraction and quantitative RT-PCR (RT-qPCR) detection of enteroviruses in solid environmental matrixes. To this end, three indirect methods and one direct method for viral recovery from sewage sludge and fluvial sediments were examined and compared. The indirect methods were based on glycine elution under high pH conditions followed by viral concentration using ultracentrifugation. For the direct method, a commercial kit was used. The best option was chosen and compared with the indirect method recommended by the US Environmental Protection Agency (EPA) for virus recovery from sewage sludge (US Environmental Protection Agency, 2003). Nucleic acid extraction was also evaluated by examining two commercial kits. Taking all the information obtained into account, a method decision tree was devised for choosing the best protocol depending on sample type and the level of viral contamination.

2. Material and methods

2.1. Solid environmental samples

Sludge samples were collected from two municipal wastewater treatment plants in the Barcelona metropolitan area (Spain). Plant 1 treats about 420,000 m^3 wastewater/day and serves a population of 2,275,000 equivalent inhabitants. In this plant, a mixture of 50% primary and 50% secondary (waste activated) sludge is subjected to mesophilic anaerobic digestion. Plant 2 has a treatment capacity of 64,000 m^3 wastewater/day and serves a population of 385,000 equivalent inhabitants. In this plant, a mixture of 60% primary and 40% secondary sludge is anaerobically digested under mesophilic conditions. Different sludge samples were taken from the entire treatment process, including: primary and secondary sludge, untreated mixed sludge, digested mixed sludge and digested mixed sludge subjected to dewatering. Raw wastewater samples were also taken. Fluvial sediment samples were collected from the final stretch of the Llobregat River (NE Spain), downstream from a drinking water treatment plant. This river runs through a heavily urbanized area and is subjected to the influence of effluent from several sewage treatment plants. Sediments were taken at different distances from the water, in the four seasons of a year, and under dry and wet weather conditions.

2.2. Viral strain

The viral strain used for sample inoculation and for positive control in RT-qPCR experiments was human coxsackievirus B3 (genus *Enterovirus*, family *Picornaviridae*, order *Picornavirales*) and it was previously isolated from wastewater samples (Costán-Longares et al., 2008).

2.3. Sediment sample inoculation

To obtain a homogeneous distribution of the viral inoculum in the sediment samples, the method used was as follows. First, 500 ml of distilled water was inoculated with $4.16 \cdot 10^6$ colony-forming unit (CFU) of human coxsackievirus B3 and this water was placed in an Erlenmeyer flask. Next, 100 *g* of sediment was added and the flask was agitated with an orbital shaker at 250 rpm for 2 h. After 24 h of decantation at 4 °C to allow viral attachment to sediment particles, the water was removed and the sediment was collected for analysis.

2.4. Viral extraction methods

Three indirect methods (M_1, M_2 and M_3) and one direct method (M_4) were assayed. For the indirect methods, a glycine buffer solution was prepared by diluting glycine (Sigma-Aldrich, MO, USA) at 0.25 M in double-distilled sterile water. The pH of the buffer was adjusted to 9.0 or 9.5 by adding NaOH.

M_1. A) Viral elution: 5 g of sample was diluted in a 1/10 ratio with 45 ml of glycine buffer solution, pH 9.5. Viruses attached to solid particles were subsequently eluted by agitation with a wrist-action shaker for 20 min at 900 osc/min. The sample was clarified with centrifugation at 1,800 *g* for 15 min. The pellet was discarded and the pH of the supernatant was adjusted to 7.0 by slowly adding 1 M HCl. The supernatant was filtered using 0.22 µm pore-size polyethersulfone non-protein-binding membrane filters (Millipore, MA, USA) and this constituted the viral extract. B) Viral particle concentration: viruses were concentrated from the viral extract using a Beckman Coulter Optima L–90 K ultracentrifuge and a 70.1 Ti rotor (Beckman Coulter, CA, USA). For this, 12.5 ml of viral extract was centrifuged at 100,000*g* for 1 h at 4 °C. The resulting pellet was resuspended in 3.5 ml of glycine buffer, pH 9.5 and was maintained on ice for 30 min, with periodical manual shaking. The pH was then neutralized by the addition of 3.5 ml of doubly concentrated phosphate buffer saline (PBS 2 ×), pH 7.2. The sample was then centrifuged at 1,800 *g*. The supernatant was subjected to a new ultracentrifugation at 100,000 *g* for 1 h at 4 °C. Finally, the pellet containing viral particles was resuspended in phosphate buffer saline (PBS 1 ×), pH 7.2 to a final volume of 140 µl, constituting the viral concentrate.

M_2. A) Viral elution: identical to M_1. B) Viral particle concentration: modification of M_1 in which all the supernatant obtained after the 1,800 *g* clarification (approximately 45 ml of viral extract) was subjected to the subsequent ultracentrifugation steps.

M_3. A) Viral elution: 5 g of sample was diluted in a 1/5 ratio in 20 ml of glycine buffer solution, pH 9.0. Viruses attached to solid particles were subsequently eluted by agitation with a wrist-action shaker for 15 min at 900 osc/min. The homogenate was then maintained on ice for 30 min, with periodical manual shaking. The pH was neutralized by the addition of 20 ml PBS 2 × , pH 7.2. The sample was then clarified by centrifugation at 9,100 *g* for 15 min at 4 °C. The resulting supernatant constituted the viral extract. B) Viral particle concentration: the entire viral extract (approximately 45 ml) was subjected to