Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Virological Methods 125 (2005) 111–118
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Nested multiplex PCR assay for detection of human enteric viruses in shellfish and sewage

Meritxell Formiga-Cruz[a], Ayalkibet Hundesa[a], Pilar Clemente-Casares[a], Nestor Albiñana-Gimenez[a], Annika Allard[b], Rosina Girones[a,*]

[a] *Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain*
[b] *Department of Virology, Umeå University Hospital, S-90185 Umeå, Sweden*

Received 27 July 2004; received in revised form 20 December 2004; accepted 11 January 2005
Available online 9 February 2005

Abstract

Environmental samples and contaminated shellfish present frequently low concentrations of more than one viral species. For this reason, a nested multiplex RT-PCR was developed for the detection of adenoviruses, enteroviruses and hepatitis A viruses in different environmental samples such as urban sewage and shellfish. This assay will save time and cost for detection of these enteric viruses with a smaller sample volume, which otherwise can be a limiting factor in routine analysis. The limit of detection was approximately 1 copy for adenovirus and 10 copies for enterovirus and hepatitis A virus per PCR reaction using titrated cell-cultured viruses as template material. In shellfish and environmental samples, this multiplex PCR was optimized to detect all three viruses simultaneously when the concentration of each virus was equal or lower than 1000 copies per PCR reaction. This is the level found predominantly in the environment and in shellfish when the numbers of fecal bacterial and phage indicators are low. The detection of human adenoviruses by PCR has been suggested as a molecular index of fecal contamination of human origin in the environment and food and the multiplex assay developed may be a tool for evaluating the presence of viral contamination in shellfish and water and to expand microbiological control to include viral markers.

Keywords: Multiplex; Adenovirus; Enterovirus; Hepatitis A virus; Shellfish; Sewage

1. Introduction

Viral pathogens are the most common cause of gastroenteritis in industrialized countries (Lopman et al., 2003). Enteric viruses, which are excreted in large numbers in feces even by asymptomatic carriers, can cause outbreaks of illness such as gastroenteritis and hepatitis A. Food (particularly, shellfish) and waterborne infections are of particular importance since these outbreaks may involve a large number of people and wide geographical areas (Halliday et al., 1991; Sánchez et al., 2002).

The traditional method for detection of enteric viruses involves cell culture, which is expensive, labor-intensive and time-consuming. There is also a lack of efficient cell lines to isolate some of the epidemiologically most important enteric viruses such as hepatitis A virus (HAV), adenovirus 40 and 41 (Ad40, Ad41) and norovirus. For these reasons, nucleic acid-based methods such as PCR and hybridization have been used extensively. However, in routine laboratories the use of PCR is limited by cost and sometimes the availability of adequate test volume sample. To overcome these limitations and also to increase the detection capability of PCR, the multiplex PCR assay has been developed. Since its first description by Chamberlain et al. (1988), multiplex PCR has been applied successfully mainly in clinical diagnostic laboratories (Elnifro et al., 2000; Yan et al., 2003). However, its application to human viruses in environmental samples is limited (Rosenfield and Jaykus, 1999; Cho et al., 2000; Fout et al., 2003).

* Corresponding author. Tel.: +34 934021483; fax: +34 934039047.
E-mail address: rgirones@ub.edu (R. Girones).

0166-0934/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2005.01.009

Enteroviruses have been used as target of PCR assay for the assessment of viral pollution, since they are well characterized, and have been shown to be abundant in sewage and shellfish (Kopecka et al., 1993; Puig et al., 1994; Pina et al., 1998; Formiga-Cruz et al., 2002). However, there are reports of lack of correlation between the presence of enterovirus and the presence of important pathogens such as hepatitis A virus in some environmental samples (Dubrou et al., 1991; Pina et al., 1998).

The detection of human adenoviruses by PCR has attracted considerable attention in relation to the evaluation of viral quality of environmental samples, because the adenovirus genome is well characterized, adenovirus is more stable in various environments and more resistant to some disinfection treatments (UV, chlorine) than enteric viruses (Gerba et al., 2003a, 2003b; Thurston-Enriquez et al., 2003a, 2003b), and adenoviruses are the most prevalent human viruses detected by PCR in sewage and shellfish (Puig et al., 1994; Pina et al., 1998; Vantarakis and Papapetropoulou, 1998; Hernroth et al., 2002; Formiga-Cruz et al., 2002). The detection of human adenoviruses has been suggested as a molecular index of viral contamination of human origin (Pina et al., 1998).

On the other hand, HAV is one of the most important pathogenic viruses in water and shellfish. HAV can be transmitted from person to person, or indirectly via food, water, or fomites contaminated with virus-containing feces or vomit. The burden of hepatitis A may increase due to a decreasing proportion in the population of naturally immune individuals and a concurrent increase in the population at risk.

Therefore, the simultaneous detection of adenovirus, enterovirus and hepatitis A virus could improve the feasibility of the control of viral contamination in shellfish and water. Hence, the aim of this study was the development and application of a nested multiplex RT-PCR, which provides a highly sensitive, rapid and cost-efficient method for the detection of adenoviruses, enteroviruses and HAV.

2. Materials and methods

2.1. Human virus suspensions

Adenovirus type 41 (Ad41) Tak prototype strain (ATCC VR-930) was cultivated on A549 cells. Cells were grown in 75-cm^2 plastic flasks in Dulbecco's minimum essential medium (D-MEM) supplemented with 2% fetal calf serum. For enterovirus, coxsackievirus type B5 was used as a control and was inoculated into Green Monkey Kidney (GMK) cells under the same conditions as the adenovirus strains. This strain has been isolated from a clinical sample, typed by a neutralization test and sequenced in the VP1 region to confirm its identity.

Hepatitis A virus vaccine strain HM175 was inoculated into Vero cells. These cells were grown in D-MEM supplemented with 10% fetal bovine serum, 20 U/ml of penicillin and 20 μg/ml of streptomycin. During this infection, no cytopathic effect (CPE) was detected. Approximately 15–20 days post-infection, HAV was harvested and the infected cell suspensions were freeze–thawed 4–5 times to release the virus particles. All viral dilution suspensions were divided into 60 μl batches to be used only once, since repeated freeze–thawing can reduce the viral content up to 50–80% (Formiga-Cruz et al., 2002).

2.2. Samples

2.2.1. Shellfish samples

Two sets of commercial mussel samples (*Mytilus galloprovincialis*) were used in experiments to determine the sensitivity of the multiplex PCR. These mussels were kept at −80 °C before being processed. In addition, three cockle samples (*Tapes decussatus*) contaminated naturally and associated with an outbreak of hepatitis A occurred in Spain were examined. Finally, nine depurated commercial samples of stripped venus [*Venus* (*Chamelea*) *gallina*], and nine depurated mussel samples were also tested. These samples were collected independently at different selling points in Tarragona (Spain) from 14 to 28 October 2003, and had been depurated previously. All shellfish samples were shipped directly to the laboratory in cold storage and processed within a 24-h period.

Shellfish were washed, scrubbed under clean running water and opened with a sterile shucking knife. Cockles and stripped venus flesh and liquor, and mussels digestive glands were collected into a sterile beaker and diluted with glycine buffer 0.25 N at pH 10 (1:5, w/v) according to the method described previously (Pina et al., 1998; Muniain-Mujika et al., 2000; Formiga-Cruz et al., 2002). The mixture was homogenized by magnetic stirring for 15 min. Once the pH was adjusted to 7 ± 0.2, the treated homogenate was clarified by centrifugation at $2170 \times g$ for 15 min at 4 °C. The supernatant was centrifuged at $39{,}800 \times g$ for 45 min at 4 °C. To pellet all viral particles, the supernatant was ultracentrifuged at $100{,}000 \times g$ for 1 h at 4 °C. The final pellet was resuspended in 200–400 μl PBS with a maximum volume of viral concentrate of 500 μl. The viral concentrate was stored at −80 °C prior to nucleic acid extraction.

2.2.2. Sewage samples

Ten independent raw domestic sewage samples collected from June 2001 to August 2003 in the sewers of Barcelona (Spain) were tested by the multiplex assay developed for this study. This water treatment plant receives 670,000 m^3/day of waste products from approximately 1.8 million inhabitants. Each sample was collected in a sterile 500-mL polyethylene container, kept at 4 °C for less than 8 h until processed. Forty-two milliliters of each sample was ultracentrifuged at $100{,}000 \times g$ at 4 °C for 1 h to form pellets of all viral particles with any suspended material. The viruses retained in the pellet were eluted by mixing it with 3.5 ml of 0.25 N glycine buffer, pH 9.5, on ice for 30 min and then 3.5 ml of PBS 2× (phosphate buffer saline, double concentration) were