Food Microbiology 46 (2015) 161–167

Contents lists available at ScienceDirect

Food Microbiology

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

A comparison of virus concentration methods for molecular detection and characterization of rotavirus in bivalve shellfish species

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ARTICLE INFO

Article history:
Received 24 March 2014
Received in revised form
9 July 2014
Accepted 30 July 2014
Available online 12 August 2014

Keywords:
Rotavirus
Genotype
Virus concentration method
Bivalve shellfish

ABSTRACT

The objectives of this study were to develop a method for concentrating rotavirus, to assess the detection rate, and to characterize the genotype of naturally occurring rotavirus in bivalve shellfish species; including oysters (*Saccostrea forskali*), cockles (*Anadara nodifera*), and mussels (*Perna viridis*). The results demonstrated that an adsorption-twice elution-extraction method was less-time consuming method of concentrating the spiked rotavirus, yielding high sensitivity of 1.14 genome copies/g of digestive tissues from all three shellfish species, as detected using an RT-nested PCR. In seeding experiments, rotavirus as low as 1.39 genome copies was able to be detected in 4 g of digestive tissues or per sample. In the period of August 2011 to July 2012, of the 300 bivalve shellfish samples collected and tested, 24 (8.0%) were found to be contaminated with rotavirus, the figures being: oysters, 13/100 samples; mussels, 10/100 samples; and cockles, 1/100 samples. By DNA sequencing of the RT-nested PCR products and phylogenetic analysis, the rotaviruses detected were classified into G1, lineage II (4 samples); G3 (10 samples): lineage I (3 samples), lineage IIIc (3 samples), lineage IIId (3 samples), lineage IV (1 sample); G9 (6 samples); and G12, lineage III (1 sample). These findings suggest that this virus concentration method provides high sensitivity for the detection of rotavirus from the three bivalve shellfish species. The prevalence of rotavirus and the identified genotypes contribute to the molecular epidemiology of rotavirus in different shellfish species.

1. Introduction

Group A rotaviruses are the major cause of acute diarrhea in young children worldwide (Centers for Disease Control and Prevention, 2011). Rotavirus infection also occurs in adult patients with acute gastroenteritis (Anderson and Weber, 2004). The viruses are transmitted via fecal-oral route, excreted in large numbers in the feces of infected individuals and spread around the environment. Group A rotaviruses belong to a genus *Rotavirus* of the family *Reoviridae* and the rotavirus virion is a triple-layered icosahedral particle containing 11 segments of double-stranded RNA (Estes and Kapikian, 2007). By using the genotyping classification system, to date 27 G genotypes and 37 P genotypes have been identified in humans and animals (Matthijnssens et al., 2011; Trojnar et al., 2013). Rotaviruses from human and animal fecal excreta can contaminate the aquatic environment and consequently accumulate in bivalve shellfish (Bagordo et al., 2013). The presence of rotavirus in both fecal and shellfish samples, associated with a shellfish-borne outbreak, has been reported (Le Guyader et al., 2008). Rotavirus virus-like particles can persist in shellfish tissues at 22° C for 5–12 weeks (Loisy et al., 2005). Rotaviruses are detected in molluscan shellfish such as oysters (Le Guyader et al., 2000; Rigotto et al., 2010), mussels (Gabrieli et al., 2007; Keller et al., 2013; Le Guyader et al., 2000), and clams (Gabrieli et al., 2007; Hansman et al., 2008). However, only group A rotaviruses in various bivalve shellfish are described without genotype identification.

Because the number of viruses is often quite low, the concentration method and sensitive molecular techniques are required for the detection of viruses in shellfish. However, the presence of polymerase chain reaction (PCR) inhibitors in shellfish is an obstacle in virus detection by the molecular technique (Schwab et al., 1998). Thus, virus extraction and concentration methods have been

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http://dx.doi.org/10.1016/j.fm.2014.07.020
0740-0020/

developed in an effort to enhance the detection rate of enteric viruses in shellfish. Among the virus extraction protocols described, three methods are most commonly used: virus elution followed by concentration, direct extraction of the viral RNA, and the extraction of viruses by proteinase K treatment (Le Guyader et al., 2009; Lowther et al., 2012; Pintó et al., 2009; Stals et al., 2012). Currently, an international standard method for the detection and quantification of HAV and norovirus GI and GII in foods using real-time RT-PCR adopted by the European Committee on Normalization (CEN) has been proposed as CEN ISO/TS 15216: 2013 (www.iso.org).

It has long been recognized that the best methods for virus extraction from food samples will be those that are simple, rapid, inexpensive and reproducible. Recently, a virus extraction and detection method has been established in our laboratory, the process of which is composed of virus extraction and concentration by adsorption-twice elution-twice concentration-twice extraction followed by virus identification using highly sensitive RT-nested PCR. The method has been shown to be applicable to detect rotavirus contamination in oysters (*Crassostrea belcheri*) (Kittigul et al., 2008, 2014). However, this method is time-consuming, labor-intensive and requires multi-step processes. A more rapid virus concentration method, therefore, should be considered to aid in virus detection in shellfish. The present study aimed to develop a method for concentrating rotavirus and to determine rotavirus genotypes present in oysters, cockles, and mussels. Three main extraction and concentration protocols were compared initially by seeding rotavirus into rotavirus-free bivalve shellfish and detected using an RT-nested PCR. The most appropriate protocol was then used to establish the presence of rotavirus contamination in those shellfish. Molecular characterization of rotavirus genotypes was also undertaken in rotavirus-positive shellfish samples.

2. Materials and methods

2.1. Shellfish sampling

In the laboratory experiments, three species of bivalve shellfish, including oysters (*Saccostrea forskali*), cockles (*Anadara nodifera*) and mussels (*Perna viridis*), were collected from local markets in Bangkok, Thailand. These shellfish samples were transported to the laboratory and dissected immediately on arrival. The digestive tissues of shellfish were removed and processed for virus extraction and concentration. A known rotavirus-positive fecal sample was added to the digestive tissue concentrates after processing or added initially to the digestive tissues prior to virus processing for the purposes of detecting the presence of the virus in sensitivity assays. In the field study, a total of 300 bivalve shellfish samples (100 samples of each of oysters, cockles, and mussels) were collected from two local markets in a one-year period from August 2011 to July 2012. Four grams of digestive tissues from each sample consisting of 6 individual oysters, 10 cockles, and 5 mussels were processed and analyzed for rotavirus.

2.2. Rotavirus positive control

A rotavirus-positive fecal sample, 5.69×10^6 genome copies/ml, was used as a positive control for the sensitivity assays of virus detection using the RT-nested PCR. The quantification of rotavirus as genome copies/ml in a fecal sample was determined using the commercial quantitative real-time RT-PCR kit (Shanghai ZJ Bio-Tech, Shanghai, China) according to the European Authorized Representative Obelis S.A. (Brussels, Belgium). Rotavirus DNA positive control (1×10^8 genome copies/ml) provided with the kit was serially diluted ten-fold ($1 \times 10^3 - 1 \times 10^7$ copies/ml) and tested using the real-time RT-PCR. A standard curve for rotavirus copy numbers versus a threshold cycle (Ct) was generated and rotavirus in the fecal sample was quantified from Ct values obtained and compared with the standard curve.

2.3. Virus extraction and concentration

The bivalve shellfish (oysters, mussels, and cockles) were scrubbed and shucked aseptically. The digestive tissues from each sample were dissected and weighed at 4 g. Chilled and sterilized distilled water (150 mL) was added to the digestive tissues. They were then homogenized using a high speed blender (Waring, Torrington, CT) twice for 45 s each. The homogenates from the digestive tissues were processed according to the three different methods, described thus:

Method A: an adsorption-twice elution-extraction was performed by adjusting the shellfish homogenate to pH 5.0 with 1 N HCl, shaken at 200 rpm for 15 min on ice, and centrifuged at 2900 × *g* for 15 min at 4 °C. The supernatant was decanted and discarded. The pellet was suspended in 4 mL of 2.9% tryptose phosphate broth (TPB) containing 6% glycine, pH 9.0, shaken at 215 rpm for 15 min on ice and centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant (S_1) was collected and the pellet was re-suspended in 4 mL of 0.5 M arginine-0.15 M NaCl, pH 7.5. The suspension was shaken at 230 rpm for 15 min on ice and centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant (S_2) was decanted, combined with S_1 and adjusted to pH 7.5 with 1 N HCl. The virus was purified by extraction using 30% chloroform, and mixed by vortex for 2 min. The tube was then centrifuged at 3000 × *g* for 15 min at 4 °C, and the top layer of the aqueous phase was collected. The volume of concentrate was reduced to approximately 0.8 mL using a vacuum centrifuge (UNIEQUIP Laborgeratebau und-vertriebs GmbH, Munich, Germany) for 6–8 h at 3 °C and stored at −80 °C until nucleic acid extraction was performed.

Method B: an adsorption-twice elution-precipitation-twice extraction was performed in a similar fashion to Method A with the addition of precipitation and one further extraction step. Briefly, after eluting twice and adjusting to pH 7.5, the virus was precipitated by adding 12.5% polyethylene glycol (PEG) 8000 in 1.9% NaCl (PEG-NaCl solution) to the supernatant. The mixture was shaken at 120 rpm for 2 h on ice, refrigerated overnight, and then centrifuged at 10,000 × *g* for 1 h at 4 °C. The pellet was re-suspended in 4 mL of 0.05 M phosphate-buffered saline (PBS), pH 7.5. After that the suspension was extracted using 30% chloroform and the top layer of the aqueous phase (A_1) was collected. The pellet was re-extracted with one volume (wt/vol) of 0.5 M arginine-0.15 M NaCl, pH 7.5 and mixed for 2 min. Then, the tube was centrifuged at 3000 × *g* for 15 min at 4 °C. The top layer of the aqueous phase (A_2) was collected, and combined with A_1. The volume of concentrate was reduced to approximately 0.8 mL using a vacuum centrifuge and stored at −80 °C until nucleic acid extraction was performed.

Method C: an adsorption-twice elution-twice precipitation-twice extraction was carried out according to the method previously described by Kittigul et al. (2008). This method was performed similar to Method B with one extra PEG precipitation step. Briefly, after the PEG precipitation step, the pellet was re-suspended in 4 mL of 0.05 M PBS, pH 7.5 and precipitated again with PEG-NaCl solution. The mixture was shaken at 120 rpm for 2 h on ice and then centrifuged at 10,000 × *g* for 20 min at 4 °C. The pellet was dissolved in 2 mL of PBS and extracted twice with 30% chloroform followed by 0.5 M arginine-0.15 M NaCl, pH 7.5. The aqueous phase was collected and the volume of concentrate was reduced to approximately 0.8 mL using a vacuum centrifuge and stored at −80 °C until nucleic acid extraction was performed.