Development of a method for concentrating and detecting rotavirus in oysters

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Abstract

Identification of enteric viruses in outbreak-implicated bivalve shellfish is difficult because of low levels of contamination and natural inhibitors present in shellfish tissue. In this study, the acid adsorption–alkaline elution method developed in our laboratory was proposed for the detection of rotavirus from oyster samples. The acid adsorption–alkaline elution process included the following steps: acid adsorption at pH 4.8, elution with 2.9% tryptose phosphate broth containing 6% glycine, pH 9.0, two polyethylene glycol precipitations, chloroform extraction and reconcentration using speedVac centrifugation. Oyster concentrates were extracted for RNA and examined for rotavirus using reverse transcription-nested polymerase chain reaction (RT-nested PCR). A comparison of SuperScript™ One-Step RT-PCR system and RT followed by PCR before the nested PCR reaction showed the former detecting four-fold lower concentration of rotavirus (78.12 plaque forming units [PFU]/ml or 0.26 PFU/assay) than the latter (3.12×10² PFU/ml or 1.04 PFU/assay). In the seeding experiment, the developed acid adsorption–alkaline elution gave high sensitivity of rotavirus detection (125 PFU/g of oyster). From August 2005 to February 2006, 120 oyster samples (Crassostrea belcheri) were collected from local markets and oyster farms, concentrated, and tested for naturally occurring rotaviruses. Four oyster samples were group A rotavirus-positive. Based on phylogenetic analysis of rotavirus DNA sequences in those positive samples, the oyster samples contained the sequences associated with human rotavirus G9 (two samples), G3 (one sample), and G1 (one sample). The present study demonstrates the successful application of developed virus concentration method and RT-nested PCR for the detection of rotaviruses in naturally contaminated oyster samples. The method might be used as a tool for evaluating the presence of enteric viruses in shellfish for monitoring and control of public health.

Keywords: Virus concentration method; Rotavirus; Oyster; RT-nested PCR

1. Introduction

Enteric virus contamination in shellfish associated with outbreaks is a global public health concern (Koopmans and Duizer, 2004). Bivalve shellfish are filter-feeders and can concentrate any pathogenic microorganisms in sewage-containing water. Consumption of raw or slightly cooked shellfish such as oysters, cockles and mussels can cause gastrointestinal infection in humans. Food-borne outbreaks of enteric viruses have been reported in association with shellfish (Sanchez et al., 2002; Le Guyader et al., 2006; Shieh et al., 2007). Moreover, the viruses are resistant to physical and chemical treatment, and the depuration process may not remove viruses effectively (Croci et al., 1999; Kingsley and Richards, 2003; Loisy et al., 2005). Shellfish are contaminated with enteric viruses including norovirus or Norwalk-like virus (Boxman et al., 2006), hepatitis A virus (Croci et al., 2007), adenovirus (Formiga-Cruz et al., 2005) and rotavirus (Le Guyader et al., 2000).

Rotavirus is one of the important viruses present in water and shellfish. The virus mainly causes acute gastroenteritis in children and asymptomatic illness in adults. In Thailand, rotavirus is the most important etiologic agent causing acute diarrhea in infants and children less than five years old. Rotavirus causes 30–50% of all hospital admissions for acute diarrhea (Maneekarn and Ushijima, 2000; Jiraphongsya et al., 2005). Rotavirus is a member of family Reoviridae with segmented double-stranded RNA genome, surrounded by a
triple-layered capsid. The virus is transmitted easily from person-to-person, or indirectly via food, water, or fomites contaminated with virus-containing feces (Butz et al., 1993; Soule et al., 1999; Gallimore et al., 2005; van Zyl et al., 2006). Although rotavirus mainly infects young children, the adults with rotavirus infection can spread the virus through direct or indirect transmission to children and environment. Food- or waterborne outbreaks caused by rotavirus are uncommon, however, the findings of rotavirus present in various sources of water in Thailand may imply a health risk to humans (Kittigul et al., 2000, 2005). Furthermore, genetic analysis of rotavirus in the environment is helpful for epidemiological study and surveillance of acute gastrointestinal disease. However, detection and identification of viruses in water and shellfish is problematic because of the low density of contamination, inefficient recovery of viruses during the concentration process and the presence of natural inhibitors to detection by polymerase chain reaction (PCR). The methods commonly used to concentrate viruses from whole shellfish include direct alkaline elution (De Medici et al., 2001), and acid adsorption–neutral elution (Mullendore et al, 2001).

An efficient and sensitive method for concentrating rotavirus and detection by RT-nested PCR from water samples has been established in our laboratory. The virus concentration method was based on acid adsorption–alkaline elution and reconcentration using speedVac centrifugation (Kittigul et al, 2005). In the present study, this method was modified for improving the detection limit of rotavirus in oyster samples and compared with direct alkaline elution and acid adsorption–neutral elution. In a field trial, the developed method was used to assess the rotavirus contamination in raw oysters collected from local markets and oyster farms in Thailand.

2. Materials and methods

2.1. Virus and oyster samples

Rotavirus seeded experimentally in oyster samples was inactivated bovine rotavirus (Calf rotavirus strain 3209176) and contained approximately $10^7$ plaque forming units (PFU)/ml, as determined by cell culture before inactivation.

One hundred and twenty oyster samples (Crassostrea belcheri) were collected from various local markets (60 samples) in Bangkok and ten oyster farms (60 samples) in Surat Thani Province, located at the south of Thailand, from August 2005 to February 2006. All oyster samples were transported to the laboratory in chilled containers. On arrival, the oysters were washed, scrubbed, and the shells opened with a sterile shucking knife. The liquor or mantle fluid was drained into a discard container. The oyster meat, except the adductor muscle that was left attached to the shell, was collected, cut into small pieces, and trimmed to 25 g for analysis of rotavirus.

2.2. Oyster processing methods to concentrate rotavirus

Three different concentration methods were used to determine the detection limit of rotavirus in oyster samples. Twenty five grams of oyster meat were inoculated with known concentrations of rotavirus and left to stand for 60 min at room temperature. Direct alkaline elution was performed as described by De Medici et al. (2001) with some modifications. Briefly, seven volumes of chilled, sterilized, distilled water were added to the oyster flesh and homogenized using a blender (Hamilton Beach, Southern Pines, NC). The virus was eluted from oyster tissues with 10% tryptose phosphate broth (TPB) containing 0.05 M glycine, pH 9.0 and re-eluted with 0.5 M arginine–0.15 M sodium chloride (NaCl), pH 7.5. The pooled eluates were precipitated twice by adding polyethylene glycol (PEG 8000) and NaCl to a final concentration of 12.5% PEG–0.3 M NaCl. After dissolving the pellet, the virus was extracted with chloroform to a final concentration of 30% and re-extracted with 0.5 volume of arginine–NaCl, pH 7.5. The aqueous phase was collected and kept at −80 °C prior to assay.

The procedure of acid adsorption–neutral elution was based on the method essentially described by Mullendore et al. (2001) with some modifications. Briefly, after oyster homogenization, the conductivity of homogenates was measured and reduced to less than 2000 μS/cm. The virus was adsorbed to the homogenates at pH 4.8 and eluted from oyster tissues with 0.75 M glycine–0.15 M NaCl, pH 7.6 followed by re-elution with 0.5 M threonine–0.15 M NaCl, pH 7.5. Then, the virus was first precipitated by adding 8% PEG–0.3 M NaCl and further purified by extraction with chloroform and re-extraction with threonine-NaCl, pH 7.5. The virus was precipitated again using PEG–NaCl. After centrifugation, the pellet was stored at −80 °C until use.

The acid adsorption–alkaline elution method developed in this study was modified from the virus concentration methods as described by Kittigul et al. (2005) and Mullendore et al. (2001). Seven volumes of chilled, sterilized distilled water were added to 25 g of oyster flesh and homogenized at high speed twice. The conductivity of the homogenate was reduced to less than 2000 μS/cm by adding sterilized distilled water. The homogenate of oyster was adjusted to pH 4.8 with 1 N HCl, shaken for 15 min, and centrifuged at 2000 ×g for 20 min at 4 °C. The supernatant was discarded and the pellet was suspended in 25 ml of 2.9% TPB containing 6% glycine, pH 9.0 for elution of the virus, shaken for 15 min, and centrifuged at 10,000 ×g for 15 min at 4 °C. The supernatant (S1) was collected and the pellet was re-eluted with 25 ml of 0.5 M arginine–0.15 M NaCl, pH 7.5. The suspension was shaken for 15 min and centrifuged. The supernatant (S2) was collected, combined with S1 and adjusted to pH 7.2 with 1 N HCl. The virus in the supernatant was precipitated by adding 12.5% PEG 8000 and 0.3 M NaCl. The mixture was refrigerated overnight at 4 °C. The pellet was dissolved in 15 ml of 0.05 M phosphate-buffered saline (PBS), pH 7.5 and precipitated again with PEG–NaCl. The mixture was stirred for 2 h at 4 °C and then centrifuged at 10,000 ×g for 10 min. The pellet was dissolved in 5 ml of PBS. The virus was extracted with chloroform at a final concentration of 30%. After centrifugation at 3000 ×g for 10 min, the top layer of the aqueous phase was collected (S3). The pellet at the interface between the solvent and the aqueous
phase was re-extracted with 0.5 volume of arginine–NaCl, pH 7.5. After centrifugation, the top layer (S4) was collected and combined with S3. The sample was reconcentrated using speedVac centrifugation to reduce the volume of the concentrate to approximate 1 ml and stored at −80 °C until nucleic acid extraction.

In seeding experiments, rotavirus was inoculated in the oyster tissues and processed by the virus concentration methods. The supernatant or pellet discarded in the concentration process was collected at each step of the acid adsorption methods. The supernatant or pellet discarded in the concentration process was collected at each step of the acid adsorption methods. The supernatant or pellet discarded in the concentration process was collected at each step of the acid adsorption methods. The supernatant or pellet discarded in the concentration process was collected at each step of the acid adsorption methods. The supernatant or pellet discarded in the concentration process was collected at each step of the acid adsorption methods. The supernatant or pellet discarded in the concentration process was collected at each step of the acid adsorption methods.

2.3. Viral RNA extraction from oyster samples

Viral RNA from oyster samples was extracted using RNasey® mini kit (Qiagen AG, Basel, Switzerland). In brief, 200 μl of the concentrated oysters was lysed and RNA was purified on the silica-based column according to the manufacturer’s protocol. RNA bound to the membrane in the column was eluted in 60 μl of warm RNase-free water.

2.4. RT-nested PCR

2.4.1. One-Step RT-PCR system

Two microliters of extracted RNA was heated at 94 °C for 4 min and placed on ice for at least 10 min. RNA was examined by SuperScript™ One-Step RT-PCR system with Platinum® Taq DNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA). One-Step RT-PCR was performed with 50 μl reaction volume. The extracted RNA sample (2 μl) was added to RT-PCR mixture (48 μl) consisting of 1× Reaction Mix (a buffer containing 0.2 mM of each dNTP, 2 mM MgSO4), SuperScript® III RT/Platinum® Taq Mix, 0.25 μM primer RV1, 0.25 μM primer RV2 (Gilgen et al., 1997) and nuclease-free water. The RT and PCR were carried out with following steps: RT at 41 °C for 60 min; PCR cycle 1–25, 94 °C for 2 min, 94 °C for 30 s, 55 °C for 30 sec, 72 °C for 60 s; final extension, 72 °C for 3 min.

2.4.2. RT followed by PCR

Two microliters of extracted RNA was heated at 94 °C for 4 min and placed on ice for at least 10 min. RNA was examined by RT followed by PCR (Promega, Madison, WI) according to the method previously described by Kittigul et al. (2005). In 20 μl reaction volume, the extracted RNA sample (2 μl) was added to RT mixture (18 μl) consisting of 10 mM Tris–HCl (pH 9.0 at 25 °C), 50 mM KCl, 0.1% Triton® X-100, 5 mM MgCl2, 1 mM each of dNTP, 20 U of Recombinant Rnasin® (Ribonuclease Inhibitor), 15 U of AMV reverse transcriptase, 1.25 μM RV1, and nuclease-free water. After incubation at 41 °C for 60 min, the sample was heated at 99 °C for 5 min, and immediately cooled at 4 °C for 5 min.

First-strand cDNA diluted 1:5 (10 μl) was added to 40 μl of PCR mixture. The final concentrations were: 0.2 mM of each dNTP, 2 mM of MgCl2, 1× PCR buffer (10 mM Tris–HCl, pH 9.0 at 25 °C, 50 mM KCl, 0.1% Triton® X-100), 0.25 μM of primer RV1, 0.25 μM of primer RV2, 2.5 U of Taq DNA polymerase. All were mixed with nuclease-free water. The PCR cycling conditions were as follows: 60 s at 94 °C followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, and the final extension step at 72 °C for 3 min.

For nested PCR, 1 μl of the first amplification reaction from One-Step RT-PCR system or RT followed by PCR was further amplified under the same conditions of amplification as for the first PCR except for changing the primer pair to RV3 and RV4 and their concentrations to 0.5 μM and the concentration of MgCl2 to 3.5 mM (Gilgen et al., 1997). PCR products were analysed by electrophoresis on 1.5% agarose gels and ethidium bromide staining. A DNA fragment of 346-bp was considered the rotavirus DNA. The sample concentrates were inoculated with various concentrations of rotavirus and used to test the sensitivity of RT-nested PCR in order to study the presence of PCR inhibitors in concentrated oyster samples.

2.5. Sequence and phylogenetic analyses of rotavirus-positive oyster samples

Amplified products (346-bp) were sequenced at the Bioservice Unit of National Science and Technology Development Agency, Bangkok, using the same forward (RV3) primer. The nucleotide sequences of VP7 gene were compared with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using BLAST (Basic Local Alignment Search Tool) server (Altschul et al., 1990). Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 3.1 (Kumar et al., 2004).

2.6. Nucleotide sequence accession numbers

The nucleotide sequence of VP7 of rotavirus-positive oyster samples; THOYS019, THOYS020, THOYS068, and THOY S108 were deposited in GenBank under the accession numbers EF687852, EF687853, EF687854, and EF687855, respectively.

2.7. Bacteriological analysis of rotavirus-positive oyster samples

The values of most probable number (MPN) fecal coliforms and E. coli in rotavirus-positive oyster samples were determined using multiple fermentation tube method in a five tube series of five dilutions ranged from 10−1 to 10−5 according to the Bacteriological Analytical Manual of the Food and Drug Administration (Hitchins et al., 1998). Fecal coliform density was determined using lactose fermentation with gas production. Numbers of gassing tubes were recorded for calculation of MPN fecal coliforms by means of MPN table. The presence of E. coli was examined by culture on MacConkey agar. The suspected colonies were picked up, and identified by TSI (Triple sugar iron) and IMViC (Indole, MR, VP, Citrate) tests. Acid slant, acid butt, and gas producing without hydrogen
sulfide indicated that organism was presumably *E. coli*. The suspected colony was identified as *E. coli* when the results of IMViC were ++−−, respectively. MPN *E. coli* was interpreted from numbers of gassing tubes that contained *E. coli* by means of MPN table.

3. Results

3.1. Sensitivity and specificity of RT-nested PCR

The rotavirus detection sensitivity was initially determined using SuperScript™ One-Step RT-PCR system and compared with RT followed by PCR. The PCR products from both assays were re-amplified using nested PCR. The assays were performed with various concentrations of the rotavirus stock in 0.05 M PBS. It was found that One-Step RT-PCR system could detect rotavirus at the lowest concentration of 78.12 PFU/ml or 0.26 PFU/assay; whereas, RT followed by PCR gave the lowest concentration of rotavirus at 3.12 × 10³ PFU/ml or 1.04 PFU/assay. The specificity of RT-nested PCR was examined with primers RV1, RV2, RV3, and RV4 employing different enteric viruses such as poliovirus type 1 (≥1.25 × 10⁵ 50% tissue culture infective dose [TCID₅₀]/ml) or formaldehyde-inactivated hepatitis A virus vaccine strain HM175 (≥7.20 × 10² enzyme-linked immunosorbent assay Units [EL.U.]/ml). The RT-nested PCR amplified the DNA of rotavirus but not for poliovirus or hepatitis A virus in both PCR procedures (Fig. 1A and B). Therefore, the sensitive One-Step RT-PCR was chosen for the subsequent studies.

To determine the sensitivity of RT-nested PCR for rotavirus detection in oysters concentrated by direct alkaline elution, acid adsorption–neutral elution, and acid adsorption–alkaline elution, various concentrations of the rotavirus stock were prepared in concentrated oysters and assayed for DNA amplification. The acid adsorption–neutral elution and acid adsorption–alkaline elution concentration methods provided the same sensitivity (78.12 PFU/ml of concentrated oysters) as found for rotavirus in 0.05 M PBS but the rotavirus detection limit in oysters concentrated by direct alkaline elution method was higher concentration (1.25 × 10³ PFU/ml) than that in PBS. This result showed the presence of PCR inhibitors in the oysters concentrated by the direct alkaline elution.

3.2. Detection limit of RT-nested PCR in seeding experiments

Three different methods for concentrating rotavirus from oysters were compared for detection of experimentally contaminated rotavirus. Various concentrations of the rotavirus stock were inoculated in oyster samples, concentrated, and determined for viral RNA using RT-nested PCR. The acid adsorption–neutral elution gave the highest sensitivity and could detect rotavirus at the lowest concentration of 3.12 × 10³ PFU/25 g or 125 PFU/g of oyster. The acid adsorption–alkaline elution and the direct alkaline elution were able to detect rotavirus at 1.25 × 10⁴ PFU/25 g and 2.5 × 10⁴ PFU/25 g, respectively (Table 1).

The residual rotavirus was determined in the supernatant or pellet that was discarded in each step of virus concentration. Using the acid adsorption–alkaline elution method, the rotavirus loss was observed in the pellet of oyster tissue after

Table 1

<table>
<thead>
<tr>
<th>Rotavirus (PFU/25 g)</th>
<th>Acid adsorption–neutral elution</th>
<th>Acid adsorption–alkaline elution</th>
<th>Direct alkaline elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 × 10⁰</td>
<td>4+</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>2.50 × 10⁰</td>
<td>4+</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>1.25 × 10⁰</td>
<td>3+</td>
<td>2+</td>
<td>−</td>
</tr>
<tr>
<td>6.25 × 10⁰</td>
<td>2+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>3.12 × 10³</td>
<td>1+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5.16 × 10³</td>
<td>−</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd, not done.

a Twenty five grams of oyster tissues were inoculated with various concentrations of rotavirus, homogenized, and concentrated by acid adsorption–alkaline elution, acid adsorption–neutral elution, or direct alkaline elution method.

b 1+, 2+, 3+, 4+, degree of intensity of DNA bands from weak to strong positive results.

c −, negative result.
re-elution (faint band) and re-extraction (intense band) steps, as shown in Fig. 2.

3.3. Examination of rotavirus in oyster samples

A total of 120 raw oyster samples collected from local markets and oyster farms were concentrated by the acid adsorption–alkaline elution method. Rotavirus RNA in the concentrated oyster samples was determined using One-Step RT-PCR followed by nested PCR. Four oyster samples (3.33%) were positive for rotavirus (Fig. 3): 5% (3/60 samples) collected from three oyster farms from the south of Thailand, and 1.67% (1/60 samples) collected from one local market in Bangkok. The amplicons of these rotavirus-positive samples were sequenced and the phylogenetic analysis revealed that the genetic sequences were associated with human rotavirus G9 (two samples), G3 (one sample), and G1 (one sample), as shown in Fig. 4. These oyster samples were collected from September to

Table 2
Characteristics of the oyster samples where rotavirus was detected

<table>
<thead>
<tr>
<th>Oyster sample</th>
<th>Date of collection</th>
<th>Source of collection</th>
<th>Rotavirus genotype</th>
<th>Fecal coliforms MPN/g</th>
<th>E. coli MPN/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>THOYS019</td>
<td>19 Sep 05</td>
<td>Oyster farm A</td>
<td>G9</td>
<td>230</td>
<td>230</td>
</tr>
<tr>
<td>THOYS020</td>
<td>19 Sep 05</td>
<td>Oyster farm B</td>
<td>G9</td>
<td>210</td>
<td>210</td>
</tr>
<tr>
<td>THOYS068</td>
<td>7 Nov 05</td>
<td>Oyster farm C</td>
<td>G3</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>THOYS108</td>
<td>12 Dec 05</td>
<td>Local market</td>
<td>G1</td>
<td>93</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* The oyster samples were examined for fecal coliforms and E. coli by multiple fermentation tube method. The acceptable level of fecal coliforms present in raw shellfish was <20 MPN/g.
December, 2005, the annual peak of rotavirus outbreaks. All four oyster samples contained both bacterial indicators for fecal contamination (fecal coliforms and E. coli) of MPN/g in higher levels than the standard level of raw shellfish (~20 MPN/g) proposed in Thailand, as shown in Table 2.

4. Discussion

Outbreaks of viral gastroenteritis have been associated with the consumption of sewage-contaminated shellfish (Lees, 2000). Two major areas of methodological developments are needed for detection of viruses in shellfish: the development of efficient methods to extract and concentrate viruses and the refinement of RT-PCR amplification methods to increase sensitivity and specificity of detecting viruses in shellfish.

The present study attempted to develop a virus concentration method using rotavirus as a model and to detect the virus in oyster samples collected from local markets and oyster farms in Thailand. RT-nested PCR was used for detection of rotavirus from oysters because of its high sensitivity. Rotavirus RNA was determined by two RT-PCR procedures: SuperScript™ One-Step RT-PCR system was more practical because of its simplicity, rapidity and lower risk of cross-contamination than RT followed by PCR. Moreover, the sensitivity of One-Step RT-PCR system was four-fold higher than that of the RT followed by PCR. A previous study showed that the SuperScript™ One-Step RT-PCR System could amplify 0.2–1 PFU/µl of hepatitis A virus and that it provided the highest sensitivity among different commercial RT-PCR kits (Ribao et al., 2004).

Direct alkaline elution, acid adsorption–neutral elution, and acid adsorption–alkaline elution were evaluated for concentration of rotavirus from oyster tissues. The acid adsorption–alkaline elution provided the lowest detection limit, about 8-fold and 4-fold lower, compared to direct alkaline elution and acid adsorption–neutral elution, respectively. The procedure of direct alkaline elution was simple and rapid but less effective in removing inhibitors. This finding was consistent with the study of Sunen et al. (2004). The acid adsorption–alkaline elution and acid adsorption–neutral elution seem to have a greater ability to remove PCR inhibitors, possibly due to the two-step process used. Nevertheless, the procedure of acid adsorption–neutral elution produced a pellet in the final step which was difficult to dissolve prior to RNA extraction. Meanwhile, the acid adsorption–alkaline elution method has several advantages. First, 2.9% TPB containing 6% glycine, pH 9.0, used for elution of virus, is the most effective eluent, as reported previously in a study on concentrating rotavirus from water samples (Kittigul et al., 2001). Second, arginine used in re-elution and re-extraction steps instead of threonine was 7 times cheaper and gave comparable sensitivity to threonine, data not shown. Third, re-concentration of the eluates using speedVac centrifugation gave high recovery of rotavirus (Kittigul et al., 2001). The evaporation combined with centrifugation simultaneously reduced the volume of the eluate and concentrated each sample 25 fold, meaning that 25 g of oyster meat will reduce to about 1 ml of concentrate. The final concentrates in soluble forms were easily extracted for RNA prior to RT-nested PCR.

A number of virus particles were still adsorbed to oyster solids after re-elution or re-extraction with 0.5 M arginine–0.15 M NaCl, pH 7.5. Virus loss at the step of elution and solvent extraction from oyster solids was previously mentioned by Mullendore et al. (2001), who studied the concentration method for hepatitis A virus. The study of concentration of norovirus from oysters also showed the crucial step of virus elution (Schultz et al., 2007). Therefore, the elution and extraction of virus from oyster tissues are important steps to allow the successful detection of enteric viruses.

Of note, the high sensitivity and simplicity of the acid adsorption–alkaline elution method and One-Step RT-PCR system followed by nested PCR make them suitable for application to examination of rotavirus in environmental oyster samples. In Thailand, the most important edible oysters are representative of the genus Crassostrea. The oysters are often eaten raw or only slightly cooked by humans. Naturally occurring rotaviruses were found in four (3.33%) out of 120 oyster samples. Three oyster samples with rotavirus contamination in oyster farms might occur during production or harvesting. The oysters were probably contaminated from sewage pollution at the source of production. The one oyster sample from the local market contaminated with rotavirus might have been contaminated during production, transportation or storage.

Computer aided DNA sequence analysis for comparison of rotavirus-positive oyster samples with data banks enabled classification of human rotavirus G9 (two samples), G3 (one sample), and G1 (one sample). Although a low number of rotavirus-positive samples was found, it is likely that human rotavirus G9 is predominant in the environment, as reported for water samples (Kittigul et al., 2005). The emergence of serotype G9 human rotavirus has been reported worldwide, including in Thailand (Khamrin et al., 2006) and might originate through genetic reassortment between human and animal rotaviruses (Gratacap-Cavallier et al., 2000; Ramachandran et al., 2000). The presence of rotavirus in natural oysters might present a potential health risk of bivalves for the consumer and support the need for appropriate controls of the viral contamination of oysters. Among the rotavirus-positive oyster samples, fecal coliforms and E. coli were also found in higher level of MPN/g than the standard level. The presence of these bacterial indicators confirmed the rotavirus-positive oyster samples were contaminated with fecal materials both at production source and during transportation or storage.

The present study is the first report of naturally occurring rotavirus contamination in oysters from Thailand and demonstrated the effectiveness of the developed method for concentrating rotavirus and highlighted RT-nested PCR for detecting the VP7 gene of rotavirus in environmental oyster samples. Improvement in sanitary quality of oysters is one preventive measure that can reduce or prevent outbreaks related to raw consumption. Shellfish quality of oysters is one preventive measure that can reduce or prevent outbreaks related to raw consumption. Shellfish should be tested for the presence of health significant enteric viruses to guarantee the virological quality of oyster products for human consumption or exportation.
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