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

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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 a RT-nested PCR. In seed 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 rotavirus detected were classified into G1, lineage II (4 samples); G3 (10 samples): lineage I (3 samples), lineage IIc (3 samples), lineage IIIa (3 samples), lineage IV (1 sample); G6 (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.

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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 virology 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 (Matthijssens et al., 2011). Trojanar 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
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-concentration-twentwice elution-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 detecting 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 (Crassostrea 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 Biotech, Shanghai, China) according to the European Authorized Representative Obelix S.A. (Brussels, Belgium). Rotavirus DNA positive control (1 × 10^9 genome copies/ml) provided with the kit was serially diluted ten-fold (1 × 10^3 - 1 × 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% trypose 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 (S1) 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 (S2) was decanted, combined with S1, 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 (UNIQUEP 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 (A1) 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 (A2) was collected, and combined with A1. 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.
2.4. Sensitivity assays

Two experiments were performed for testing the sensitivity of rotavirus detection: 1) the digestive tissue concentrates processed by Methods A, B, and C were added with serially ten-fold dilutions of a rotavirus-positive fecal sample between 10⁻⁶ and 10⁻³ (5.69 x 10⁻² to 5.69 x 10⁻⁴ genome copies/ml), or equal 1.14 x 10⁻² to 1.14 x 10⁻⁴ genome copies/g of digestive tissues and 2) in seeding experiments, serially two-fold dilutions of a rotavirus-positive fecal sample were inserted into the digestive tissues of the shellfish at 0.70, 1.39, and 2.78 genome copies/g per sample and processed for virus concentration using the aforementioned three methods. The digestive tissue concentrates and the digestive tissues without spiking rotavirus were included in the assays as negative controls. All digestive tissue concentrates from these experiments were extracted for viral RNA and examined for the presence of the rotavirus. In spiking experiments, a pool of digestive tissues (20 or 15 g) was prepared 5 or 4 aliquots for 4 dilutions of serially ten-fold or 3 dilutions of serially two-fold of a rotavirus-positive fecal sample and 1 negative control. If the shellfish are contaminated with naturally occurring rotavirus as observed in the negative control, they would be discarded. Only the shellfish provided a negative result in the control, the results of spiking rotavirus in shellfish were analyzed. The 4–8 repetitions of spiking experiments were performed in different occasions i.e. done on other days (inter-daily variations).

2.5. Nucleic acid extraction

Viral nucleic acids were extracted from 200 µL of shellfish concentrates using an RNeasy® mini kit (QIAGEN GmbH, Hilden, Germany) used according to the manufacturer’s instruction. Viral nucleic acids obtained (50–55 µL) were stored at −80 °C until used in the RT-nested PCR assay.

2.6. RT-nested PCR amplification for rotavirus

Viral nucleic acids were tested for rotavirus using the previously described RT-nested PCR assay (Kittigul et al. 2008). Briefly, for the first round of the SuperScript™ One-Step RT-PCR system with Platinum® Taq DNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA), a 2 µL of viral nucleic acids was added to 48 µL of the RT-PCR mixture consisting of 1x Reaction Mix (a buffer containing 0.2 mM of each dNTP, 2 mM MgSO₄, SuperScript™ III RT/Platinum™ TaqMix, 0.25 µM of each primer (RV1 and RV2) (Gilgen et al., 1997), and nuclease-free water. The cycling conditions consisted of reverse transcription at 41 °C for 60 min; 94 °C for 2 min, PCR of 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 3 min. In the second round, a nested PCR was performed using 0.5 µM of each primer (RV3 and RV4) (Gilgen et al., 1997), and 3.5 mM MgCl₂. The RT-PCR product (1 µL) was further amplified under the same conditions of amplification as used for the first round RT-PCR for 40 cycles. The PCR products were analyzed using 1.5% agarose gel electrophoresis and ethidium bromide staining. A DNA fragment of 346-bp was considered to be rotavirus DNA.

2.7. Sequencing of rotavirus PCR products

The RT-nested PCR products of the rotavirus were purified using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and sequenced at the Bioservice Unit of the National Science and Technology Development Agency, Bangkok, Thailand. The nucleotide sequences of the VP7 gene were compared with those of the reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search Tool) server (Altschul et al., 1990). Phylogenetic analysis was conducted using MEGA, version 5.2 (Tamura et al., 2011).

The nucleotide sequences of rotavirus obtained in bivalve shellfish samples, corresponding to fragments of the VP7 gene of rotavirus, were deposited in GenBank under the following accession numbers: KJ590137–KJ590157.

3. Results

3.1. Comparison of three methods for rotavirus concentration

The bivalve shellfish samples were initially screened for the natural presence of rotavirus using the RT-nested PCR. Rotavirus-negative shellfish concentrates processed by Methods A, B, and C were added with varied dilutions of the rotavirus to determine the sensitivities of the RT-nested PCR. It was found that the RT-nested PCR was able to detect rotavirus equally at the 10⁻⁴ dilution (1.14 genome copies/g of digestive tissues) in oysters, cockles or mussels when using any of the previously described methods. Over the course of repeated seeding experiments, the detection limits of rotavirus in oysters, cockles or mussels, concentrated by Methods A, B, and C, were equal at 139 copies/g of digestive tissues. Among three different virus concentration methods, the processing periods required in Method A was 11 h, Method B was 1 day and 9 h, and Method C was 1 day and 12 h. Because Method A was the most rapid, it was chosen to be used for concentrating the naturally occurring rotaviruses in the bivalve shellfish obtained from the markets.

3.2. Rotavirus detection in shellfish samples

For the one-year study, rotavirus was detected using RT-nested PCR in 24 out of 300 (8%) shellfish samples; 13/100 samples of oysters (13%), 10/100 samples of mussels (10%), and 1/100 samples of cockles (1%). The prevalence of rotavirus-positive shellfish samples peaked in October, however, a high frequency of rotavirus detection was also observed among samples collected in September and January (Fig. 1).

3.3. Rotavirus genotyping

Out of the 24 rotavirus-positive shellfish samples, 21 (87.5%) were successfully sequenced and analyzed. Using the BLAST

![Fig. 1. Monthly distribution of rotavirus detected in bivalve shellfish from August 2011 to July 2012. The number of rotavirus-positive samples was expressed in percentage of the number of samples collected each month.](image-url)
Fig. 2. Phylogenetic trees of partial VP7 nucleotide sequences derived from rotavirus strains detected in this study and other reference strains from the GenBank database. A, B, C, and D represent G1, G3, G9, and G12 genotypes, respectively. The trees were generated using the neighbor-joining analysis of 1000 repetitions in MEGA 5.2. The scale bar indicates nucleotide substitutions per site. Bootstrap values >80% are shown at the branch nodes. Rotavirus strains in the present study are indicated in bold.
program and phylogenetic analysis of the partial VP7 nucleotide sequences for lineage classification (Chaimongkol et al., 2012; Than et al., 2011, 2013). The rotavirus strains were able to be classified into four genotypes; G1, G3, G9, and G12. These strains had the nucleotide sequences similar to human rotavirus in 14 samples and animal rotaviruses, including canine, porcine, and equine rotaviruses, in 7 samples. Within G1 (Fig. 2A), two rotavirus strains from oysters (Oys021 and Oys088) and one strain from mussels (Mus093) showed a 99% nucleotide identity with the human rotavirus A strain RVA/Human-wt/BEL/BE1001a (JN849126) from Belgium. Another rotavirus strain (Oys042) had a 96% nucleotide identity with the rotavirus A strain 32vp7n VP7 (DQ674868) from Thailand. All four strains were clustered into lineage II of G1.

Within G3 (Fig. 2B), 10 rotavirus strains were clustered in different lineages. Two strains from oysters (Oys018 and Oys100) and one strain from cockles (Cock062) showed a 92–93% nucleotide identity with the equine rotavirus A strain RVA/Horse-wt/ARG/E3198 (X036370) from Argentina and belonging to lineage I. Three strains from mussels (Mus043, Mus092, and Mus099) were genetically similar to the rotavirus A VP7 gene strain RAC-DG5/AB526247 from Japan, with a 94–99% nucleotide identity and belonging to lineage III (sub-lineage c). One strain from oysters (Oys031) and two strains from mussels (Mus019 and Mus045) were closely related to the human rotavirus A strain CU976-KK/11 (JN706568) from Thailand with a 99% nucleotide identity and belonging to lineage III (sub-lineage d). One strain from oysters (Oys024) was similar to the genetic sequence of the porcine rotavirus A isolate CMP214 (DQ786577) from Thailand with a 93% nucleotide identity and belonging to lineage IV.

In the G9 tree (Fig. 2C), three strains from mussels (Mus011, Mus012, and Mus013) were related to the 97’S237 strain from China (AF260959) with 93% nucleotide identity, whereas three strains from oysters (Oys013, Oys083, and Oys099) were genetically similar to the strain G2275 from the USA (JF521480) with a nucleotide identity of 91–92%. Of interest, these six rotavirus strains were clustered in different branches from the identified G9 lineages. In the G12 tree (Fig. 2D), one strain from mussels (Mus047) had a 98% nucleotide identity to the human strain CU616 (JN706565) from Thailand and belonging to lineage III.

4. Discussion

Three virus concentration methods (Method A: adsorption-twingelution-extraction; Method B: adsorption-twingelution-precipitation-twingelution extraction; and Method C: adsorption-twingelution-precipitation-twingelution extraction) were compared to establish a sensitive and simple detection method for group A rotaviruses in three bivalve shellfish species (oysters, cockles and mussels). Methods A and B are modified from Method C, which is currently used in our laboratory, in order to reduce some of the processing steps. The main concentration protocol consists of the steps of acid adsorption, alkaline elution, PEG precipitation and chloroform extraction. Alkaline elution and chloroform extraction are crucial steps for the extraction of rotavirus from oyster tissues.
(Kittigul et al., 2008), and hence were performed twice in the virus processing in order to get high virus recovery. PEG precipitation is frequently used after the elution step to increase the concentration of the virus extract (Kittigul et al., 2008; Lewis and Metcalfe, 1988; Mullendore et al., 2001). However, this step was excluded from Method A because at least a 2 h, or an overnight, incubation was needed to precipitate the virus. After chloroform extraction by all three methods, the sample was centrifuged using a centrifuge with an aspirator vacuum pump and evaporated gently to reduce the sample volume. In our previous study, this re-concentration step could significantly reduce the volume of the eluate of the water sample and yielded a high rotavirus recovery (Kittigul et al., 2001). Thus, the re-concentration step was included in all of the virus processing methods in this study.

The same sensitivities of RT-nested PCR are displayed for the detection of rotavirus in those bivalve shellfish or in the seeding experiments of digestive tissues using three methods. Method C may give more a purified virus because of the extra steps in the virus processing, however, shows the same sensitivity as Methods A and B. It is postulated that some virus loss might occur during concentrating process through multi-steps of the Method C but very low numbers and did not alter the sensitivity of RT-nested PCR. More important, all three methods keep the re-elution step which was found to affect virus loss (Kittigul et al., 2008), thus it probably supports the same sensitivity of the three methods. These findings indicate that all three virus concentration methods can help in rotavirus detection in the three bivalve shellfish species. However, Method A is simpler and quicker than Methods B and C. Since Method A provides the same sensitivity of rotavirus detection as Method C, which was previously found to be able to remove PCR inhibitors (Kittigul et al., 2008), it is proposed that this method might have a similar capability to Method C in PCR inhibitors removal.

Using Method A to extract rotavirus from bivalve shellfish samples and subsequently examined by RT-nested PCR, the prevalence of rotavirus is highest in oysters (13%), followed by mussels (10%) and cockles (1%). The detection rate of rotavirus in oysters (S. forskali), in this study, is higher than that in the previous studies of different oyster species (C. belcheri) in Thailand, which were reported at 3.3% (Kittigul et al., 2008) and 5.4% (Kittigul et al., 2014). In France, 27% of oyster samples were contaminated with rotavirus, whereas mussel samples were more contaminated (52%) because the mussels were collected in areas which were highly impacted by human sewage (Le Guyader et al., 2000). Rotavirus was found in mussels at a level of 25.5% in Italy (Gabrieli et al., 2007) and 100% in Brazil (Keller et al., 2013). The different detection rates may be in part due to the difference in sample collection sites and the species of bivalve shellfish studied. The yield from both markets in this study were obtained from shellfish production areas in the eastern coast of Thailand. The harvesting areas are located in the industrial area and surrounded by congested communities. Human sewage from surrounding communities probably drains into the harvesting areas. The three shellfish species were cultivated differently in seawater: oysters (Saccostrea) in floating cages, mussels (Perna) on ropes attached to stocks and cockles (Anadara) in the sediment of the lagoon. To the best of our knowledge, this study allows, for the first time, the demonstration of the presence of rotavirus in cockles, although the contamination rate is low.

So far, the prevalence of rotavirus in shellfish has been documented, but only a few studies identified the rotavirus genotype. In this present study, four genotypes (G1, G3, G9, and G12) of rotavirus were classified in mussels, three genotypes (G1, G3, and G9) in oysters and one genotype (G3) in cockles. Rotavirus G3 was found predominantly in bivalve shellfish, which is in agreement with our previous study (Kittigul et al., 2014). In contrast to environmental water, which is a source of animal fecal excreta, the bivalve shellfish sampled in this study are contaminated with the human strains of rotavirus at a higher detection rate than animal strains. This may be due to the harvesting areas of bivalve shellfish being impacted by human waste.

Rotavirus G1, G9 and G12 strains are in a single lineage of each genotype, whereas rotavirus G3 strains show co-circulating lineages. Rotavirus G1 strains, which are the most common genotype in humans worldwide (Santos and Hoshino, 2005), were also detected in oyster and mussel samples clustering in the same lineage. Rotavirus G3 strains present in shellfish are more diverse in different lineages than other genotypes and are related to both human and animal strains (such as canine, porcine, and equine). Three genetic sequences in lineage I of G3 are similar to the unusual equine rotavirus A strain RVA/Horse-wt/ARG/E3198, which has been occasionally detected in horses and suggested as being an interspecies transmitted rotavirus, possibly by reassortment, from a feline, canine or related host species to a horse (Mino et al., 2013). Rotavirus G9 has become recognized as one of the most widespread emerging genotypes causing acute diarrhea (Santos and Hoshino, 2005). The G9 genetic sequences of six rotavirus strains in shellfish are distantly related to other reference strains. Since the present study uses partial sequences (254 bp) of rotavirus strains for phylogenetic analyses, the full VP7 sequences are required to further study of G9 lineage classification for confirmation. Rotavirus G12, which is similar to the human strains detected in this study, has not been reported in bivalve shellfish, whereas G1, G3, and G9 have been registered in oysters (Kittigul et al., 2008, 2014). Continued surveillance of rotavirus strains circulating in both humans and the environment should be carried out to study the molecular epidemiology and the emergence of new rotavirus strains.

5. Conclusions

The current study provides an alternative method for rotavirus extraction and concentration requiring less effort than the other methods discussed in this paper. The approaches taken in the methodologies for virus detection in foods can be applied to other enteric viruses for molecular epidemiological investigations of food-borne outbreaks and screening a variety of foods under active monitoring. The finding of rotavirus strains in various shellfish species revealed the harvesting areas had been fecally polluted by humans and/or animals. As a result, bivalve shellfish may be potential vehicles for the transmission of rotaviruses and consequently cause the sort of acute gastroenteritis outbreaks associated with raw consumption.

Acknowledgments

This work was supported by a research grant from the Thai Government Budget through Mahidol University, fiscal year 2010-2012. This study was partially supported for publication by the China Medical Board (CMB), Faculty of Public Health, Mahidol University, Bangkok, Thailand. We thank Mr. Kevin Kirk, The Language Center, Faculty of Graduate Studies, Mahidol University for editorial assistance.

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