Food Environ Virol (2016) 8:133–140
DOI 10.1007/s12560-016-9228-6

ORIGINAL PAPER

Prevalence and Molecular Genotyping of Noroviruses in Market Oysters, Mussels, and Cockles in Bangkok, Thailand

Leera Kittigul[1] · Anyarat Thamjaroen[1] · Suwat Chiawchan[1] · Porntip Chavalitshewinkoon-Petmitr[2] · Kannika Pombubpa[1] · Pornphan Diraphat[1]

Received: 8 December 2015 / Accepted: 29 January 2016 / Published online: 12 February 2016

Abstract Noroviruses are the most common cause of acute gastroenteritis associated with bivalve shellfish consumption. This study aimed to detect and characterize noroviruses in three bivalve shellfish species: oysters (*Saccostrea forskali*), cockles (*Anadara nodifera*), and mussels (*Perna viridis*). The virus concentration procedure (adsorption-twice elution-extraction) and a molecular method were employed to identify noroviruses in shellfish. RT-nested PCR was able to detect known norovirus GII.4 of 8.8×10^{-2} genome copies/g of digestive tissues from oyster and cockle concentrates, whereas in mussel concentrates, the positive result was seen at 8.8×10^{2} copies/g of digestive tissues. From August 2011 to July 2012, a total of 300 shellfish samples, including each of 100 samples from oysters, cockles, and mussels were collected and tested for noroviruses. Norovirus RNA was detected in 12.3 % of shellfish samples. Of the noroviruses, 7.7 % were of the genogroup (G) I, 2.6 % GII, and 2.0 % were mixed GI and GII. The detection rate of norovirus GI was 2.1 times higher than GII. With regards to the different shellfish species, 17 % of the oyster samples were positive, while 14.0 and 6.0 % were positive for noroviruses found in mussels and cockles, respectively. Norovirus contamination in the shellfish occurred throughout the year with the highest peak in September. Seventeen norovirus-positive PCR products were characterized upon a partial sequence analysis of the capsid gene. Based on phylogenetic analysis, five different genotypes of norovirus GI (GI.2, GI.3, GI.4, GI.5, and GI.9) and four different genotypes of GII (GII.1, GII.2, GII.3, and GII.4) were identified. These findings indicate the prevalence and distribution of noroviruses in three shellfish species. The high prevalence of noroviruses in oysters contributes to the optimization of monitoring plans to improve the preventive strategies of acute gastroenteritis.

Keywords Norovirus · Genotype · Bivalve shellfish · RT-nested PCR

Introduction

Noroviruses are recognized as the most common cause of acute gastroenteritis associated with bivalve shellfish consumption (Bellou et al. 2013). Shellfish-borne outbreaks of norovirus gastroenteritis have been reported (Fitzgerald et al. 2014; Lodo et al. 2014; Loury et al. 2015). Noroviruses belong to the family *Caliciviridae* and are classified into six genogroups, of which genogroup (G) I, II, and IV infect humans. Within each genogroup, one or more genotypes have been identified, with 9 genotypes in GI, 22 in GII, and 2 in GIV (Kroneman et al. 2013; Mesquita et al. 2010). Norovirus strains within the same genogroup share 55–85 % amino acid identity, while strains within the same genotype share >85 % amino acid identity (Zheng et al. 2006). GII strains; genotype GII.4 are more commonly associated with person-to-person transmission, while GI strains; genotypes GI.2 and GI.4 are more frequently identified in shellfish-associated outbreaks (Verhoef et al. 2010). Norovirus-infected patients shed a high concentration of viral particles in feces and this is distributed in the

✉ Leera Kittigul
leera.kit@mahidol.ac.th

[1] Department of Microbiology, Faculty of Public Health, Mahidol University, 420/1 Ratchawithi Road, Bangkok 10400, Thailand

[2] Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand

surrounding environment (Lopman et al. 2012). As a consequence, noroviruses can contaminate water sources and shellfish-growing areas. The viruses have been detected in a variety of shellfish species including oysters, cockles, mussels, and clams (Bigoraj et al. 2014; Brake et al. 2014; Li et al. 2014; Polo et al. 2015; Suffredini et al. 2014). However, only a few studies report the norovirus genotype present in bivalve shellfish (Manso and Romalde 2013; Verhoef et al. 2010).

An international standard method for the detection of norovirus in foods, adopted by the European Committee on Normalization (CEN), has been proposed as CEN ISO/TS 15216: 2013 (www.iso.org). Recently, a developed virus concentration method has been used to detect rotavirus with high sensitivity in various shellfish species (Kittigul et al. 2015). The application of the virus concentration method for other enteric viruses such as norovirus is of interest in the surveillance of virus contamination in foods. Therefore, this study aimed to examine norovirus contamination in three shellfish species (oysters, mussels, and cockles) using the virus concentration method and reverse transcription-nested polymerase chain reaction (RT-nested PCR). Molecular genotyping of noroviruses detected in shellfish samples was also undertaken. The presence of norovirus genotypes in different shellfish species would be beneficial for active epidemiological surveillance of norovirus gastroenteritis.

Materials and Methods

Shellfish Sampling

Oysters (*Saccostrea forskali*), mussels (*Perna viridis*), and cockles (*Anadara nodifera*) were collected fresh from 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 norovirus-positive fecal sample was added to the digestive tissue concentrates for the sensitivity assays. A total of 300 shellfish samples (100 samples of each of oysters, cockles, and mussels) were collected from two fresh markets in a 1-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 for the detection of noroviruses.

Virus Extraction and Concentration

The shellfish 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 USA) twice for 45 s each. The homogenates from the digestive tissues were processed using a rapid virus concentration method called the adsorption-twice elution-extraction method, as described by Kittigul et al. (2015). Briefly, the shellfish homogenate was adjusted 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. Chloroform, with a final concentration of 30 %, was added 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 (UNI-EQUIP Laborgeratebau und-vertriebs GmbH, Munich, Germany) for 6–8 h at 3 °C and stored at −80 °C until the nucleic acid extraction was performed.

Nucleic Acid Extraction and RT-Nested PCR

Viral nucleic acids were extracted from 200 µL of shellfish concentrates using an RNeasy® mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Viral nucleic acids (50–55 µL) were obtained and tested for noroviruses using the previously described RT-nested PCR assay (Kittigul et al. 2011). Briefly, the assay was performed in a 50 µL reaction volume with a separate tube for norovirus GI and norovirus GII. RNA extract (2 µL) was heated at 94 °C for 4 min and placed on ice for at least 10 min. Then 2 µL of denatured RNA was added to 48 µL of the RT-PCR mixture consisting of 1× Reaction Mix (a buffer containing 0.2 mM each of dNTP and 1.6 mM $MgSO_4$), SuperScript™ III RT/Platinum®-*Taq*Mix (Invitrogen, Life Technologies, Carlsbad, CA), 0.33 µM of each primer for norovirus GI (COG1F and G1-SKR) and for norovirus GII (COG2F and G2-SKR) (Kageyama et al. 2003; Kojima et al. 2002), and Ultrapure™ distilled water. The reaction tube was inserted into a thermocycler (Thermo Hybaid, Franklin, MA USA). RT-PCR was carried out using the following steps: RT at 42 °C for 60 min; initial denaturation at 94 °C for 2 min; PCR of 35 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min; and a final extension at 72 °C for 3 min.

Nested PCR was performed in a 50 μL reaction volume. The first PCR amplification product (2 μL) was added to the reaction mixture (48 μL): 1X PCR Buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.63 U of *Taq* DNA polymerase (Invitrogen) for norovirus GI or 2.5 U of *Taq* DNA polymerase for norovirus GII, 0.33 μM of each primers for norovirus GI (G1-SKF and G1-SKR) and for norovirus GII (G2-SKF and G2-SKR) (Kageyama et al. 2003; Kojima et al. 2002), and Ultrapure™ distilled water. The cycling conditions were as follows: initial denaturation at 94 °C for 3 min; PCR 35 cycles for norovirus GI or 30 cycles for norovirus GII at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 15 min. The PCR products were analyzed using 1.5 % agarose gel electrophoresis and ethidium bromide staining. DNA fragments of 330 bp and 344 bp were considered to be norovirus GI and norovirus GII, respectively. A positive control of norovirus and two negative amplification controls, Ultrapure™ distilled water and the PCR mixture for RT-PCR and nested PCR, were included in each amplification series.

Sensitivity Assay

The digestive tissue concentrates were added with serial ten-fold dilutions of a norovirus GII.4-positive fecal sample between 10^{-2} and 10^{-8} (2.2×10^{-2} to 2.2×10^{4} genome copies/mL), or equal $8.8. \times 10^{-3}$ to 8.8×10^{3} genome copies/g of digestive tissues. Norovirus RNA in the concentrates was extracted and examined using RT-nested PCR. The negative control was the digestive tissue concentrate without any added norovirus.

Sequencing of Norovirus PCR Products

The RT-nested PCR products of the noroviruses GI and GII were purified using a QIAquick PCR Purification Kit or QIAquick gel extraction 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 capsid 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 6.0 (Tamura et al. 2013).

The nucleotide sequences of noroviruses GI and GII obtained in shellfish samples, corresponding to fragments of the capsid gene of noroviruses, were deposited in GenBank under the following Accession Numbers: KU220625-KU220641.

Results

Sensitivity of RT-Nested PCR for Norovirus Detection

RT-nested PCR was able to detect norovirus GII.4 from a fecal sample at 10^{-7} dilution or $8.8. \times 10^{-2}$ genome copies/g of digestive tissues in oyster concentrates. For cockle concentrates, the same sensitivity was observed after the norovirus RNA was 1:2 diluted in nuclease-free water prior to RT-nested PCR assay. However, in mussel concentrates, the positive result was seen at end point fecal dilution of 10^{-3} or 8.8×10^{2} copies/g of digestive tissues. These results demonstrated the higher sensitivity of RT-nested PCR for norovirus detection in oyster and cockle concentrates than in mussel concentrates.

Prevalence of Norovirus in Three Shellfish Species

For the one-year study, 12.3 % (37/300 samples) of the shellfish samples tested positive for noroviruses. The frequency of noroviruses in oysters (17.0 %, 17/100 samples) was higher than that in mussels (14.0 %, 14/100 samples) and cockles (6.0 %, 6/100 samples). Of the norovirus-positive samples, 23 (7.7 %), 8 (2.6 %), and 6 (2.0 %) harbored GI, GII, and mixed GI + GII, respectively. Mixed noroviruses GI + GII were found in five oyster samples and one mussel sample. Analysis of the shellfish samples contaminated with either norovirus GI or GII revealed that norovirus GI (29 samples) occurred 2.1 times more frequently than norovirus GII (14 samples). The distribution of noroviruses GI and GII in the three shellfish species is shown in Table 1.

A monthly analysis of norovirus-positive shellfish samples showed the distribution of noroviruses in the three shellfish species throughout the year (Fig. 1). Norovirus contamination in the shellfish occurred year round, except in June, with the highest peak (45.8 %) in September. The prevalence of norovirus-positive shellfish samples that peaked in September was found in oysters, cockles, and mussels. Noroviruses GI, GII, and/or mixed GI + GII strains were detected in oyster, mussel, and/or cockle

Table 1 Presence of naturally occurring noroviruses in bivalve shellfish samples

Bivalve	Total	No. of norovirus-positive samples (%)			
Shellfish	No.	GI	GII	GI + GII	Total
Oyster	100	10 (10.0)	2 (2.0)	5 (5.0)	17 (17.0)
Mussel	100	9 (9.0)	4 (4.0)	1 (1.0)	14 (14.0)
Cockle	100	4 (4.0)	2 (2.0)	–	6 (6.0)
Total	300	23 (7.7)	8 (2.6)	6 (2.0)	37 (12.3)

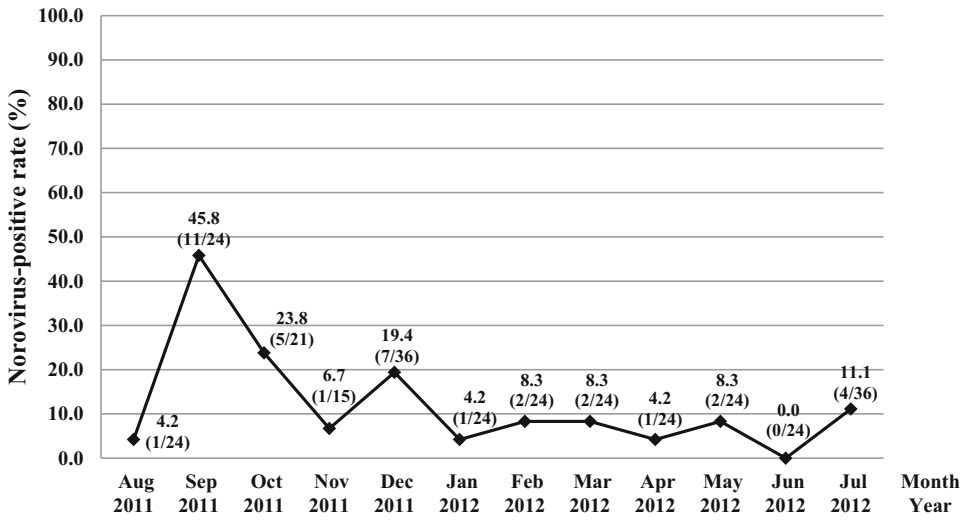


Fig. 1 Monthly distribution of noroviruses detected in bivalve shellfish from August, 2011 to July, 2012. The norovirus-positive rate represents the percentage of noroviruses detected in shellfish samples during each month. Norovirus-positive samples/total shellfish samples collected are shown in *brackets*

samples on all sampling occasions throughout the sampling period, except in June.

Genetic Characterization and Genotyping of Noroviruses Detected in Bivalve Shellfish

Seventeen norovirus-positive PCR products were subjected to DNA sequencing and the partial nucleotide sequences of the capsid gene were analyzed. Norovirus GI sequences (7 samples) detected in oysters, cockles, and mussels revealed similar nucleotide sequences (98–99 % nucleotide identity) to the norovirus strains, which are available in GenBank, found in humans, oyster, and water. Norovirus GII sequences (10 samples) detected in oysters and mussels showed similar nucleotide sequences to human noroviruses (90–99 % nucleotide identity).

The partial capsid nucleotide sequences of noroviruses were classified according to the phylogenetic clustering method (Vinjé 2015; Zheng et al. 2006). Five genotypes of noroviruses GI and four of GII could be classified and the phylogenetic trees are shown in Fig. 2. For norovirus GI, the nucleotide sequence of Coc072 was clustered in the same branch as the reference Southampton strain (L07418) belonging to GI.2. The norovirus sequence of Mus050 was distantly clustered in the reference Otofuke (AB187514) of GI.3c. Norovirus sequences of Oys058 and Oys028 were clustered in the same branches as the references Chiba407 (AB042808) and SzUG1 (AB039774) strains belonging to GI.4, and GI.5b, respectively. Interestingly, three norovirus sequences of Oys009, Oys034, and Oys042 were found to be most closely related to the norovirus sequence from a wastewater sample in South Africa (GP; KC495626) and clustered in a separate branch in the tree. These norovirus sequences are proposed as GI.9 (Murray et al. 2013).

For norovirus GII, the nucleotide sequence of Oys042 was clustered in the same branch as the reference Hawaii strain (U07611) belonging to GII.1. Of note, the nucleotide sequences of Oys028 and Oys034 exhibited separate branches from the reference Hawaii strain (U07611) of GII.1 and the reference Toronto strain (U02030) of GII.3a. These two noroviruses were classified as GII.1-like and GII.3-like, respectively. The norovirus sequence of Mus011 was clustered in the same branch as the reference Melksham strain (X81879) and belonged to GII.2. A majority of identified norovirus GII sequences were GII.4: one sequence (Oys033) was clustered in the GII.4 2006b variant (Nijmegen115/2006/NZL; EF126966), whereas two sequences (Mus035 and Mus036) were clustered in the GII.4 2009 variant (New Orleans 1805/2009/USA; GU445325). Although three norovirus sequences (Oys023, Oys024, and Mus076) were clustered in a separate branch of the GII.4, these sequences were probably GII.4 2009 variants.

Discussion

Bivalve shellfish have been implicated in norovirus outbreaks worldwide (Fitzgerald et al. 2014; Lodo et al. 2014; Loury et al. 2015). The shellfish are able to accumulate and concentrate norovirus particles by the filtration of fecal-contaminated water. The bioaccumulation may vary with the shellfish species, norovirus genogroups or genotypes, and environmental conditions (Le Guyader et al. 2012). Different detection rates of norovirus in a variety of shellfish species have been reported (Suffredini et al. 2014; Polo et al. 2015). Human consumption of raw or slightly cooked shellfish is associated with norovirus-related gastroenteritis outbreaks (Prato et al. 2004).

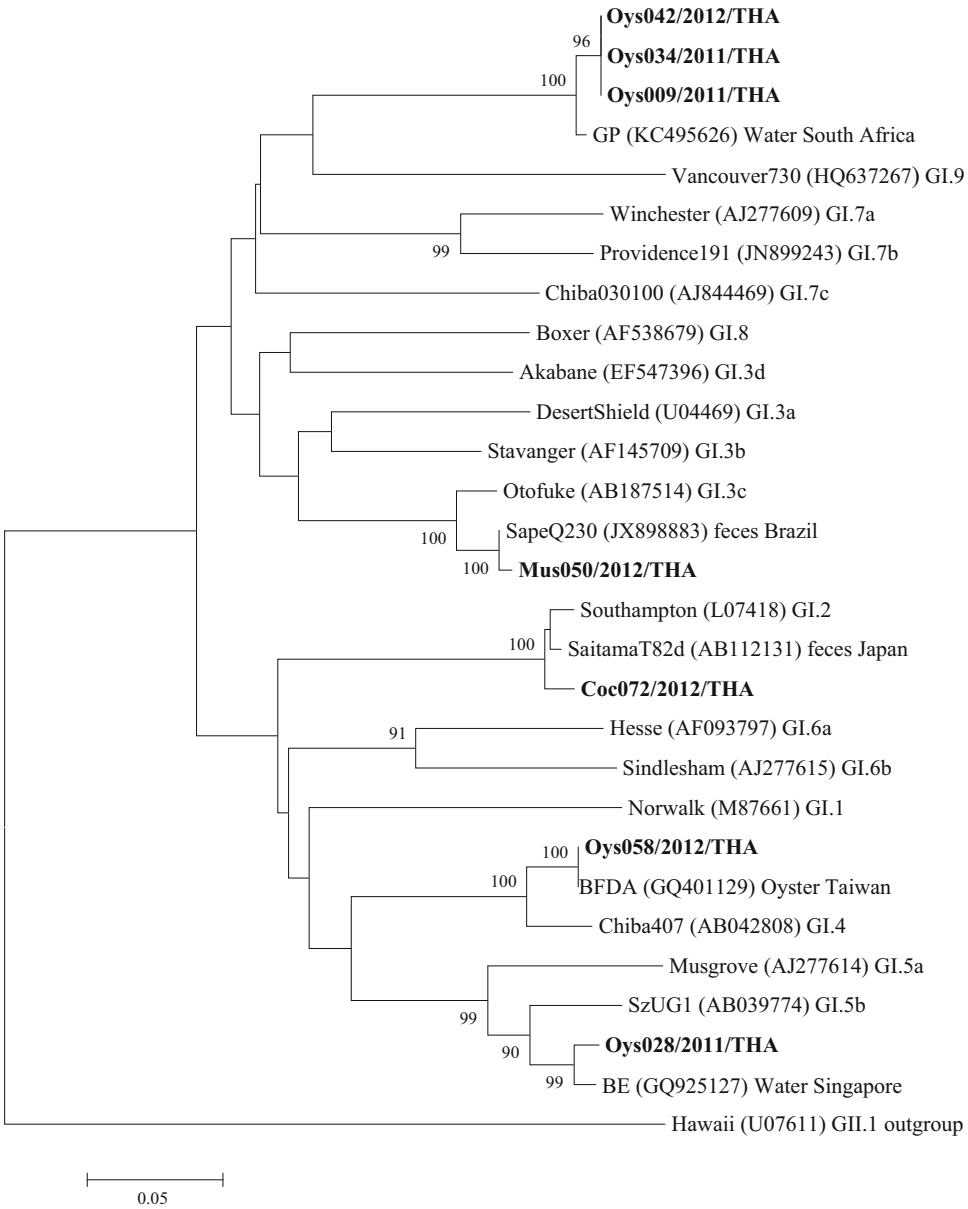
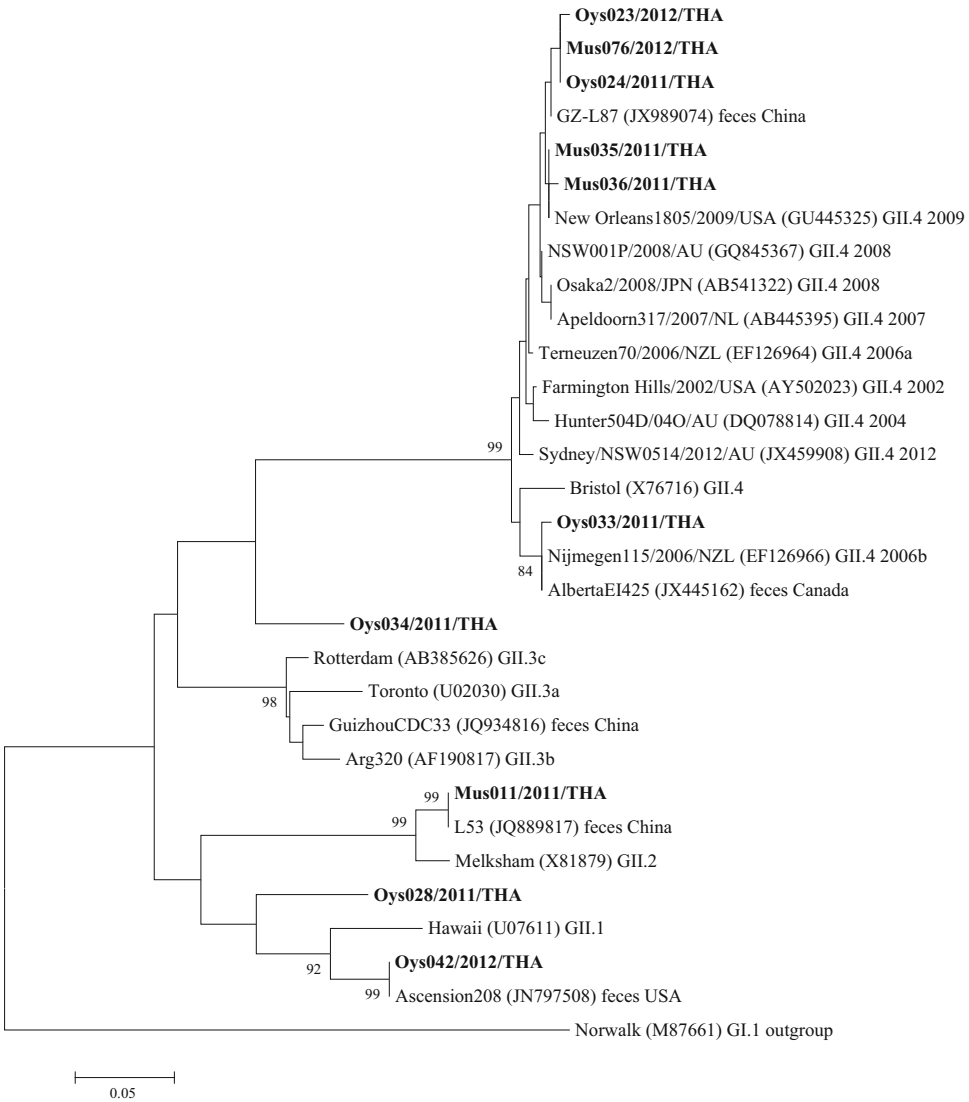


Fig. 2 Phylogenetic tree of partial nucleotide sequences derived from norovirus GI (**a**) and GII (**b**) strains detected in this study and other reference strains from the GenBank database. The tree was generated using the neighbor-joining analysis of 1000 repetitions in MEGA 6.0. The *scale bar* indicates nucleotide substitutions per site. Bootstrap values >80 % are shown at the branch nodes. Norovirus GI and GII strains in the present study are indicated in *bold*

The present study applied the developed virus concentration method and RT-nested PCR for testing for norovirus in three shellfish species. The sensitivities of RT-nested PCR were determined by spiking known norovirus GII into the norovirus-negative shellfish concentrates. The assays were carried out to assess the detection limits of RT-nested PCR for norovirus and also PCR inhibitors in different shellfish species. RT-nested PCR gave the same sensitivity of norovirus GII in oyster and cockle concentrates (8.8×10^{-2} genome copies/g of digestive tissues) when the RNA, obtained from the cockle concentrate, was diluted 1:2. However, the lower sensitivity (8.8×10^{2} copies/g of digestive tissues) was observed in mussel concentrates. Although RNA extracts of norovirus GII.4 from mussel concentrates were diluted at 1:2, 1:5, and 1:10 in nuclease-free water, the RT-nested PCR assay showed negative results. It is likely that PCR inhibitors were present in mussel concentrates and the method used in the current study could not remove them. PCR inhibitors, including polysaccharides and organic compounds, have been recognized as causing major problems in the application of RT-PCR to shellfish samples because they inhibit the *Taq* DNA polymerase for the amplification of viral RNA (Atmar et al. 1993). The presence of PCR inhibitors was reported in certain species of mussels (*Mytilus* spp.) (Casas and Sunen 2001). The content of PCR inhibitors in shellfish may vary between different species and harvesting areas (Schultz et al. 2007). Additionally, the type of virus might

Fig. 2 continued



be of concern since the study of rotavirus demonstrated no effect of inhibitors in the PCR reaction among these oysters, cockles, and mussels (Kittigul et al. 2015). Nevertheless, in the field study, noroviruses could be detected in collected mussel samples.

Naturally occurring noroviruses were found in 12.3 % of shellfish samples. The presence of noroviruses in shellfish is variable according to several studies conducted in different geographical areas due to the difference in collection sites, and in particular the fecal contamination sources (Benabbes et al. 2013; Lowther et al. 2012; Mesquita et al. 2011; Suffredini et al. 2012). Regarding the distribution of norovirus among the three shellfish species tested, the results highlighted a higher detection rate of norovirus in oysters than mussels, and cockles. The high prevalence of norovirus in oysters might be due to the shellfish samples being collected in polluted sites or sites heavily impacted by human sewage. The shellfish species is one factor affecting norovirus prevalence since the different shellfish species might have different rates of bioaccumulation for norovirus. Environmental factors such as temperature and salinity also influence the bioaccumulation of noroviruses in shellfish (Maalouf et al. 2011).

Norovirus GI was predominant in all three shellfish species at a higher frequency than GII. GI was more often implicated in shellfish-related outbreaks than GII (Le Guyader et al. 2006; Nenonen et al. 2009) and is the most prevalent virus in shellfish (Boxman et al. 2006; Polo et al. 2015). GI might be more stable in water environments than GII (da Silva et al. 2007). Nevertheless, norovirus GII which is the main cause of acute gastroenteritis in humans was also found predominantly in shellfish (Croci et al. 2007; Suffredini et al. 2012). Different norovirus strains show different specificities for their receptors or histo-

blood group antigens in oysters. GI.1 recognizes the A-like ligand of the digestive tract, whereas GII.4 recognizes a sialic acid-containing ligand, which is present in all tissues including the digestive tracts of oysters (Maalouf et al. 2010).

Norovirus sequences from all three shellfish samples in the present study were closely related to the norovirus strains found in fecal, oyster, and water samples worldwide. These findings demonstrate noroviruses circulating in humans and the environment and the shellfish might be a potential vehicle for norovirus transmission. Based on RT-nested PCR, the current study identified five norovirus GI genotypes (GI.2, GI.3, GI.4, GI.5, and GI.9), and four GII genotypes (GII.1, GII.2, GII.3, and GII.4) in the shellfish samples. This is the first study of norovirus genotype distributions in different species of bivalve shellfish in Thailand. In addition, GI.5 and recently classified GI.9 were first identified in oyster samples. The norovirus GII.4 2006b variant identified in the oyster sample was also reported in the United States (Woods and Burkhardt 2010) and in mussel samples from Spain (Manso and Romalde 2013). The well-known GII.4 2006b variant, which caused gastroenteritis outbreaks worldwide during the years 2006–2007 (Siebenga et al. 2009), continued in prevalence through this study period (2011–2012). Interestingly, the GII.4 variant strains (New Orleans 2009) emerging and causing acute gastroenteritis in humans were also found in two mussel samples. Three strains from oyster and mussel samples with genetically diverse sequences are similar to a novel norovirus strain GZ2010-L87 found in China which belongs to the new cluster GII.4-2009 as identified in Asia and the United States since 2009 (Xue et al. 2013).

The present study demonstrates both the prevalence and characterization of norovirus in three different shellfish species. Norovirus contaminated oysters may be a major source of health risks upon norovirus infection associated with raw or slightly cooked consumption. The norovirus variants found in this study confirm that the shellfish can harbor different norovirus strains, including strains that are frequently involved in norovirus gastroenteritis. Hence, the introduction of strategic plans in wastewater treatment systems, the improvement in the sanitary quality of bivalve shellfish, and good personal hygiene are suggested as preventive measures for food safety.

Acknowledgments This work was supported by a research grant from the Thai Government Budget through Mahidol University, Bangkok, Thailand, for the fiscal year 2010–2012. The 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.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403–410.

Atmar, R. L., Metcalf, T. G., Neill, F. H., & Estes, M. K. (1993). Detection of enteric viruses in oysters by using the polymerase chain reaction. *Applied and Environmental Microbiology, 59*(2), 631–635.

Bellou, M., Kokkinos, P., & Vantarakis, A. (2013). Shellfish-borne viral outbreaks: A systematic review. *Food and Environmental Virology, 5*(1), 13–23.

Benabbes, L., Ollivier, J., Schaeffer, J., Parnaudeau, S., Rhaissi, H., Nourlil, J., et al. (2013). Norovirus and other human enteric viruses in Moroccan shellfish. *Food and Environmental Virology, 5*(1), 35–40.

Bigoraj, E., Kwit, E., Chrobocińska, M., & Rzeżutka, A. (2014). Occurrence of norovirus and hepatitis A virus in wild mussels collected from the Baltic Sea. *Food and Environmental Virology, 6*(3), 207–212.

Boxman, I. L., Tilburg, J. J., Te Loeke, N. A., Vennema, H., Jonker, K., de Boer, E., et al. (2006). Detection of noroviruses in shellfish in The Netherlands. *International Journal of Food Microbiology, 108*(3), 391–396.

Brake, F., Ross, T., Holds, G., Kiermeier, A., & McLeod, C. (2014). A survey of Australian oysters for the presence of human noroviruses. *Food Microbiology, 44*, 264–270.

Casas, N., & Sunen, E. (2001). Detection of enterovirus and hepatitis A virus RNA in mussels (*Mytilus* spp.) by reverse transcriptase-polymerase chain reaction. *Journal of Applied Microbiology, 90*(1), 89–95.

Croci, L., Losio, M. N., Suffredini, E., Pavoni, E., Di Pasquale, S., Fallacara, F., et al. (2007). Assessment of human enteric viruses in shellfish from the northern Adriatic sea. *International Journal of Food Microbiology, 114*(2), 252–257.

da Silva, A. K., Le Saux, J. C., Parnaudeau, S., Pommepuy, M., Elimelech, M., & Le Guyader, F. S. (2007). Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: Different behaviors of genogroups I and II. *Applied and Environmental Microbiology, 73*(24), 7891–7897.

Fitzgerald, T. L., Merritt, T. D., Zammit, A., McLeod, C., Landinez, L. M., White, P. A., et al. (2014). An outbreak of norovirus genogroup II associated with New South Wales oysters. *Communicable Diseases Intelligence Quaterly Report, 38*(1), E9–E15.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., et al. (2003). Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology, 41*(4), 1548–1557.

Kittigul, L., Pombubpa, K., Sukonthalux, S., Rattanatham, T., & Utrarachkij, F. (2011). Noroviruses in oysters from local markets and oyster farms in southern Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health, 42*(1), 105–113.

Kittigul, L., Singhaboot, Y., Chavalitshewinkoon-Petmitr, P., Pombubpa, K., & Hirunpetcharat, C. (2015). A comparison of virus concentration methods for molecular detection and characterization of rotavirus in bivalve shellfish species. *Food Microbiology, 46*, 161–167.

Kojima, S., Kageyama, T., Fukushi, S., Hoshino, F. B., Shinohara, M., Uchida, K., et al. (2002). Genogroup-specific PCR primers for detection of Norwalk-like viruses. *Journal of Virological Methods, 100*(1–2), 107–114.

Kroneman, A., Vega, E., Vennema, H., Vinjé, J., White, P. A., Hansman, G., et al. (2013). Proposal for a unified norovirus nomenclature and genotyping. *Archives of Virology, 158*(10), 2059–2068.

Le Guyader, F., Atmar, R. L., & Le Pendu, J. (2012). Transmission of viruses through shellfish: When specific ligands come into play. *Current Opinion in Virology, 2*(1), 103–110.

Le Guyader, F., Bon, F., DeMedici, D., Parnaudeau, S., Bertone, A., Crudeli, S., et al. (2006). Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. *Journal of Clinical Microbiology, 44*(11), 3878–3882.

Li, D., Stals, A., Tang, Q. J., & Uyttendaele, M. (2014). Detection of noroviruses in shellfish and semiprocessed fishery products from a Belgian seafood company. *Journal of Food Protection, 77*(8), 1342–1347.

Lodo, K. L., Veitch, M. G., & Green, M. L. (2014). An outbreak of norovirus linked to oysters in Tasmania. *Communicable Diseases Intelligence Quaterly Report, 38*(1), E16–E19.

Lopman, B., Gastañaduy, P., Park, G. W., Hall, A. J., Parashar, U. D., & Vinjé, J. (2012). Environmental transmission of norovirus gastroenteritis. *Current Opinion in Virology, 2*(1), 96–102.

Loury, P., Le Guyader, F. S., Le Saux, J. C., Ambert-Balay, K., Parrot, P., & Hubert, B. (2015). A norovirus oyster-related outbreak in a nursing home in France, January 2012. *Epidemiology and Infection, 143*(12), 2486–2493.

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E., & Lees, D. N. (2012). Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom. *Applied and Environmental Microbiology, 78*(16), 5812–5817.

Maalouf, H., Schaeffer, J., Parnaudeau, S., Le Pendu, J., Atmar, R. L., Crawford, S. E., et al. (2011). Strain-dependent norovirus bioaccumulation in oysters. *Applied and Environmental Microbiology, 77*(10), 3189–3196.

Maalouf, H., Zakhour, M., Le Pendu, J., Le Saux, J. C., Atmar, R. L., & Le Guyader, F. S. (2010). Distribution in tissue and seasonal variation of norovirus genogroup I and II ligands in oysters. *Applied and Environmental Microbiology, 76*(16), 5621–5630.

Manso, C. F., & Romalde, J. L. (2013). Detection and characterization of hepatitis A virus and norovirus in mussels from Galicia (NW Spain). *Food and Environmental Virology, 5*(2), 110–118.

Mesquita, J. R., Barclay, L., Nascimento, M. S., & Vinjé, J. (2010). Novel norovirus in dogs with diarrhea. *Emerging Infectious Diseases, 16*(6), 980–982.

Mesquita, J. R., Vaz, L., Cerqueira, S., Castilho, F., Santos, R., Monteiro, S., et al. (2011). Norovirus, hepatitis A virus and enterovirus presence in shellfish from high quality harvesting areas in Portugal. *Food Microbiology, 28*(5), 936–941.

Murray, T. Y., Mans, J., & Taylor, M. B. (2013). Human calicivirus diversity in wastewater in South Africa. *Journal of Applied Microbiology, 114*(6), 1843–1853.

Nenonen, N. P., Hannoun, C., Olsson, M. B., & Bergstrom, T. (2009). Molecular analysis of an oyster-related norovirus outbreak. *Journal of Clinical Virology, 45*(2), 105–108.

Polo, D., Varela, M. F., & Romalde, J. L. (2015). Detection and quantification of hepatitis A virus and norovirus in Spanish authorized shellfish harvesting areas. *International Journal of Food Microbiology, 193*, 43–50.

Prato, R., Lopalco, P. L., Chironna, M., Barbuti, G., Germinario, C., & Quarto, M. (2004). Norovirus gastroenteritis general outbreak associated with raw shellfish consumption in south Italy. *BMC Infectious Diseases, 4*, 37.

Schultz, A. C., Saadbye, P., Hoorfar, J., & Norrung, B. (2007). Comparison of methods for detection of norovirus in oysters. *International Journal of Food Microbiology, 114*(3), 352–356.

Siebenga, J., Vennema, H., Zheng, D. P., Vinje, J., Lee, B. E., Pang, X. L., et al. (2009). Norovirus illness is a global problem: Emergence and spread of norovirus GII-4 variants, 2001–2007. *The Journal of Infectious Diseases, 200*(5), 802–812.

Suffredini, E., Lanni, L., Arcangeli, G., Pepe, T., Mazzette, R., Ciccaglioni, G., et al. (2014). Qualitative and quantitative assessment of viral contamination in bivalve molluscs harvested in Italy. *International Journal of Food Microbiology, 184*, 21–26.

Suffredini, E., Magnabosco, C., Civettini, M., Rossetti, E., Arcangeli, G., & Croci, L. (2012). Norovirus contamination in different shellfish species harvested in the same production areas. *Journal of Applied Microbiology, 113*(3), 686–692.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*(12), 2725–2729.

Verhoef, L., Vennema, H., van Pelt, W., Lees, D., Boshuizen, H., Henshilwood, K., et al. (2010). Use of norovirus genotype profiles to differentiate origins of foodborne outbreaks. *Emerging Infectious Diseases, 16*(4), 617–624.

Vinjé, J. (2015). Advances in laboratory methods for detection and typing of norovirus. *Journal of Clinical Microbiology, 53*(2), 373–381.

Woods, J. W., & Burkhardt, W, I. I. I. (2010). Occurrence of norovirus and hepatitis A virus in U.S. oysters. *Food and Environmental Virology, 2*(3), 176–182.

Xue, L., Wu, Q., Kou, X., Cai, W., Zhang, J., & Guo, W. (2013). Complete genome analysis of a novel norovirus GII.4 variant identified in China. *Virus Genes, 47*(2), 228–234.

Zheng, D. P., Ando, T., Fankhauser, R. L., Beard, R. S., Glass, R. I., & Monroe, S. S. (2006). Norovirus classification and proposed strain nomenclature. *Virology, 346*(2), 312–323.