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ORIGINAL PAPER

Norovirus Monitoring in Oysters Using Two Different Extraction Methods

Thamapan Tunyakittaveeward[1] · Kitwadee Rupprom[1] · Kannika Pombubpa[1] · Nopporn Howteerakul[2] · Leera Kittigul[1]

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Abstract

Detection of noroviruses in bivalve shellfish is difficult because of the low concentration of norovirus and the presence of reverse transcription (RT)-PCR inhibitors. This study aimed to assess the presence of noroviruses in oysters extracted using a proteinase K extraction (ISO 15216 method) and an adsorption–elution method. Seventy oyster samples were extracted using the two extraction methods and evaluated using RT-nested PCR. The results showed norovirus detection rates at an equal frequency of 28.6%, of which a total of 48 (68.6%) samples had corresponding positive or negative results, while there were 22 (31.4%) samples with discrepant results. Norovirus genogroup (G)I, GII, and mixed GI and GII were detected in 20%, 4.3%, and 4.3% of samples, respectively, by the proteinase K extraction method, which comprised of GI.2, GI.5b, GI.6b, GII.4, and GII.17 genotypes. With the adsorption–elution method noroviruses were detected in 17.1%, 8.6%, and 2.9% of samples, respectively, which comprised of GI.2, GII.2, GII.4, and GII.17 genotypes. All norovirus-positive oyster samples were further estimated for genome copy number using RT-quantitative PCR. The oyster samples processed using the adsorption–elution method contained norovirus GI of 3.36×10^{1}–1.06×10^{5} RNA copies/g of digestive tissues and GII of 1.29×10^{3}–1.62×10^{4} RNA copies/g. Only GII (2.20×10^{1} and 7.83×10^{1} RNA copies/g) could be quantified in samples prepared using the proteinase K extraction method. The results demonstrate the different performance of the two sample-processing methods, and suggest the use of either extraction method in combination with RT-nested PCR for molecular surveillance of norovirus genotypes in oysters.

Keywords Norovirus · Genotype · Proteinase K extraction · Adsorption–elution · Oyster

Introduction

Noroviruses are the most common cause of acute gastroenteritis in humans of all age, and are associated with foodborne outbreaks. The viruses account for one-fifth of all cases of acute gastroenteritis in children less than 5 years of age, and norovirus infections are believed to cause an estimated $4.2 billion in direct health system costs globally each year (Atmar et al. 2018). Noroviruses belong to the genus *Norovirus* in the family *Caliciviridae*. There are currently seven genogroups, of which genogroups (G) I, II, and IV infect humans. Genogroups are further subdivided into genotypes: 9 GI (GI.1–GI.9), 19 GII (GII.1–GII.10, GII.12–GII.17, GII.20–GII.22) and 1 GIV (GIV.1) based upon viral capsid (VP1) sequences (Vinjé 2015). Norovirus GII.4 variants continue to cause epidemics worldwide, although GII.17 became the predominant genotype during 2014–2016 (Chan et al. 2017), and a GII.P16–GII.2 strain emerged in various countries in 2016–2017 (Liu et al. 2017; Tohma et al. 2017).

Where norovirus foodborne outbreaks have been reported the most commonly implicated food vehicle in the outbreaks were oysters (Hardstaff et al. 2018). Detection of noroviruses associated with oyster-related outbreaks has been continually documented (Iritani et al. 2014; Loury et al. 2015; Woods et al. 2016; Le Mennec et al. 2017). The strategy for detection of foodborne viruses in food samples consists of virus extraction, purification of the viral RNA, and molecular

✉ 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 Epidemiology, Faculty of Public Health, Mahidol University, Bangkok, Thailand

identification. Norovirus detection in bivalve shellfish samples based on proteinase K extraction and reverse transcription-quantitative PCR (RT-qPCR) method has recently been implemented in the revised ISO 15216-1:2017 (Anonymous 2017), which supersedes ISO/TS 15216-1:2013 (Anonymous 2013), which is widely used for analysis of noroviruses in bivalve shellfish (Farkas et al. 2017; La Rosa et al. 2017; Lowther et al. 2018; Purpari et al. 2019). Nevertheless, an improvement of extraction procedures has been reported for the detection of noroviruses in shellfish (Quang Le et al. 2018). Meanwhile, virus extraction by elution method has been evaluated for detection of norovirus in deli meats (da Silva Luz and Miagostovich 2018), fresh produce including berries and vegetables (Bartsch et al. 2016; Cheng et al. 2018; Summa and Maunula 2018), and dairy products (Hennechart-Collette et al. 2017). An acid adsorption–alkaline elution method and RT-nested PCR have been used to extract and examine noroviruses in several bivalve shellfish species such as oysters, cockles, and mussels (Kittigul et al. 2016) as well as in oyster digestive tissues, gills and mantle (Lowmoung et al. 2017). In the present study, to achieve high performance, the adsorption–elution method and the proteinase K extraction method were evaluated for the molecular detection and characterization of norovirus in oyster samples using RT-nested PCR. Genotyping of norovirus recovered from the oyster samples processed by the two extraction methods was carried out to assess norovirus RNA remained to be identified after processing steps. Norovirus levels in positive oyster samples were determined by RT-qPCR. Detection of different norovirus genotypes and concentrations of the virus in oyster samples prepared by the two different extraction methods demonstrated the different performance of the virus-processing methods.

Materials and Methods

Norovirus-Positive Control Sample

A norovirus-positive fecal sample was used as a positive control for determination of the sensitivity and the detection limit of norovirus in oysters using RT-nested PCR. The fecal sample contained norovirus GII.4 at 4.01×10^9 RNA copies/mL.

Oyster Samples

A total of 70 oyster (*Crassostrea belcheri*) samples (three oysters/sample) were collected at 6–12 samples/month from one seafood retailer in a local market in Bangkok, Thailand from August 2015 to March 2016. Digestive tissues of three oysters were excised and pooled (approximately 4 g/sample). A total of 2 g of digestive tissues was extracted using both proteinase K and adsorption–elution methods.

Proteinase K Extraction Method

The digestive tissues of oyster samples were processed by a proteinase K extraction method (Jothikumar et al. 2005) as described in ISO 15216-1:2017 with slight modification of the procedure. Briefly, proteinase K solution (2 mL, 100 µg/mL) was added to the digestive tissues (2 g) of the oyster sample and the sample was vortexed for 5 min. The sample was incubated at 37 °C with shaking (200 rpm) for 75 min and further incubated at 60 °C for 15 min. The sample was centrifuged at 3000×*g* for 5 min. The soluble homogenate (2.5 mL) was collected and the pellet was discarded. The homogenate was stored at − 80 °C until nucleic acid extraction. In this method, the final volume was always higher than the initial volume of proteinase K solution added due to the contribution of fluid from the tissues.

Adsorption–Elution Method

The digestive tissues of oyster samples were processed using an adsorption–elution method as previously described by Kittigul et al. (2016). Briefly, the digestive tissues (2 g) was added with deionized water (150 mL) and homogenized using a blender (Hamilton Beach, Southern Pines, NC) at high speed twice for 45 s each. The 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. After centrifugation, the virus in the pellet was eluted twice using 2 mL of 2.9% tryptose phosphate broth containing 6% glycine, pH 9.0 followed by 2 mL of 0.5 M arginine-0.15 M NaCl, pH 7.5. The soluble fraction was collected and chloroform added to a final concentration of 30% followed by mixing and centrifugation. The aqueous phase was collected and reduced the volume to approximately 2.5 mL. This solution, termed as a concentrate, was stored at − 80 °C until nucleic acid extraction.

Extraction of Nucleic Acids and RT-Nested PCR

A total of 200 µL of digestive tissue oyster homogenate from the proteinase K extraction method or oyster concentrate from the adsorption–elution method was used to extract RNA using the RNeasy® Mini kit (QIAGEN, GmbH, Hilden, Germany) according to product manufacturer's instructions. Primers COG1F, G1-SKF, G1-SKR, and COG2F, G2-SKF, G2-SKR (Kojima et al. 2002; Kageyama et al. 2003) were used for the amplification of sequences from the capsid genes (region C) of norovirus GI and GII, respectively. RT-nested PCR was performed using the SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA

Polymerase (Invitrogen, Carlsbad, CA) in a 50 µL reaction volume each for norovirus, with GI and GII analyzed in separate tubes (Kittigul et al. 2016). Briefly, RNA (2 µL) was heated to 94 °C for 4 min and spun down briefly, then placed on ice for 10 min. The denatured RNA was added into a RT-PCR mixture (48 µL) consisting of 1X Reaction Mix (a buffer containing 0.2 mM each of dNTP, 1.6 mM $MgSO_4$), SuperScript™ III RT/Platinum®*Taq* Mix, 0.33 µM of each pair of primers: COG1F (CGYTGGATGCGNTTYCATGA) and G1-SKR (CCAACCCARCCATTRTACA) for norovirus GI; COG2F (CARGARBCNATGTTYAGRTGGATGAG) and G2-SKR (CCRCCNGCATRHCCRTTRTACAT) for norovirus GII, and nuclease-free water. The reaction tube was placed into a thermocycler (Thermo Hybaid, Frankin, MA). RT-PCR was carried out with following steps: RT at 42 °C for 60 min, initial denaturation at 94 °C for 2 min, PCR 35 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 3 min. The RT-PCR amplification product (2 µL) was added into a reaction mixture (48 µL) consisting of 1X PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.63 U for norovirus GI or 2.5 U for norovirus GII of *Taq* DNA polymerase, 0.33 µM of each pair of primers: G1-SKF (CTGCCCGAATTYGTAAATGA) and G1-SKR for norovirus GI; G2-SKF (CNTGGGAGGGCGATCGCAA) and G2-SKR for norovirus GII, and nuclease-free water. The reaction tube was placed into a thermocycler. The cycling conditions were as follows: initial denaturation at 94 °C for 3 min, PCR for 35 cycles for norovirus GI or 30 cycles for norovirus GII at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and final extension at 72 °C for 15 min. PCR amplicons of noroviruses GI and GII were analyzed by agarose gel electrophoresis. DNA amplicons of size 330 bp or 344 bp were considered as indicative of norovirus GI and GII, respectively.

Determination of the Sensitivity and Detection Limit of RT-Nested PCR

Digestive tissues of oyster samples processed by the proteinase K extraction method or by the adsorption–elution method were initially screened for the natural presence of noroviruses GI and GII using RT-nested PCR. Serial tenfold dilutions of the norovirus GII-positive fecal sample were added in a norovirus-negative oyster homogenate or concentrate (200 µL each) at final norovirus concentrations of 5×10^{-1}–5×10^{2} RNA copies/g. RNAs were extracted and analyzed for the sensitivity of the RT-nested PCR. In seeding experiments, serial tenfold dilutions of the norovirus GII-positive fecal sample at concentrations of $3.3–3.3\times10^{3}$ RNA copies/g were inoculated (100 µL each) into digestive tissues (2 g each) of oysters before the start of processing, and samples were incubated at room temperature for 1 h, after which the samples were processed by the proteinase K extraction or the adsorption–elution method. Norovirus RNAs in homogenate or concentrate (200 µL each) were extracted and examined for the detection limit using RT-nested PCR.

RT-PCR Inhibitors Test in Oyster Samples

RT-PCR inhibitors in oyster samples were tested in two separate experiments undertaken as previously described by Lowmoung et al. (2017). First, a 1 µL of known norovirus GI RNA (2.31×10^{2} RNA copies/mL) was added to 2 µL of RNAs from 30 GI-negative oyster samples (13 samples from proteinase K extraction and 17 samples from adsorption–elution) and a 1 µL of known norovirus GII RNA (4.01×10^{2} RNA copies/mL) was added to 2 µL of RNAs from 38 GII-negative oyster samples (19 samples from proteinase K extraction and 19 samples from adsorption–elution). These oyster samples were tested for noroviruses using RT-nested PCR. Second, 1 µL each of known norovirus GI (2.31×10^{2} RNA copies/mL) and GII RNAs (4.01×10^{2} RNA copies/mL) was added to 2 µL each of RNAs from oyster samples for GI (30 samples; 15 from each extraction method) and GII (30 samples; 15 from each extraction method) in parallel with control tubes of known GI and GII RNAs, respectively. The RNAs with known GI and GII in control tubes and sample tubes were qualitatively tested using RT-nested PCR.

Reverse Transcription-Quantitative PCR

Norovirus RNA copy numbers in oyster samples which were positive for norovirus GI or GII by RT-nested PCR were determined using RT-qPCR assays previously described by Rupprom et al. (2018). A one-step TaqMan real-time RT-PCR was carried out in a separate tube for norovirus GI or GII. Briefly, each real-time RT-PCR reaction mixture of 20 µL contained 5 µL of extracted RNA, 1X LightCycler® RNA Master Hybprobe with *Tth* DNA polymerase, reaction buffer, and dNTPs (with dUTP instead of dTTP) (Roche Diagnostics, Mannheim, Germany), 3.25 mM $Mn(OAc)_2$, 0.4 µM forward primer (GITF, CGYTGGATGCGITTC CAT for GI; GIITF, TGGAITTTTAYGTGCCCAG for GII), 0.4 µM reverse primer (GITR, TCCTTAGACGCCATCATC ATT for GI; GIITR, CGTCAYTCGACGCCATCT for GII), 0.2 µM probe (GIT-TP, FAM-TGGRCAGGAGAYCGC-MGB-BHQ for GI; GIIT-TP, FAM-AGATTGCGATCG CCCT-MGB-BHQ for GII), and PCR grade water. Thermocycling was performed in a LightCycler® 96 Real-Time PCR System (Roche Diagnostics) and included 30 min RT of RNA at 58 °C followed by a denaturation step at 95 °C for 4 min, and 45 cycles of amplification consisting of 95 °C for 15 s and 55 °C for 1 min. A quantification cycle (Cq) value below 45 and a significant increase in fluorescence

distinguishable from background were considered as positive. Standard curves for quantification were generated using GI.2 (5×10^1–5×10^7 RNA copies per reaction) and GII.4 (5–5×10^5 RNA copies per reaction) RNA transcripts. The analytical sensitivity of the TaqMan real-time RT-PCR assay for GI was 50 and for GII 5 RNA copies per reaction. Norovirus RNA copy numbers of GI and GII in oyster samples were determined from Cq values obtained and compared with the standard curves of norovirus GI and GII RNA transcripts, respectively.

DNA Sequencing

RT-nested PCR products of noroviruses GI and GII were purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) or QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's protocol. The purified PCR products were subjected directly to DNA sequencing. The nucleotide sequences of the capsid genes were compared with those of reference strains available in the GenBank database using the BLAST (Basic Local Alignment Search Tool) server. Phylogenetic neighbor-joining analysis of noroviruses GI and GII was performed using MEGA (Molecular Evolutionary Genetic Analysis), version 6.0 (Tamura et al. 2013).

GenBank Accession Numbers

The nucleotide sequences of the GI and GII noroviruses obtained from the oyster samples in this study were deposited in GenBank and assigned Accession Numbers MK616594 - MK616617.

Data Analysis

A Chi square test was used to compare the detection rate of norovirus extracted from oysters using the proteinase K extraction and the adsorption–elution method. p value <0.05 was considered statistically significant. The agreement rate between two extraction methods for the detection of noroviruses in oyster samples were determined by kappa analysis. The kappa value was interpreted as almost perfect ($k=0.81$–1.00), substantial ($k=0.61$–0.80), moderate ($k=0.41$–0.60), fair ($k=0.21$–0.40), slight ($k=0.00$–0.20), and poor ($k=<0$) agreement (Cohen 1960). Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) version 18.

Results

Sensitivity and Detection Limit of RT-Nested PCR for Norovirus in Oysters

In three replicates, the sensitivity of RT-nested PCR detection of norovirus GII in oyster concentrate processed using the adsorption–elution method was 5 RNA copies/g of digestive tissues, which was tenfold higher than processing samples using the proteinase K extraction method, in which the sensitivity was 50 RNA copies/g. However, in seeding experiments the amount of norovirus RNA detected in oyster samples before processing was equal to 3.3×10^2 RNA copies/g by both methods. When the sensitivity of norovirus detection in oysters after processing was compared with the amount of norovirus RNA detected in oyster samples before processing, the virus recovery rate of norovirus GII obtained from the proteinase K extraction method (~ 15%) was approximately ten times higher than that obtained from the adsorption–elution method (~ 1.5%).

Presence of Noroviruses in Oyster Samples Processed by Two Extraction Methods

The frequency of noroviruses in oyster samples extracted by the proteinase K extraction method or the adsorption–elution method was equal at 28.6% (20/70 samples) (Table 1). Norovirus GI occurred 2.8 times more frequently than GII by the proteinase K extraction method and 1.8 times by the adsorption–elution method. Of 70 oyster samples, 48 (68.6%) revealed corresponding positive (9 samples) or negative (39 samples) results. The discrepant results were found in 22 (31.4%) samples (Table 2). By statistical analysis, the adsorption–elution method was not significantly different from the proteinase K extraction method in detecting noroviruses ($\chi^2=3.70$, p value $=0.054$). The agreement rate determined by kappa analysis was fair ($k=0.230$). The addition of known GI or GII RNAs into RNAs from norovirus-negative oyster samples showed positive results in the RT-nested PCR assay. Alternatively, RNAs from norovirus-negative oyster samples added to known GI or GII RNAs in sample tubes showed the results as positive as in the control tubes, while

Table 1 Detection rates of noroviruses in oyster samples extracted by two different extraction methods

Virus extraction method	Total tested	Norovirus-positive oyster samples, No. (%)			
		GI	GII	GI + GII	Total
Proteinase K	70	14 (20.0)	3 (4.3)	3 (4.3)	20 (28.6)
Adsorption–elution	70	12 (17.1)	6 (8.6)	2 (2.9)	20 (28.6)

Table 2 Comparison of proteinase K extraction and adsorption–elution method for detection of noroviruses in oyster samples using RT-nested PCR

Virus extraction method	Proteinase K		Total	*p* value[a]
	Positive	Negative		
Adsorption–elution				
Positive	9	11	20	0.054
Negative	11	39	50	
Total	20	50	70	

[a]Association determined by Chi square test

the oyster samples containing naturally occurring noroviruses gave positive results with more intense DNA amplicons in the sample tubes than in the control tubes (data not shown).

Quantification of Norovirus in Oyster Samples

All 31 norovirus-positive oyster samples by RT-nested PCR were evaluated for norovirus genome copy number using RT-qPCR. Two repeated experiments were performed in some oyster samples to confirm the negative results. Norovirus GI levels in the oyster samples processed by the adsorption–elution method were in the range of 3.36×10^{1}–1.06×10^{5} RNA copies/g in 7/14 (50%) of samples, whereas norovirus GI in 17 oyster samples processed by the proteinase K extraction method could not be quantified. Norovirus GII levels in the samples processed by the proteinase K extraction method were 2.20×10^{1} and 7.83×10^{1} RNA copies/g in 2/6 (33.3%) samples, while those processed by the adsorption–elution method were in the range of 1.29×10^{3}–1.62×10^{4} RNA copies/g in 6/8 (75%) samples. One oyster sample processed by both methods provided different norovirus GII concentrations of 2.20×10^{1} RNA copies/g by the proteinase K extraction method and 1.58×10^{4} RNA copies/g by the adsorption–elution method.

Characterization of Norovirus Genotypes in Oyster Samples

Partial capsid nucleotide sequences of norovirus GI (11 samples) and GII (13 samples) strains were determined and identified for genotype using phylogenetic analysis. Six GI and GII RNA samples were obtained using the proteinase K extraction method (designated OysP) while five GI and seven GII RNA samples were obtained using the adsorption–elution method (designated OysA). For norovirus GI, seven oyster samples (OysP14, OysP30, OysA31, OysA34, OysA39, OysA48 and OysA51) contained virus belonging to GI.2. One oyster sample (OysP43) contained virus belonging to GI.5b. Three oyster samples (OysP51, OysP52 and OysP53) contained virus belonging to GI.6b. For norovirus GII, one oyster sample (OysA04) had virus belonging to GII.2. Four oyster samples (OysP13, OysP21, OysA23 and OysA34) had virus belonging to GII.4. Eight oyster samples (OysP07, OysP09, OysP15, OysP17, OysA08, OysA09, OysA11 and OysA25) had virus belonging to GII.17. These norovirus GII.17 strains clustered in the same branch as the outbreak norovirus Kawasaki323 strain (AB983218) (Fig. 1).

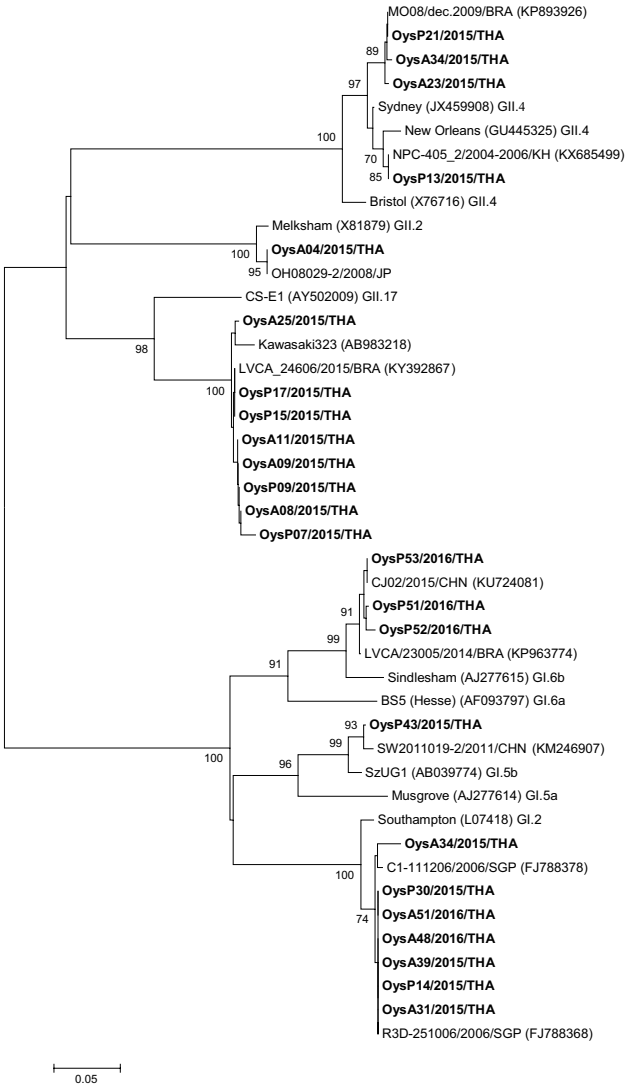

Fig. 1 Phylogenetic tree of partial nucleotide sequences of the capsid genes derived from norovirus GI (267 bp) and GII (242 bp) strains detected in oyster samples processed using a proteinase K extraction method (OysP07/2015–OysP53/2016) and an adsorption–elution method (OysA04/2015–OysA51/2016), and other reference strains from the GenBank database. The tree was generated using the neighbor-joining analysis of 1000 repetition in MEGA 6.0. The scale bar indicates nucleotide substitutions per site. Bootstrap values ≥ 70% are shown at the branch nodes. Norovirus strains in the present study are indicated in bold

Between the two extraction methods, a similar frequency of norovirus genotypes identified was found using the proteinase K extraction method (52.2%; 12/23 samples) and the adsorption–elution method (54.5%; 12/22 samples). Of 24 identified noroviruses, 2 (8.3%) were derived from the same oyster samples; one sample processed by both extraction methods demonstrated the same norovirus genotype of GII.17 (OysP09 and OysA09), however, the other sample exhibited different norovirus genotype of GI.6b (OysP51) and GI.2 (OysA51) when processed by the proteinase K extraction and adsorption–elution methods, respectively. Most oyster samples could be characterized for norovirus genotypes by either method. Norovirus GI.2, GII.4, and GII.17 strains could be found after both extraction methods. GI.5b and GI.6b strains were identified only by the proteinase K extraction method, whereas GII.2 was identified only by the adsorption–elution method.

Discussion

Noroviruses are an important cause of viral gastroenteritis in humans, and are responsible mainly for foodborne outbreaks worldwide. The limitations for monitoring norovirus contaminated bivalve shellfish and investigating outbreaks are the difficulty in detection of low concentration of norovirus and the presence of RT-PCR inhibitors (Le Guyader et al. 2009). Virus extraction is the first step needed for the process of virus concentration and for removal of RT-PCR inhibitors from shellfish samples. This study assessed the presence of norovirus in oysters processed using two different extraction methods, namely a proteinase K extraction method modified from the ISO 15216-1:2017 method and an adsorption–elution method. RT-nested PCR was used to detect the presence of noroviruses in oyster samples and to compare the efficiency of the extraction methods rather than using RT-qPCR which is defined in the ISO 15216 method since RT-nested PCR is more sensitive than RT-qPCR (Ruprom et al. 2018) and the amplicons from RT-nested PCR could be characterized for the norovirus genotypes. The sensitivity of RT-nested PCR for detection of known norovirus GII in oysters processed by the adsorption–elution method was similar to that in RNase-free water (data not shown) and tenfold higher than that by the proteinase K extraction method. Any potential RT-PCR inhibitors in the oyster concentrates processed by the adsorption–elution method did not interfere with interpretation of RT-nested PCR results. Seeding experiments of norovirus GII at a stage prior to virus processing showed the same detection limit by both extraction methods. A higher virus recovery rate was found in oysters processed by the proteinase K extraction method due to less virus loss during the processing steps. The proteinase K extraction method is more rapid in processing time (3 h) than the adsorption–elution method (6 h). Since the adsorption–elution method requires several steps to extract virus, RT-PCR inhibitors might be removed effectively. The present study was performed with seeded viruses and genome detection, which cannot differentiate between viable and non-viable viruses. Langlet et al. (2018) showed some protective effect of shellfish digestive glands that had an impact when using proteinase K digestion on capsid integrity and infectivity of viruses.

The frequency of noroviruses in collected oyster samples extracted by the proteinase K extraction method was equal to that by the adsorption–elution method albeit with different detection rates amongst genogroups. Norovirus GI was more prevalent than GII by both extraction methods which are consistent with our previous studies on GI predominance in bivalve shellfish (Kittigul et al. 2016) and environmental samples (Kittigul et al. 2019). On a global scale, a higher proportion of GI has been observed in oyster-related outbreaks than in non-oyster-related outbreaks (Yu et al. 2015). Farkas et al. (2018) has shown greater persistence of norovirus GI in the environment. The differential accumulation efficiency of norovirus GI and GII in bivalve shellfish provides a possible explanation for the higher proportion of GI associated with shellfish-related outbreaks (Le Guyader et al. 2012). However, acute gastroenteritis reported in humans is mainly caused by norovirus GII strains (Atmar et al. 2018). Differences of norovirus prevalence in patients with acute gastroenteritis and bivalve shellfish may be due to several factors such as relative concentration, specific or non-specific binding to shellfish, and relative persistence of noroviruses.

In this study, a process control virus was not available to be included in the assays. Norovirus GII was recovered at > 1% in seeding experiments using both the proteinase K extraction method (15%) and the adsorption–elution method (1.5%) implying the utility of both extraction processes. According to the ISO method (15216-1:2017), the proteinase K extraction method uses 500 µL of shellfish homogenate and mixed magnetic silica solution for extraction of the viral RNA. In this study, the modified proteinase K extraction method uses 200 µL of oyster homogenate and the silica on column (the RNeasy® Mini kit) for extraction of the viral RNA which may result in a loss of RNA recovery due to clogging of the column. RT-PCR inhibitors were not observed in oyster samples processed by either the proteinase K extraction method or the adsorption–elution method as evaluated using RT-nested PCR, suggesting that both extraction methods may remove any possible RT-PCR inhibitors. In support of this, our previous study of RT-PCR inhibitors using RT-qPCR found high percentage of acceptable oyster samples when processed by both the adsorption–elution method (73%) and the proteinase K extraction method (69%), in which RT-PCR amplification efficiency values

were ≥ 25% according to ISO/TS 15216-1 (2013) guidelines for norovirus quantification (Rupprom et al. 2018). Some of the acceptable oyster samples were used in this study and also showed no PCR inhibitors using a qualitative RT-nested PCR method.

Although the two extraction methods could extract noroviruses which were identified for norovirus genotypes at a similar frequency based on the partial capsid region C, only 2 in 24 identified genotypes derived from the same oyster samples demonstrating the different performance of the extraction methods. One norovirus-positive oyster sample was identified as GI.6b using the proteinase K extraction method and GI.2 using the adsorption–elution method. This oyster sample might contain mixed norovirus genotypes which are generally difficult to identify in complex environmental samples. The findings of common GI.2, GII.4, and GII.17 genotypes in oysters processed by both extraction methods are consistent with other studies in Japan (Imamura et al. 2018), Vietnam (Nguyen et al. 2018) as well as Thailand (Kittigul et al. 2016; Lowmoung et al., 2017) consistent with their transmission via oysters, and are possibly the causative agents of gastroenteritis outbreaks in south Korea (Cho et al. 2016), Australia (Fitzgerald et al. 2014), the US (Woods et al. 2016) and several coastal countries (Yu et al. 2015).

The newly emerged GII.17 norovirus strains have caused gastroenteritis outbreaks in Asia (de Graaf et al. 2015) and became the predominant genotype during 2014–2016 (Chan et al. 2017). In the same period (2015–2016), this study obtained GII.17 sequences from oysters clustering in the same branch as the novel GII.17 (Kawasaki323) strain, similar to previous studies in fecal samples from Brazil (Andrade et al. 2017), and in oyster digestive tissues (Lowmoung et al. 2017), and sewage sludge samples (Kittigul et al. 2019) from Thailand. These results indicate that the GII.17 Kawasaki323 strain is circulating in environment and may consequently cause gastroenteritis illness at a population level.

Of interest, the same frequency of norovirus detection was registered by both extraction methods albeit with different norovirus genogroups and genotypes. A possible explanation might be the different efficiencies of the extraction methods, and the low level of norovirus present in naturally contaminated oysters producing the discrepant results. This was supported by the finding of norovirus GI present in oyster samples in the range of 10^1–10^5 RNA copies/g processed by the adsorption–elution method but was undetectable after the proteinase K extraction method. Norovirus GII concentrations in the oyster samples processed by the adsorption–elution method (10^3–10^4 RNA copies/g) were higher than that detected after proteinase K extraction method (10^1 RNA copies/g). The detection limits of norovirus RNA transcripts do not always reflect the genome copy numbers of naturally contaminated noroviruses in oysters. The estimated genome copy numbers of noroviruses in oysters below the theoretical limit of detection for RT-qPCR were observed. This finding is consistent with a previous study as described by Fuentes et al. (2014). The levels of noroviruses GI and GII in oyster samples in this study were comparable to a previous study undertaken in the United Kingdom (Lowther et al. 2012). Notably, high and low concentrations of norovirus determined in the same oyster sample using the adsorption–elution and proteinase K extraction methods, respectively, might emphasize the different efficiency of these methods for norovirus extraction.

The present study highlights the prevalence, genotyping, and quantification of noroviruses in oyster samples processed by two different extraction methods including proteinase K extraction and adsorption–elution. Both virus extraction methods have the potential to be employed for virus analysis. This study suggests the use of either extraction method in combination with RT-nested PCR for molecular characterization of norovirus in oysters. The importance of oysters in the transmission of norovirus in the environment has significant implications for norovirus surveillance in oyster samples.

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