DEVELOPMENT OF ONE-STEP TAQMAN QUANTITATIVE RT-PCR ASSAY FOR DETECTION OF NOROVIRUS GENOGROUPS I AND II IN OYSTER

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Abstract. Noroviruses are important causes of acute gastroenteritis in humans, and consumption of raw bivalve shellfish has been frequently linked to norovirus disease. We developed a one-step TaqMan quantitative (q)RT-PCR assay for norovirus genogroup (G)I and GII using RNA extracted from oyster samples. Assay sensitivity was 5×10^2 RNA copies for noroviruses GI and GII, 1,000-fold higher than two in-house qRT-PCR methods for norovirus GI but 10-fold lower for GII. The detection range of the new assay was 3.4×10^1 - 1.1×10^5 and 7.8×10^1 - 6.4×10^3 RNA copies / g oyster of norovirus GI and GII, respectively. Using the assay developed, norovirus GI was detected at a higher frequency than GII (31% *vs* 20%) in oyster samples. However, an in-house assay detected norovirus GII at a higher frequency than GI (67% *vs* 0%). The new assay was able to detect common circulating norovirus genotypes, such as GI.2 and GII.4. Replacing in-house qRT-PCR assays of norovirus GI with the developed TaqMan method will result in an improved assay for this genogroup, which can be used for routine detection of norovirus in both clinical as well as oyster samples.

Keywords: norovirus, genogroup, oyster, quantitative RT-PCR

INTRODUCTION

Noroviruses constitute a common cause of epidemic and endemic acute gastroenteritis in humans of all ages (Lopman *et al*, 2016). These viruses are transmitted via a fecal-oral route and infections often result from consumption of contaminated food, such as bivalve shellfish, and resulting in large outbreaks (Bellou *et al*, 2013; Loury *et al*, 2015; Woods *et al*, 2016). Noroviruses are non-enveloped and icosahedral in shape with a single-stranded, positive-sense RNA genome of 7.5 kb, and they form a separate genus in the family Caliciviridae (Green, 2013). They are classified into at least seven genogroups (GI-GVII), of which GI, GII and GIV infect humans, and the virus can be further divided into >40 genotypes (Vinjé, 2015). Since 2002, the majority of gastroenteritis outbreaks in humans have been caused by GII.4 norovirus. In 2014, a new GII.17 norovirus emerged and became the pre-

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dominant genotype in East Asia followed in 2016 by a new recombinant GII.2 strain (de Graaf *et al*, 2015; Liu *et al*, 2017).

Bivalve shellfish are often consumed raw, thereby posing a food safety risk owing to their ability to concentrate viruses when present in contaminated water habitats (Hassard *et al*, 2017). Over the past decade quantitative (q)PCR or qRT-PCR has become the gold standard for virus detection in food, including shellfish. A standard method that combines proteinase K pre-treatment with qRT-PCR is widely used for detection and quantification of noroviruses (ISO 15216-1:2017). This ISO procedure was employed for investigation of norovirus gastroenteritis outbreaks (Le Mennec *et al*, 2017) and surveillance of norovirus contamination in shellfish (Suffredini *et al*, 2014; Polo *et al*, 2015; La Bella *et al*, 2017). An alternative method employing adsorption-elution of virus was applied for the study of norovirus in various shellfish species, particularly in oysters, mussels and cockles (Kittigul *et al*, 2016).

As noroviruses are genetically and antigenically diverse, with new strains emerging frequently, continuous assessment and validation of current molecular methods are required to detect new emerging genotypes. Three qRT-PCR assays for detection and quantification of norovirus GI and GII in clinical samples have been evaluated and an in-house qRT-PCR assay using primers and probes of Kageyama *et al* (2003) gives the highest efficiency, but the detection rate of GI noroviruses is low (Rupprom *et al*, 2017). Although the majority of norovirus outbreaks are caused by GII.4 viruses (Vega *et al*, 2014), GI.2 and GI.4 noroviruses are relatively more frequently detected in foodborne outbreaks (Verhoef *et al*, 2010). Thus, improvement in detection of GI and GII genogroups, especially at low levels expected in such food as oyster, is of high necessity.

This study compared a one-step TaqMan qRT-PCR assay employing a set of new primers and probes targeting the most conserved region of GI and GII norovirus genomes with two existing in-house qRT-PCR assays (Kageyama *et al*, 2003; da Silva *et al*, 2007) for quantitative detection at low levels of noroviruses in oyster. In addition, two different virus extraction methods, namely adsorption-elution and proteinase K treatment, and three different oyster tissues, namely, digestive tissue, gill and mantle, were evaluated to obtain an optimal source of norovirus for the qRT-PCR methods.

MATERIALS AND METHODS

Oyster samples

A panel of 64 oyster (*Crassostrea belcheri*) samples (46 GI and 18 GII) giving positive results for noroviruses by RT-nested PCR method (Lowmoung *et al*, 2017) was selected to evaluate the three different norovirus qRT-PCR assays, to compare qRT-PCR efficiencies using samples from two extraction methods (Group I) and three different oyster tissues (Group II). Group I oyster samples (n=28) included 15 (8 GI and 7 GII genogroups) oyster digestive tissue samples extracted using the adsorption-elution method (Kittigul *et al*, 2016) and 13 (10 GI and 3 GII) using the proteinase K method (ISO 15216-1:2017). Oyster digestive tissue samples (4 g) were equally divided for processing by each of the extraction methods. Group II oyster samples (n=36) consisted of 13 digestive tissues (9 GI and 4 GII), 10 gills (7 GI and 3 GII), and 13 mantles (12 GI and 1 GII) extracted by the adsorption-elution method (Lowmoung *et al*, 2017).

Norovirus RNA transcript standards preparation

RNA samples were prepared from noroviruses GI.2- and GII.4-positive fecal samples obtained from patients with acute gastroenteritis (Kittigul *et al*, 2010). Viral RNA was extracted using QIAamp® viral RNA extraction kit (QIAGEN, Hilden, Germany). In brief, a 140 µl aliquot of fecal sample diluted 1:10 with 0.05 M phosphate-buffered saline was incubated with 565 µl of AVL-carrier RNA buffer for 30 minutes, then mixed with 560 µl of absolute ethanol, centrifuged in a spin column at 8,000*g* for 1 minute and washed with AW1 buffer followed by AW2 buffer. The tube was centrifuged at 15,000*g* for 3 minutes and RNA eluted from the spin column with 60 µl of AVE buffer by centrifugation at 8,000*g* for 1 minute. RNA was stored at -80°C until used.

Norovirus RNA targets were amplified by RT-PCR modified from procedures of Kageyama *et al* (2003) and Kittigul *et al* (2010) using primers (for GI) G1FF1, G1FF2, G1FF3, and G1-SKR, and (for GII) G2FB1, G2FB2, G2FB3, and G2-SKR (Kojima *et al*, 2002; Kageyama *et al*, 2003). Amplicons (597 bp of GI; 468 bp of GII) were inserted into pCR™4 TOPO® vector (Invitrogen, Carlsbad, CA) and transfected into One-shot TOP10 *Escherichia coli* (Invitrogen). Recombinant plasmids were purified using PureLink® Quick Plasmid Miniprep Kit (Invitrogen) and linearized with *Mss*I. *In vitro* RNA transcription then was performed using RiboMAX™ Large Scale RNA Production Systems-T7 (Promega, Madison, WI). After DNase treatment, RNA transcripts were purified using RNeasy Mini Kit (QIAGEN, Hilden, Germany) and quantified (NanoDrop™ 2000 Spectrophotometer; Thermo Fisher Scientific, Wilmington, DE). Concentration of RNA transcript was calculated using the formula: RNA copy/µl = [(g/µl RNA)/(length of RNA × 340)] × (6.022×10^{23}) (Troxler *et al*, 2011). RNA transcript stocks of noroviruses GI and GII were 4.50×10^{11} and 5.03×10^{11} copies/µl, respectively, and were stored at -80°C until used.

Oyster RNA extraction and qRT-PCR assay

Virus RNA was extracted from 200 µl aliquot of oyster concentrate obtained from adsorption-elution (Kittigul *et al*, 2016) or proteinase K (ISO 15216-1:2017) method using RNeasy® Mini Kit (QIAGEN) and tested for norovirus by three different qRT-PCR assays. New norovirus GI and GII primers and probes (Table 1) were designed based on alignments of selected sequences from norovirus strains from Thailand and reference sequences deposited in the NCBI (National Center for Biotechnology Information) GenBank database. Multiple-sequence alignments of noroviruses GI and GII were performed using a MUSCLE program of MEGA 5.0 software (Tamura *et al*, 2011). Oligo Analyzer software of Integrate DNA Technologies (IDT) website (http://www.idtdna.com/analyzer/applications/oligoanalyzer/) was used to analyze primers and probes for GC content, melting temperature, hairpin loop, and self- and heterodimers. TaqMan norovirus probes were labeled at the 5′ terminus with reporter 6-carboxyfluorescein (FAM) and at the 3′ terminus with quencher Minor Groove Binder-Black Hole Quencher (BHQ) (Biolegio, Nijmegen, The Netherlands).

Reaction mixture (20 µl), prepared in separate tubes for norovirus GI or GII, contained 5 µl of RNA, 7.5 µl of 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 $Mn(OAc)_2$, 0.4-0.5 µM

Table 1
Primers and TaqMan probes used in quantitative RT-PCR assays for detection of norovirus genogroup (G)I and GII.

Primer/probe	Sequence (5′⟶3′)[a]	Polarity	Location[b]	Reference
Norovirus GI				
QNIF4	CGCTGGATGCGNTTCCAT	+	5291-5308	da Silva *et al* (2007)
NV1LCR	CCTTAGACGCCATCATCATTTAC	–	5376-5354	Svraka *et al* (2007)
NVGG1p	FAM-TGGACAGGAGAYCGCRATCT-BHQ[c]	+	5321-5340	Svraka *et al* (2007)
COG1F	CGYTGGATGCGNTTYCATGA	+	5291-5310	Kageyama *et al* (2003)
COG1R	CTTAGACGCCATCATCATTYAC	–	5375-5358	Kageyama *et al* (2003)
RING1a-TP	FAM-AGATYGCGATCYCCTGTCCA-TAMRA[c]	–	5359-5340	Kageyama *et al* (2003)
RING1b-TP	FAM-AGATCGCGGTCTCCTGTCCA-TAMRA[c]	–	5359-5340	Kageyama *et al* (2003)
GITF	CGYTGGATGCGITTCCAT	+	5291-5308	This study
GITR	TCCTTAGACGCCATCATCATT	–	5377-5357	This study
GIT-TP	FAM-TGGRCAGGAGAYCGC-MGB-BHQ[c]	+	5321-5335	This study
Norovirus GII				
QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA	+	5012-5037	Loisy *et al* (2005)
QNIFs	FAM-AGCACGTGGGAGGGCGATCG-BHQ[c]	+	5042-5061	Loisy *et al* (2005)
COG2F	CARGARBCNATGTTYAGRTGGATGAG	+	5003-5023	Kageyama *et al* (2003)
COG2R	TCGACGCCATCTTCATTCACA	–	5100-5080	Kageyama *et al* (2003)
RING2-TP	FAM-TGGGAGGGCGATCGCAATCT-TAMRA[c]	+	5048-5067	Kageyama *et al* (2003)
GIITF	TGGAITTTTAYGTGCCCAG	+	4983-5001	This study
GIITR	CGTCAYTCGACGCCATCT	–	5106-5089	This study
GIIT-TP	FAM-AGATTGCGATCGCCCT-MGB-BHQ[c]	–	5067-5052	This study

[a]B = C, G or T; I = inosine; N = A, C, G, or T; R = A or G; W = A or T; Y = C or T. [b]Nucleotide positions are taken from reference norovirus GI (Norwalk virus 68; GenBank accession no. M87661) and norovirus GII (Lordsdale; GenBank accession no. X86557). [c]BHQ, Black Hole Quencher; FAM, 6-carboxyfluorescein; MGB, minor groove binder; TAMRA, 6-carboxy-tetramethylrhodamine.

forward primer, 0.4-0.9 μM reverse primer, 0.2-0.25 μM probe for norovirus GI or GII, and PCR grade water. Thermocycling was conducted in a LightCycler® 96 Real-Time PCR System (Roche Diagnostics) for 45 cycles as described in Table 2. In-house assay 1 was performed as described by Kageyama *et al* (2003), in-house assay 2 according to the method of da Silva *et al* (2007) with some modifications and the new qRT-PCR assay of norovirus GI.2 and GII.4 RNA transcripts by optimizing RT temperature, concentrations of $Mn(OAc)_2$, *Tth* DNA polymerase, primers and probes, and PCR annealing-extension steps. Quantification cycle (Cq) value below 45 and a significant increase in fluorescence distinguishable from background are considered as positive. Amplicon size obtained from the in-house assay 1, in-house assay 2 and new qRT-PCR assay was 85, 86 and 87 bp for norovirus GI and 98, 89, and 124 bp for norovirus GII, respectively.

Sensitivity and amplification efficiency determination

Sensitivity of each of the three qRT-PCR assays was determined using a 10-fold serial dilutions of GI.2 and GII.4 RNA transcripts at an initial amount of 5×10^7 RNA copies per reaction. Sensitivity of the assay is defined as the highest dilution of GI or GII transcript positive by qRT-PCR. Standard curves were constructed using a 10-fold dilution series of GI and GII RNA transcripts ranging from 5×10^1 - 5×10^7 and 5 - 5×10^5 RNA copies per reaction, respectively. Cq values were plotted against log RNA amounts and the slope and regression coefficient (R^2) values were determined. Amplification efficiency was calculated using the equation: efficiency (E) = $10^{-1/\text{slope}}$ - 1 (Bustin *et al*, 2009).

Table 2
Primer and probe concentrations and thermocycling profiles of three quantitative (q)RT-PCR assays for detection of norovirus genogroup (G)I and GII.

qRT-PCR	New in-house assay	In-house assay 1[b]	In-house assay 2[c]
Primer and probe[a] concentrations	Norovirus GI 0.4 μM GITF 0.4 μM GITR 0.2 μM GIT-TP	Norovirus GI 0.4 μM COG1F 0.4 μM COG1R 0.2 μM RING1a-TP 0.2 μM RING1b-TP	Norovirus GI 0.5 μM QNIF4 0.9 μM NV1LCR 0.25 μM NVGG1p
	Norovirus GII 0.4 μM GIITF 0.4 μM GIITR 0.2 μM GIIT-TP	Norovirus GII 0.4 μM COG2F 0.4 μM COG2R 0.2 μM RING2-TP	Norovirus GII 0.5 μM QNIF2 0.9 μM COG2R 0.25 μM QNIFs
Thermocycling conditions			
Reverse transcription	58°C for 30 minutes	58°C for 30 minutes	55°C for 30 minutes
Initial denaturation	95°C for 4 minutes	95°C for 4 minutes	95°C for 5 minutes
Denaturation	95°C for 15 seconds	95°C for 15 seconds	95°C for 15 seconds
Annealing-extension	55°C for 1 minute	56°C for 1 minute	60°C for 1 minute

[a]From Table 1. [b]Modified from Kageyama *et al* (2003). [c]Modified from da Silva *et al* (2007).

RT-PCR inhibitors determination

In order to determine presence of RT-PCR inhibitors in oyster concentrate samples, 1 µl aliquot of norovirus GI or GII RNA transcript containing 10^7 RNA copies (external control, EC) was added to 5 µl of sample RNA or 5 µl of PCR-grade water. In order to calculate percent amplification efficiency (E) the following equation was employed: $E = (10^{\Delta Cq/slope}) \times 100$, where ΔCq = (Cq value of EC-spiked sample) - (Cq value of EC-spiked water). Oyster concentrate samples with E values ≥25% were considered acceptable for analysis of the results (ISO/TS 15216-1:2013).

RESULTS

Evaluation of in-house qRT-PCR assays

Sensitivity of detection of norovirus GI and GII by the three in-house qRT-PCR assays were determined using a 10-fold serial dilutions of norovirus GI and GII RNA transcripts in repeated experiments. The limit of detection of the new (developed) in-house assay for norovirus GI was 3 orders of magnitude lower than that of in-house assays 1 and 2 (5×10^2 *vs* 5×10^5 and 5×10^5 RNA copies per reaction), with an amplification efficiency of 88% and a 6-log range of linearity compared to amplification efficiency of 91% and 87% for assay 1 and 2, respectively and a 3-log range of linearity for both (Fig 1A). However, for norovirus GII, the limit of detection of the new in-house assay was comparable to that of in-house assay 1 (5×10^2 RNA copies per reaction), but one order of magnitude less sensitive compared to in-house assay 2 (Fig 1B); the amplification efficiency of the new assay was 105% with a 4-log range of linearity, while that of assay 1 was 101% with the same range of linearity and of assay 2 was 106% with, as expected, a 5-log range of linearity.

qRT-PCR detection efficiency of norovirus in oyster

Of the 64 oyster samples tested positive for norovirus infection using RT-nested-PCR, 35 (55%) had acceptable E values ≥25% determined by the new in-house qRT-PCR assay, and of the 29 remaining oyster samples with unacceptable E values (<25%), 6 samples showed positive results for noroviruses (3 samples giving positive results after RNA samples were diluted 1:2 with RNase-free water) (Table 3). The frequency of acceptable oyster samples processed by adsorption-elution method was 73% and that by proteinase K procedure 69%. The highest frequency of acceptable oyster samples was found in digestive tissue (77%), followed by mantle (31%) and gill (10%).

Quantification of norovirus in oyster samples

Of the 41 norovirus-positive (by RT-nested PCR) oyster samples (35 with acceptable and 6 unacceptable E values), only the new in-house qRT-PCR assay detected norovirus GI in 8/26 (31%) samples (range=3.4×10^1-1.1×10^5 RNA copies/g) (Table 4). The new assay detected norovirus GII in 3/15 (20%) samples (range =7.8×10^1-6.4×10^3 RNA copies/g), while assay 1 detected 3/15 (20%) (range= 1.3×10^3-7.7×10^3 RNA copies/g) and assay 2 detected 10/15 (67%) (range=2.2×10^1-4.3×10^4 RNA copies/g). Noroviruses GI.2 and GII.4 were detected by the new in-house assay, GII.4 and GII.17 by in-house assay 1, and GII.2, GII.4 and GII.17 by the in-house assay 2 (data not shown).

DISCUSSION

Several qRT-PCR assays for the detection of norovirus in bivalve shellfish have been reported (Suffredini *et al*, 2014; Polo *et al*, 2015; La Bella *et al*, 2017; Le Mennec

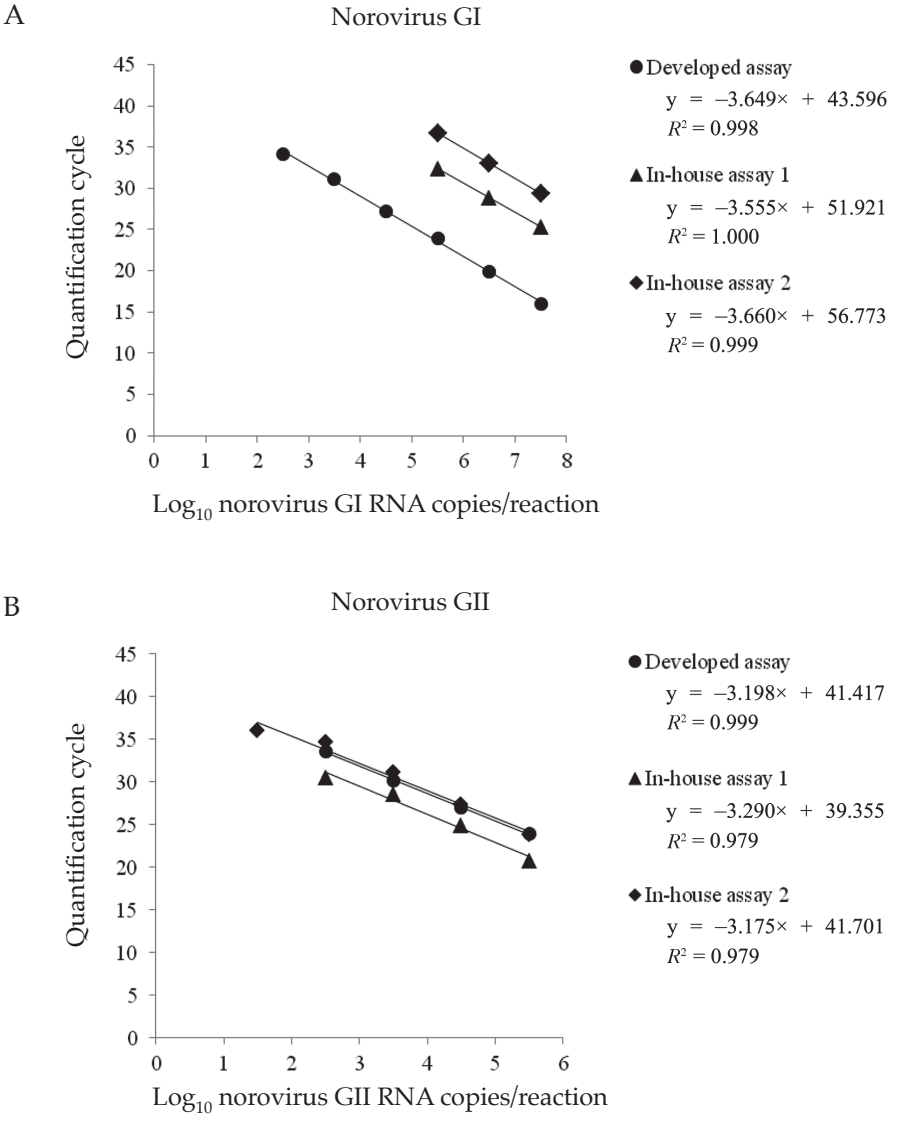

Fig 1-Linearity of developed in-house one-step TaqMan quantitative (q)RT-PCR, in-house qRT-PCR assay 1 and qRT-PCR assay 2 for detection of noroviruses GI (A) and GII (B). Norovirus RNAs of known genogroups were reverse-transcribed, cDNAs cloned and RNA transcripts quantified by spectrophotometry. TaqMan norovirus probes were labeled at the 5′ terminus with reporter 6-carboxyfluorescein and at the 3′ terminus with quencher Minor Groove Binder-Black Hole Quencher. Thermocycling was performed in a LightCycler® 96 Real-Time PCR System (Roche Diagnostics).

et al, 2017). In this study, we compared a newly developed TaqMan qRT-PCR assay with two in-house qRT-PCR assays, namely, assay 1 that showed good performance (Rupprom *et al*, 2017) and assay 2, similar to the ISO 15216 method (ISO 15216-1:2017). The most sensitive qRT-PCR assay for norovirus GI RNA transcript

Table 3
Quantitative (q)RT-PCR detection efficiency of norovirus genogroup (G)I and GII determined by one-step TaqMan qRT-PCR assay.

Virus extraction method and oyster tissue type	Total number of samples	Number of samples with acceptable qRT-PCR efficiency[a]			Number of samples with unacceptable qRT-PCR efficiency[a]		
		GI[b]	GII	Total (%)	GI	GII	Total (%)
Virus extraction method							
Adsorption-elution	15	5	6	11 (73)	3	1	4 (27)
Proteinase K	13	6	3	9 (69)	4	-	4 (31)
Oyster tissue[c]							
Digestive tissue	13	6	4	10 (77)	3	-	3 (23)
Gill	10	1	-	1 (10)	6	3	9 (90)
Mantle	13	4	-	4 (31)	8	1	9 (69)
Total (%)	64 (100)	22 (34)	13 (21)	35 (55)	24 (37)	5 (8)	29 (45)

[a]Acceptable level of qRT-PCR efficiency ≥25%; unacceptable level <25%. [b]Genogroup determined using RT-nested-PCR. [c]Processed by adsorption-elution method.

Table 4
Comparison of three different quantitative RT-PCR assays for norovirus genogroup (G)I and GII in oyster samples.

Quantitative RT-PCR method	Number of positive samples/total[a] (%)	Median Cq value (range)	Median RNA copies/g (range)
Norovirus GI			
New assay	8/26 (31)	40 (33 - 45)	7.6×10^2 (3.4×10^1 - 1.1×10^5)
In-house assay 1	0/26 (0.0)	-	-
In-house assay 2	0/26 (0.0)	-	-
Norovirus GII			
New assay	3/15 (20)	40 (35 - 43)	3.0×10^2 (7.8×10^1 - 6.4×10^3)
In-house assay 1	3/15 (20)	31 (30 - 32)	1.7×10^3 (1.3×10^3 - 7.7×10^3)
In-house assay 2	10/15 (67)	36 (32 - 45)	1.5×10^4 (2.2×10^1 - 4.3×10^4)

[a]Oyster samples positive for norovirus GI and GII using RT-nested PCR. Cq, quantification cycle.

was the TaqMan assay and that for norovirus GII RNA transcript was the in-house assay 2. All three qRT-PCR assays exhibited similar amplification efficiency for both norovirus genogroups. The new assay was highly specific, with no cross-reactions among norovirus genogroup or any of other enteric viruses tested, such as

hepatitis A virus, poliovirus and rotavirus (data not shown). Previously the primers and probes used (Kageyama *et al*, 2003) in the in-house assay 1 show less sensitive detection of norovirus GI (Loisy *et al*, 2005; Van Stelten *et al*, 2011); however, the modified primers and probes used in the present study increased sensitivity for GI. Specificity of the new assay corresponds to that of in-house assay 1 previously reported (Rupprom *et al*, 2017).

The detection of virus in bivalve shellfish samples is difficult because of the low amounts of virus, which has been solved to a certain extent with the introduction of PCR-based assay methods (Lees and CEN WG6 TAG4, 2010) and the presence of RT and/or DNA polymerase inhibitors can lead to false negative results (Le Guyader *et al*, 2009). The inclusion of external RNA controls in shellfish samples has been widely used to monitor qRT-PCR inhibition (Le Guyader *et al*, 2009; Lowther *et al*, 2012; Suffredini *et al*, 2014; Polo *et al*, 2015; La Bella *et al*, 2017).

It is worth noting in the present study ~20% of oyster samples with unacceptable amplification efficiency levels (<25%) gave positive results for the presence of norovirus using the TaqMan qRT-PCR assay. This is in line with a previous study of norovirus in wastewater samples, in which there is a number of samples with unacceptable qRT-PCR amplification efficiency (da Silva *et al*, 2007). Thus, the determination of an acceptable amplification efficiency level for qRT-PCR should be conducted with caution, otherwise the detection rate of norovirus in test samples could be underestimated. Oyster samples with an amplification efficiency <25% that show negative results become positive after the RNA samples are diluted 1:2 but negative at 1:10 dilution (Rajko-Nenow *et al*, 2012; Suffredini *et al*, 2014; La Bella *et al*, 2017). Dilution of RNA samples reduces the amount of qRT-PCR inhibitor(s), but excessive dilution also could decrease virus amounts to below the level of detection. The majority of the oyster samples processed by adsorption-elution procedure showed similar (≥25%) amplification efficiency to proteinase K method demonstrating the robustness of both virus extraction methods. Previous findings of norovirus distribution in oyster tissues suggest gill and mantle can also be successfully used as compared to digestive tissue for detction of norovirus (Lowmoung *et al*, 2017). However, in the current study, inhibition of qRT-PCR was highest in gill samples followed by mantle and digestive tissue.

In oyster samples, the TaqMan qPCR assay detects norovirus GI significantly better compared to the other two in-house assays due to the higher sensitivity of the former method. The amounts of norovirus GI detected in oyster can vary widely (Nishida *et al*, 2003; Lowther *et al*, 2012; Suffredini *et al*, 2014). On the other, in-house assay 2 had a higher efficiency for detection of norovirus GII compared to the other two assays. The TaqMan qPCR assay and in-house assay 1 detected norovirus GII at similar frequency but in different oyster samples. These observations might be due to the low amounts of norovirus GII in the samples, or to different genotypes present in each oyster sample. The new in-house assay and in-house assay 2 were able to detect norovirus GII at a lower level than in-house assay 1. The levels of norovirus GII in oyster samples were comparable to those reported in previous studies (Nishida *et al*, 2003; Lowther *et al*, 2012; Suffredini *et al*, 2014). A number of oyster samples positive by RT-nested PCR showed negative results by qRT-PCR assays, indicating the higher sensitivity of the former assay method.

The TaqMan assay was capable of detecting noroviruses GI.2 and GII.4. These norovirus genotypes are implicated in shellfish-related outbreaks (Verhoef *et al*, 2010; Woods *et al*, 2016). The sensitivity of detecting different norovirus genotypes may vary among the assays. Further studies are needed to determine the frequency of norovirus genotypes detected by various qRT-PCR assays. A multiplex qRT-PCR assay has been reported capable of simultaneous quantitative detection of norovirus GI and GII (Fuentes *et al*, 2014); however, its application to detect naturally occurring norovirus-contaminated food and water samples is limited due to very low norovirus genome copy numbers (below the limit of detection).

In summary, we have developed a new TaqMan qRT-PCR assay with improved sensitivity for detection of norovirus genogroup I in oyster, but this new assay had a similar sensitivity as in-house assay 1 (Rupprom *et al*, 2017) for detecting norovirus GII while in-house, similar to ISO 15216 method, was 10-fold more sensitive. Thus, in order to obtain an optimal sensitive method for norovirus detection and quantification in oyster, the new in-house assay should be employed for norovirus GI and in-house assay 2 for GII. A thorough evaluation of the sensitivity of various qRT-PCR assays for norovirus should be evaluated to select the optimal sensitive detection method of each norovirus genogroup present in bivalve shellfish, which, when applied in routine monitoring, may assist in reducing the risk of consumption of norovirus contaminated shellfish.

ACKNOWLEDGEMENTS

The research was supported by a grant from The Thailand Research Fund (TRF) through the Royal Golden Jubilee PhD program (grant no. PHD/0085/2554), and the Thai Government Budget through Mahidol University, fiscal year 2015-2017. The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention, USA.

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