NOROVIRUSES IN OYSTERS FROM LOCAL MARKETS AND OYSTER FARMS IN SOUTHERN THAILAND

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Abstract. One hundred and eighteen oyster samples collected from local markets and oyster farms in southern Thailand were examined for noroviruses (NoVs) and bacterial indicators of fecal contamination (fecal coliforms and *Escherichia coli*). Using a virus concentration procedure followed by RT-nested PCR, NoVs were detected in 38% of the samples. Oysters collected from oyster farms were found with NoVs at a higher detection rate (25/53 samples) than oysters from local markets (20/65 samples). Of the 45 NoV-positive oyster samples, 67% belonged to NoV genogroup I (GI), 15% to GII, and 18% to both GI and GII. DNA sequencing showed that 2 NoVs belonged to NoV GI-2 genotype. Fecal coliforms in NoV-positive oyster samples were in the range of <3.0 to 1.5 x 10^4 most probable number (MPN)/g and 33% of NoV-positive oyster samples contained fecal coliforms within the standard acceptable level of raw shellfish (<20 MPN/g). *E. coli* was found in the range of <3.0 to 1.5 x 10^4 MPN/g and 9% of NoV-positive oyster samples were within acceptable levels of *E. coli* contamination (<3 MPN/g). These findings indicate that NoV contamination in oysters obtained from both markets and oyster farms might pose a potential risk of acute gastroenteritis associated with raw oyster consumption. Examination for both fecal bacterial indicators and enteric viruses should be conducted for microbiological food safety of shellfish.

Keywords: norovirus, oyster, RT-nested PCR, fecal coliforms, *Escherichia coli*

INTRODUCTION

Noroviruses (NoVs) have been recognized as the major causative agent of outbreaks of non-bacterial acute gastroenteritis in humans worldwide and play a role in epidemic and sporadic gastrointestinal illnesses (Glass *et al*, 2009). These viruses belong to the genus *Norovirus* in the family *Caliciviridae* and are nonenveloped, positive-sense, single-stranded RNA viruses. They are classified into 5 distinct genogroups (GI, GII, GIII, GIV, and GV), of which GI, GII, and GIV are associated with gastroenteritis in humans (Green, 2007). The viruses are highly infectious and transmitted via the fecal-oral route, primarily through ingestion of contaminated food or water. Frequently, secondary infection results from person-to-person contact (CDC, 2001). Outbreaks of acute gastroenteritis caused by NoVs occur in closed environments including healthcare settings (Buesa *et al*, 2008), restaurants (Mesquita and Nascimento, 2009), college campuses (CDC, 2009), and cruise

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ships (Koopmans *et al*, 2006).

Because filter-feeding shellfish can concentrate any pathogenic microorganisms from sewage-containing water, consumption of raw or undercooked shellfish may lead to a health risk of gastrointestinal infection in humans. Various outbreaks associated with oyster consumption have been described and NoVs have been identified as being the most significant etiologic agent (Gallimore *et al*, 2005; Le Guyader *et al*, 2006; David *et al*, 2007; Webby *et al*, 2007; Nenonen *et al*, 2009). In Thailand, NoVs have been reported as an important cause of acute gastroenteritis in hospitalized patients of all ages, affecting mainly children and adults, but also the elderly (Hansman *et al*, 2004; Khamrin *et al*, 2007; Kittigul *et al*, 2010b). However, data on foodborne illnesses related to NoVs are limited because virus identification has not been undertaken routinely and laboratory detection of viruses in food is hampered by technical difficulty and time constraints. A procedure for the concentration and extraction of viruses from oysters was developed in our laboratory and subsequently applied to samples of naturally contaminated oysters (Kittigul *et al*, 2008, 2010a).

In the present study, the established method was used to monitor NoVs in oyster samples, which were collected from local markets and oyster harvesting areas. In the samples, bacterial indicators of fecal contamination (fecal coliforms and *Escherichia coli*) were also identified and their relation with the presence of NoVs was determined.

MATERIALS AND METHODS

Oyster sampling and processing for virus identification

Between August and December, 2005, a total of 118 oyster samples (*Crassostrea belcheri*) were collected from local markets (65 samples) in Bangkok, and from oyster farms (53 samples) in Surat Thani Province located in the south of Thailand. Oyster samples were transported in chilled containers to the laboratory. On arrival, oysters were washed, scrubbed and the shells opened using sterile shucking knife. The mantle fluid was discarded and oyster meat from 3 oysters per sample was pooled and cut into small pieces of 25 g for analysis of NoVs and 25 g for fecal bacteria.

Virus in the oyster samples was concentrated following the method previously described (Kittigul *et al*, 2008). In brief, oyster meat (25 g) in chilled distilled water was homogenized and adjusted to pH 4.8 with 1 N HCl. After centrifugation, virus in the pellet was extracted by adding 2.9% tryptose-phosphate broth containing 6% glycine, pH 9.0. Virus was precipitated by adding polyethylene glycol (PEG) 8000 to a final concentration of 12.5% and 0.3 M NaCl. The sample was centrifuged and the pellet was dissolved in 0.05 M phosphate-buffered saline (PBS), pH 7.5. Virus was extracted with chloroform at a final concentration of 30%. After centrifugation, the upper aqueous phase was collected and reconcentrated using SpeedVac centrifugation to reduce the volume to approximately 1 ml, which was stored at -80ºC until used.

RNA extraction and amplification

Extraction of viral RNA from oyster concentrate was performed using RNeasy® mini kit (Qiagen AG, Basel, Switzerland) following the manufacturer's instruction. In brief, a 200 µl of the oyster concentrate was used for RNA purification on a silica-based column. RNA was eluted in 60 µl of warm Rnase-free water and stored at -80ºC until further analysis.

For the detection of NoV, reverse transcription-nested polymerase chain reaction (RT-nested PCR) was used with the previously described specific primers located in the capsid gene (region C): for NoV GI, COG1F (5′-CGY TGG ATG CGN TTY CAT GA-3′), G1SKF (5′-CTG CCC GAA TTY GTA AAT GA-3′), and G1SKR (5′-CCA ACC CAR CCA TTR TACA-3′), and for NoV GII, COG2F (5′-CAR GAR BCN ATG TTY AGR TGG ATG AG-3′), G2SKF (5′-CNT GGG AGG GCG ATC GCA A-3′), and G2SKR (5′-CCR CCN GCA TRH CCR TTR TAC AT-3′); Y = C or T, R = A or G, B = C, G or T, H = A, C or T, N = A, T, G or C (Kojima *et al*, 2002; Kageyama *et al*, 2003). RT-PCR was performed with 50 µl reaction volume using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In brief, a 4 µl aliquot of RNA extract (diluted 1:2) was heated at 94ºC for 4 minutes and put on ice for 10 minutes. The denatured RNA was added to RT-PCR mixture (46 µl) consisting of a buffer containing 0.2 mM each dNTP, 1.6 mM $MgSO_4$, SuperScript™ III RT / Platinum® *Taq* Mix, 0.33 µM specific primers (COG1F/G1SKR for NoV GI or COG2F/G2SKR for NoV GII), and nuclease-free water. Thermocycling conditions were as follows: at 42ºC for 60 minutes; at 94ºC for 2 minutes; 35 cycles at 94ºC for 1 minute, 50ºC for 1 minute, 72ºC for 1 minute; and a final heating at 72ºC for 3 minutes.

Nested PCR was performed in 50 µl reaction volume containing 2 µl of RT-PCR product, 2.5 mM $MgCl_2$, 1x *Taq* buffer, 0.2 mM each dNTP, 0.33 µM specific primers (G1SKF/G1SKR for NoV GI or G2SKF/G2SKR for NoV GII), 0.63 Units of *Taq* polymerase for NoV GI or 2.5 Units of *Taq* polymerase for NoV GII, and nuclease-free water. Thermocycling conditions were as follows: 94ºC for 3 minutes; 35 cycles for NoV GI, or 30 cycles for NoV GII at 94ºC for 1 minute; 50ºC for 1 minute; 72ºC for 2 minutes; and a final heating at 72ºC for 15 minutes. Nested PCR amplicons were separated by 1.5% agarose gel-electrophoresis and stained with ethidium bromide. Amplicon of 330 bp and 344 bp was indicative of NoV GI and NoV GII, respectively.

Sequence analysis

Amplicons were purified using QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced at the Bioservice Unit of the National Science and Technology Development Agency, Bangkok. Nucleotide sequences were compared with those of NoV strains deposited in National Center for Biotechnology Information GenBank database using BLAST program (Altschul *et al*, 1990). Phylogenetic relationships of NoVs were examined using ClustalX program and a phylogenetic tree was constructed according to the neighbor-joining method using MEGA version 3.1 (Kumar *et al*, 2004). Alignment of the nucleotide sequences or amino acids was performed using BioEdit and GENEDOC programs. The nucleotide sequences of the NoV GI strains were deposited in GenBank (accession nos. GU320061 and GU320062).

Bacteriological analysis

The most probable number (MPN) values of fecal coliforms and *E. coli* in oyster samples were determined using multiple fermentation tube method according to the Bacteriological Analytical Manual of the Food and Drug Administration (Hitchins *et al*, 1998). In brief, oyster meat (25 g) was added to 225 ml of PBS and shaken in a stomacher (Seward, Lab system, London, England). The mixture was

serially diluted from 10^{-1} to 10^{-5}. Fecal coliform density was determined using lactose fermentation with gas production. The number of gassing tubes was recorded for calculation of fecal coliforms MPN by means of the MPN table. The presence of *E. coli* was examined by isolation and identified by biochemical tests (Hitchins *et al*, 1998). MPN of *E. coli* was interpreted from the number of gassing tubes that contained *E. coli* by means of the MPN table.

Table 1
Presence of noroviruses in oyster samples tested by RT-nested PCR.

Source of oyster	No. of samples	No. of norovirus (%)		
		GI	GII	GI+GII
Local markets	65	15 (23)	4 (6)	1 (1)
Oyster farms	53	15 (28)	3 (6)	7 (13)

RESULTS

Norovirus detection in oyster samples

Based on the genogroup-specific RT-nested PCR, NoV RNA was detected in 45 of the 118 (38%) oyster samples. The oysters collected from oyster farms, which are harvesting areas, tested positive for NoVs at a higher detection rate (47%, 25/53 samples) than oysters from local markets (31%, 20/65 samples) (Table 1). Of the 45 detected NoVs, 30 (67%) belonged to GI, 7 (15%) to GII, and 8 (18%) to both GI and GII. Analysis of the oyster samples contaminated with either NoV GI or GII revealed that NoV GI occurred 2.5 times more frequency than NoV GII (38 versus 15). During this study period, NoVs appeared in oysters between September and December. NoV GI and mixed GI+GII strains were found at a high frequency in October, and NoV GII in September (data not shown).

Genetic analysis of NoV-positive oyster samples

NoV GI and NoV GII yielded amplicons. Only 2 NoV GI strains (one from an oyster farm and the other from a market) could be sequenced successfully. Using BLAST program and phylogenetic analysis of the partial capsid region according to the clustering method (Zheng *et al*, 2006), the nucleotide sequences from the oyster farm sample (THOYS019/2005) and from the market sample (THOYS079/2005) were within the same cluster as the reference Southampton strain (L07418). These sequences belonged to NoV GI-2 as shown in Fig 1. The alignment of the nucleotide sequences (183 nucleotides) revealed that both sequences had nucleotide changes at 5 positions relative to the Southampton sequence (NoV GI-2). Two nucleotide changes caused a change from valine to isoleucine, whereas three nucleotide changes did not result in any amino acid changes (data not shown).

Bacteriological analysis of NoV-positive oyster samples

To assess the relationship between NoVs found in oysters and bacterial indicators of fecal contamination, fecal coliforms and *E. coli* were determined. MPN of fecal coliforms in 45 NoV-positive oyster samples were in the range of <3.0/g to 1.5 x 10^4/g and 15 (33%) contained acceptable levels of fecal coliforms according to the standard level of raw shellfish (National legislation, <20 MPN/g). Geometric mean of fecal coliforms higher than the standard level in oyster samples containing NoVs GI, GII, and mixed GI+GII strains was 954.9, 994.5, and 93 MPN/g for the market samples, and 247.1, 35, and 173.8 MPN/g for the oyster farms samples, respectively. MPNs of *E. coli* in the 45 NoV-

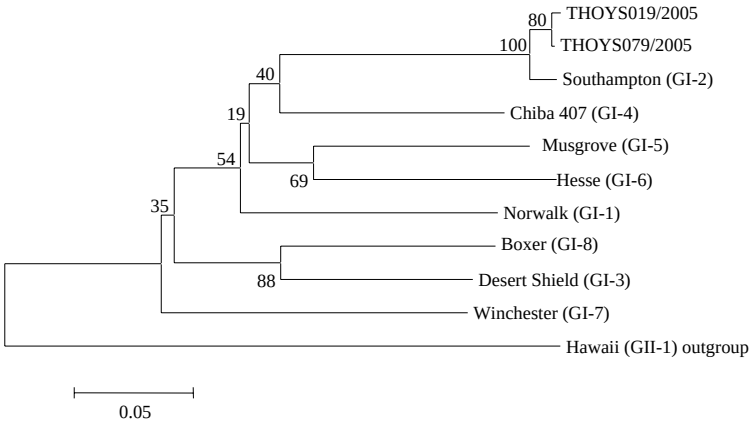

Fig 1-Phylogenetic analysis of partial capsid sequences of NoV GI (THOYS019/2005 and THOYS079/2005) detected in oyster samples. GenBank accession numbers for the 8 known GI strains are GI-1 Norwalk (M87661); GI-2 Southampton (L07418); GI-3 Desert Shield (U04469); GI-4 Chiba 407 (AB042808); GI-5 Musgrove (AJ277614); GI-6 Hesse (AF093797); GI-7 Winchester (AJ277609); and GI-8 Boxer (AF538679); with GII-1 Hawaii (U07611) as the outgroup. The tree was generated based on the neighbor-joining method and the numbers at each branch indicate bootstrap values.

Table 2
Bacterial indicators of fecal contamination in norovirus-positive oyster samples.

Source of oyster	Norovirus (No. of samples)	Fecal coliforms, MPN/g		*E. coli*, MPN/g	
		≥20 [GM][a] (No.)	<20[b] (No.)	≥3.6 [GM] (No.)	<3[b] (No.)
Local markets	GI (15)	23-1.5x10^4 [954.9] (9)	<3-16 (6)	3.6-1.5x10^4 [125.4] (14)	<3 (1)
	GII (4)	4.3x10^2-2.3x10^3 [994.5] (2)	3.6-9.1 (2)	3.6-2.3x10^3 [75.4] (4)	-
	GI + GII (1)	93 [93] (1)	-	15 [15] (1)	-
Oyster farms	GI (15)	23-2.3x10^3 [247.1] (12)	<3-15 (3)	3.6-2.3x10^3 [125] (14)	<3 (1)
	GII (3)	35 [35] (1)	3.6 (2)	3.6-35 [11.2] (2)	<3 (1)
	GI + GII (7)	23-7.5x10^3 [173.8] (5)	<3-11 (2)	6.2-7.5x10^3 [55.9] (6)	<3 (1)
	Total (45)	23-1.5x10^4 [347.9] (30)	<3-16 (15)	3.6-1.5x10^4 [89.4] (41)	<3 (4)

[a]Geometric mean
[b]Standard acceptable level of fecal coliforms (<20 MPN/g) or *E. coli* (<3 MPN/g) in raw shellfish.

positive oyster samples were in the range of <3.0/g to 1.5 x 10^4/g and 4 (9%) contained acceptable levels of *E. coli* (<3 MPN/g). Geometric mean of *E. coli* higher than the standard level in oyster samples containing NoVs GI, GII, and mixed GI+GII strains was 125.4, 75.4, and 15 MPN/g, respectively, for the market samples, and 125, 11.2, and 55.9 MPN/g, respectively, for the oyster farm samples (Table 2).

DISCUSSION

The present study focused on the prevalence of NoVs in raw oysters collected from both production areas and market places. Oysters obtained from the production areas contained NoVs at a higher detection rate than those from regional markets. The presence of NoV strains in the oysters (38%) implies fecal contamination of human origin. This is the first report from Thailand of NoV contamination in raw oysters. High prevalence of NoVs is found in various other countries, such as Tunisia (35%) (Elamri *et al*, 2006), Japan (14.6%) and China (25.3%) (Phan *et al*, 2007) where RT-PCR was used for detection of NoVs. In the current study, RT-nested PCR, which exhibits a higher sensitivity than RT-PCR for the detection of NoVs in clinical samples (Kittigul *et al*, 2010b) was employed. However, an amplification technique with a nested step might generate false positive results due to DNA carryover. A NoV positive control was not included in the experiments, whereas a negative control was included in the assays throughout the study. A number of oyster samples were re-amplified separately for confirmation of the results.

The oyster samples used in this study had been tested previously for rotaviruses (Kittigul *et al*, 2008) and hepatitis A virus (Kittigul *et al*, 2010a), showing 3 samples, one with presence of both NoV GI and rotavirus, one with NoV GII and rotavirus, and another with NoV GI, GII, and hepatitis A virus. These findings correspond to previous studies showing pathogenic human enteric viruses able to persist in the environment (Le Guyader *et al*, 2000; Elamri *et al*, 2006; Croci *et al*, 2007). Among enteric viruses, NoV is the most important etiologic agent causing disease outbreaks associated with oyster consumption (Le Guyader *et al*, 2006; Nenonen *et al*, 2009).

Regarding the genogroups of NoV present in oysters studied, the majority of strains belonged to NoV GI, whereas most human gastrointestinal disease is caused by NoV GII strains (Siebenga *et al*, 2009; Kittigul *et al*, 2010b). The predominance of NoV GI is also found in water samples collected in Thailand (unpublished data) confirming this study's assertion on the relatively high stability of NoV GI strain in the environment. Previous findings of NoV GI predominance in shellfish have been documented (Boxman *et al*, 2006; Nenonen *et al*, 2008). NoV GI is quite stable in bivalves (Boxman *et al*, 2006; Nenonen *et al*, 2009) and is resistant to wastewater treatment (da Silva *et al*, 2007). Nevertheless, several studies reported all or most of the strains found in bivalve shellfish belong to NoV GII (Nishida *et al*, 2003; Croci *et al*, 2007; Phan *et al*, 2007; Le Guyader *et al*, 2009). Further studies on NoV circulation and stability in the environment are required in order to obtain the clearer understanding of NoV epidemiology.

Two NoV GI strains could be characterized successfully and were associated with the reference Southampton strain (GI-2). Unrelated sequences that differed in homology from all of the NoV sequences found in humans have been reported in oysters (David *et al*, 2007). Therefore, it is

possible that among environmental samples, various genotypes of NoV are present. Multiple NoV genotypes in oysters and in patient stools have been characterized from oyster-associated outbreaks, indicating a common feature and the possibility of genetic recombination (Gallimore *et al*, 2005; Le Guyader *et al*, 2006; Symes *et al*, 2007; Nenonen *et al*, 2009).

NoVs were detected in oyster samples (33%) meeting the accepted limit for fecal coliforms (<20 MPN/g). The oyster samples which complied with the *E. coli* standard (<3 MPN/g) were contaminated with NoVs at a lower frequency (9%). These findings have shown the inadequacy of fecal coliforms/*E. coli* as indicator of shellfish quality. Several studies reported the presence of viruses in shellfish with acceptable levels of fecal coliforms or *E. coli* (Lodder-Verschoor *et al*, 2005; Croci *et al*, 2007; Kittigul *et al*, 2010a). Thus, the presence of fecal indicators is not related to the presence of enteric viruses in bivalve shellfish.

In summary, this study demonstrates the naturally occurring NoV contamination in oysters and the predominance of NoV GI strain. The high frequency of NoVs found in oysters might be a potential health risk for acute gastroenteritis associated with raw oyster consumption. In addition to fecal coliforms and *E. coli*, enteric viruses should be used as indicators for microbiological food safety of shellfish.

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