Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.81). Phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets was generated using neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Melksham/89/UK (X81879), SaitamaU201/98/J (AB067542), Arg320/95/AR (AF190817), Lordsdale/93/UK (X86557), Bristol/93/UK (X76716), SaitamaU3/97/JP (AB039776), Leeds/90/UK (AJ277698), SaitamaKU80aGII (AB058582).

3. Results

3.1. Cross-reactivity of Poly Ab against rVLPs in ELISA

The cross-reactivity of Poly Ab raised against rVLP GII/3 or GII/4 was checked in ELISA system (Table 1). Poly Ab raised against rVLP GII/3 reacted broadly with many genotypes including the ones in genogroup I and the titer, expressed as the reciprocal of the highest dilution of each antibody for positive results, generally ranged high against many genotypes. On the other hand, Poly Ab raised against rVLP GII/4 had the tendency to react only with the homologous genotype, GII/4, showing a very high titer compared with the titers against other genotypes.

3.2. Cross-reactivity of immunochromatography against various genotypes of rVLP and NoV in stored clinical samples

A panel of various genotypes of rVLP and NoV in stored clinical samples was used to test the cross-reactivity of the immunochromatography (Table 2). Although highly concentrated rVLP of each genotype was applied, none of heterogeneous genotypes reacted with Immunochromatography-GII/4 and only few did with Immunochromatography-GII/3. This tendency was also observed in the assessment using the stored clinical samples where few samples containing GII/1 or GII/6 NoV were weakly reactive with Immunochromatography-GII/3.

Table 1
Cross-reactivity of polyclonal antibody in enzyme-linked immunosorbent assay

	Genogroup	Genotype	Poly Ab	
			GII/3	GII/4
rVLP (90 ng/well)	I	1	102,400	3,200
		3	25,600	<400
		4	204,800	12,800
		8	204,800	51,200
	II	1	>819,200	51,200
		2	>819,200	25,600
		3	>819,200	6,400
		4	409,600	>819,200
		5	>819,200	25,600
		6	409,600	25,600
		7	>819,200	12,800
		12	204,800	800
		13	409,600	25,600
		14	>819,200	6,400
		15	3,200	25,600

Titers were expressed as reprocical dilution that gave the positive results. Titers more than 409 600 were painted by .

3.3. Detection limit of immunochromatography

Stock solution of both rVLP GII/3 and GII/4, as well as stool samples with known viral copy number were serially diluted to determine the minimal concentration of antigenic protein and virus copy number by the immunochromatography. For

Table 2
Reactivity of immunochromatography with recombinant virus-like particles and clinical stool samples

Genogroup	Genotype	IC-GII/3		IC-GII/4	
		rVLP (750 ng/ml)	No. of positive stools/no. of tested stools	rVLP (750 ng/ml)	No. of positive stools/no. of tested stools
I	1	–	0/3	–	0/3
	3	–	NT	–	NT
	4	–	0/1	–	0/1
	8	–	NT	–	NT
II	1	–	1/2	–	0/2
	2	±	0/8	–	0/2
	3	+	18/19	–	0/10
	4	–	0/10	+	26/31
	5	–	0/1	–	0/1
	6	±	2/4	–	0/2
	7	±	NT	–	NT
	12	±	NT	–	NT
	13	–	0/2	–	0/2
	14	±	0/1	–	0/1
	15	–	NT	–	NT

Note: NT, not tested; +, positive; ±, weakly positive; –, negative.

Immunochromatography-GII/3, the detection limit of rVLP was 3.0×10^{-3} ng/μl and that of viral load was 3.5×10^{7} copies/g of stool, whereas Immunochromatography-GII/4 gave the values for rVLP of 7.5×10^{-3} ng/μl and for viral load of 4.6×10^{6} copies/g of stool. The assays with rVLPs were performed in triplicate and showed the same results, giving a κ-value of 1.0.

3.4. Sensitivity, specificity and agreement rate based on RT-PCR

NoV in clinical stool samples collected during the winter season in Japan was examined by both the current immunochromatography test and RT-PCR and the kit was evaluated based

Table 3
Sensitivity, specificity, and agreement of immunochromatography and RT-PCR[a]

IC	Detection (no. of samples) by RT-PCR		
	+	−	Total
+	30[b]	4[c]	34
−	13	60	73
Total	43	64	107

+, positive; −, negative.
[a] Sensitivity = 69.8% (30/43); specificity = 93.7% (60/64); agreement = 84.1% (90/107).
[b] Nineteen was positive by Immunochromatography-GII/3 and 11 by Immunochromatography-GII/4.
[c] These samples were positive as determined by semi-nested PCR.

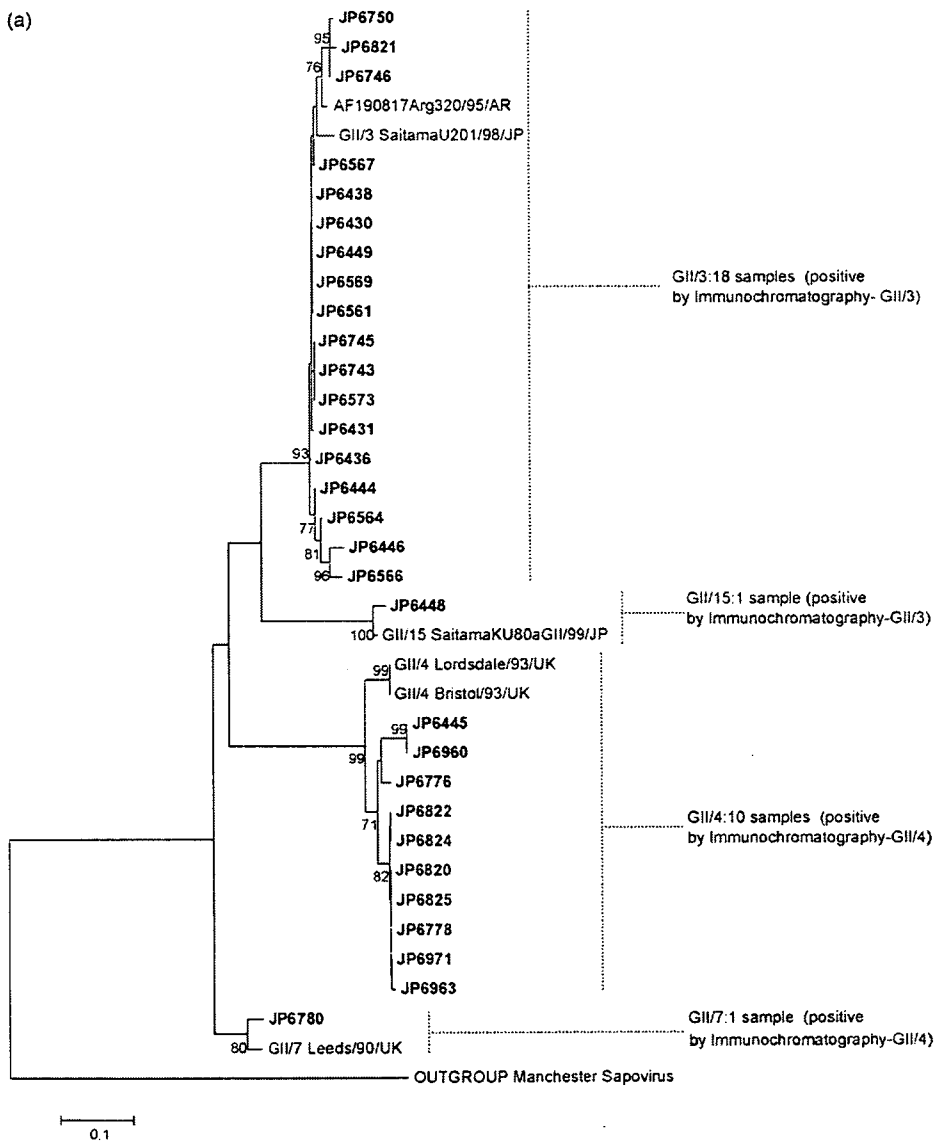

Fig. 3. (a) Phylogenetic tree of nucleotide sequences of NoV in samples positive by both immunochromatography and RT-PCR. Reference strains selected from the DDBJ/GenBank database under the accession numbers indicated in the text. Study NoV was highlighted in bold. Manchester strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. (b) Phylogenetic tree of nucleotide sequences of NoV in samples negative by immunochromatography but positive by RT-PCR.

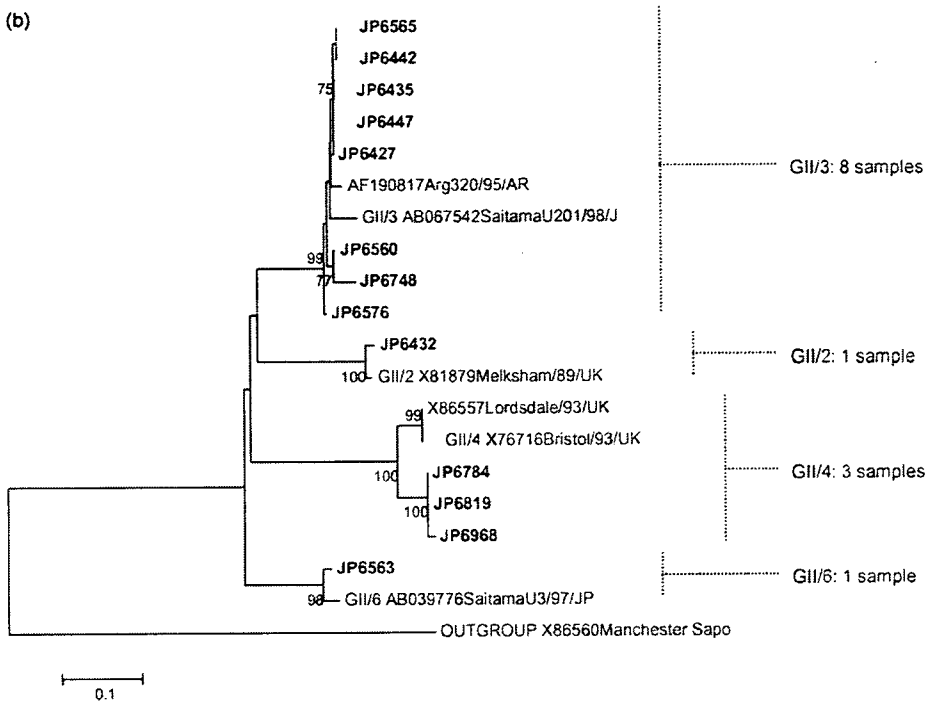

Fig. 3. (*Continued*).

on RT-PCR (Table 3). Among 107 samples, 43 became positive by RT-PCR from which 30 were determined correctly as positive by the immunochromatography and the sensitivity was calculated as 69.8%. Nineteen samples were recorded positive by Immunochromatography-GII/3, whereas 11 samples by Immunochromatography-GII/4 and the former appeared mainly in December, the latter did in March, indicating genotype change during the study period (data not shown). Sixty-four samples were negative by RT-PCR, among which 60 were negative by the immunochromatography, giving the specificity as 93.7%. The agreement rate between the immunochromatography and RT-PCR was calculated as 84.1%. Four samples were determined to be positive by the immunochromatography but negative by RT-PCR. Subsequently, these samples were subjected to semi-nested PCR which gave positive results for all the four samples.

3.5. Genotyping of positive samples by RT-PCR

Stool samples found to be positive for NoV by RT-PCR were analyzed further by sequencing to identify their genotypes. The phylogenetic tree for samples positive for both the immunochromatography and RT-PCR is shown in Fig. 3a, whereas that for negative samples for the immunochromatography but positive with RT-PCR is shown in Fig. 3b. All the GII/3 and GII/4 samples found positive by immunochromatography were done by the homologous immunochromatography. Interestingly, one GII/15 sample became positive with Immunochromatography-GII/3 and one GII/7 sample positive with Immunochromatography-GII/4. Thirteen false negative samples, which were negative by the immunochromatography but positive with RT-PCR, included not only heterogeneous genotypes such as GII/2 and GII/6 but also, unexpectedly, two homogeneous genotypes GII/3 and GII/4. The nucleotide identities of 282 bp which was used for genotyping ranged 98–100% between false negative and true positive samples of GII/3 or GII/4 (data not shown).

3.6. Cross-reactivity with other common enteric viruses

During the study period, several enteric viruses were encountered in pediatric patients. The results of RT-multiplex PCR and the immunochromatography are shown in Table 4. Since no sample was positive for astrovirus during this period, two samples known to be positive for astrovirus as described in Section 2.7 were tested and the results of these samples are also summarized in Table 4. Of note, the immunochromatography succeeded in determining positive result for a sample of mix infection between NoV GII and sapovirus. The immunochromatography gave correctly negative results for other single viral infections, such as group A rotavirus, sapovirus, adenovirus and astrovirus, which implied the high specificity of the immunochromatography kit.

Table 4
Cross-reactivity of immunochromatography with other enteric viruses

Viruses	Number of positive samples	Results of immunochromatography
Group A rotavirus	10	All negative
Sapovirus	5	All negative
Adenovirus	1	Negative
Astrovirus	2	Both negative
Mix infection[a]	1	Positive

[a] Mix infection between norovirus GII and sapovirus.

4. Discussion

With increasing number of reports on NoV and estimated increase in the number of patients with NoV infection (Estes et al., 2006), the demand of offering rapid diagnosis of this infectious disease is expanding dramatically. The results of studies evaluating commercial ELISAs as rapid diagnostic tests, which still take more than 4 h to obtain the results, differed greatly depending on the type of kits and researchers; sensitivity ranged from 36.0% to 76.3% and specificity ranged from 69.0% to 100% (Burton-MacLeod et al., 2004; De Bruin et al., 2006; Dimitriadis et al., 2006; Okitsu-Negishi et al., 2006).

In this study, simple, easy-to-read, and rapid detection test for NoV using immunochromatography membrane strip was developed. This took a shorter time; approximately 30 min to complete the assay with limited equipment needed, and the results were reproducible with the κ-value of 1.0. Based on RT-PCR using the freshly collected samples in winter when NoV infection reaches its peak, the agreement rate was 84.1%, sensitivity 69.8%, and specificity 93.7%. The high percentage of specificity and no cross-reactivity with other enteric viral pathogens, such as group A rotavirus, sapovirus, and adenovirus clearly indicated the potential applicability of the immunochromatography in screening samples for NoV infection.

The false negative samples, which were negative by immunochromatography but RT-PCR positive included GII/2, GII/6, GII/3, and GII/4. The first two genotypes were reasonable as the cross-reactivity of immunochromatography with rVLP had been checked initially and it was found that the broad cross-reactivity observed by the ELISA assessment of Poly Ab raised against rVLP GII/3 did not necessarily appear after applying the immunochromatography system. However, the latter two genotypes were the ones used as antigens to immunize rabbit and expected to react strongly with the Poly Ab in the immunochromatography. The possible reason was that the viral loads in these false negative samples were smaller than those in the true positive samples. On the other hand, the genotypes of four samples, which were positive by immunochromatography and by the semi-nested PCR, were three GII/3 and one GII/4. Then these samples may contain a smaller viral load than monoplex PCR positive samples. This phenomenon was also described elsewhere (Okitsu-Negishi et al., 2006), suggesting that the factors other than viral load cause the lower sensitivity in the immunological detection test. Recently, Hansman et al. (2006) reported that the helix structure in amino acid residues 219–237 from the start codon of VP1 may play an important role in influencing the reactivity of GII/3 NoV in stool samples and Poly Ab raised against rVLP GII/3. Secondary structural prediction by software PSIPRED (McGuffin et al., 2000) revealed that our strain 3229, belonging to GII/3 and being used as an antigen for Poly Ab, possessed the helix structure in the above-mentioned site (data not shown). Hence, there is a possibility of increasing the sensitivity against GII/3 if Poly Ab raised against a strain without such a structure is also applied for the immunochromatography test.

Another possible explanation for lower sensitivity of immunochromatography might be inner epitopes of NoV or the existence of inhibitors in mucous in the human stools. Of interest, the genotype-specific sensitivity for GII/3 and GII/4 with stored stool samples were 18/19 = 94.7% and 26/31 = 83.9%, respectively and these were higher than those with freshly collected samples, which were 18/26 = 69.2% for GII/3 and 10/13 = 76.9% for GII/4. Bon et al. (2007) reported the change in OD of ELISA for rotavirus with stored samples; OD of 14 samples decreased whereas those of 9 samples increased, and speculated that freezing and thawing as well as repeated centrifugation might have affected the immunological detection system. In our assessment, extra freezing and thawing as well as centrifugation performed for previous tests might have revealed the inner epitopes or removed inhibitors easily from the stool suspension with stored samples, resulting in better sensitivity.

Determining the detection limit of immunochromatography to rVLP is of importance especially because NoV cannot be cultured in any cell line, and therefore cannot give the minimal $TCID_{50}$ for positive results which is usually considered a meaningful index for quality constituency between different batches of the kit (Okitsu-Negishi et al., 2006). In this study, the detection limit of the rVLP used as an antigen for each type of immunochromatography was clearly showed and this might be useful as a reference point for future attempts using various genotypes. The viral load of NoV GII in the clinical stool samples are reported to range mostly around 10^8 copies/g of stool (Chan et al., 2006). Based on the results in the current study, the immunochromatography can theoretically detect 1/100 to 1/10 of viral load found in clinical samples, which is almost equivalent to the detection power of electron microscopy (Atmar and Estes, 2001), and might be justified to use for screening the stool samples.

Regarding the cross-reactivity among heterogeneous genotypes, broad reactivity of Immunochromatography-GII/3 was expected to be observed according to the titration result of Poly Ab raised against GII/3 in ELISA. This cross-reactivity was also confirmed in the sandwich ELISA where Poly Ab was used for both capture and detection antibody just like the combination used in the immunochromatography system (data not shown). In the assessment of immunochromatography using the panel of various genotypes of NoV, however, the discordant results were found in reactive genotypes from those in ELISA. Although immunochromatography and ELISA are both classified as immunological methods, several factors such as pH level in the reaction buffer and size of pore in the immunochromatographic membrane might influence the reactivity in these methods. Lessons learnt from this observation would indicate the need for optimizing the reaction buffer or materials of immunochromatography after screening broadly reactive antibody in ELISA system. Additional attempts for simplifying the process of immunochromatography would also be needed by applying the mixture of antibodies raised against several genotypes.

In conclusion, a simple and rapid detection kit with immunochromatographic system was developed using a polyclonal antibody against rVLP for the two most prevailing genotypes of NoV, and a panel of various genotypes of rVLP as well as clinical stool samples were evaluated with this kit. Excellent specificity and detection limit of virus copy number

supported the idea of applying the kit for screening of samples for NoV infection, whereas the lower sensitivity required further efforts in optimizing many factors, such as establishing broadly reactive mono/polyclonal antibodies, selection of antigen with several types of ideal secondary structure, and pretreatment of stool samples.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Sciences and Technology and the Ministry of Health, Labor and Welfare, Japan.

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International Journal of Food Microbiology 122 (2008) 204–210
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Development of a method for concentrating and detecting rotavirus in oysters

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Received 23 June 2007; received in revised form 13 November 2007; accepted 27 November 2007

Abstract

Identification of enteric viruses in outbreak-implicated bivalve shellfish is difficult because of low levels of contamination and natural inhibitors present in shellfish tissue. In this study, the acid adsorption–alkaline elution method developed in our laboratory was proposed for the detection of rotavirus from oyster samples. The acid adsorption–alkaline elution process included the following steps: acid adsorption at pH 4.8, elution with 2.9% tryptose phosphate broth containing 6% glycine, pH 9.0, two polyethylene glycol precipitations, chloroform extraction and reconcentration using speedVac centrifugation. Oyster concentrates were extracted for RNA and examined for rotavirus using reverse transcription-nested polymerase chain reaction (RT-nested PCR). A comparison of SuperScript™ One-Step RT-PCR system and RT followed by PCR before the nested PCR reaction showed the former detecting four-fold lower concentration of rotavirus (78.12 plaque forming units [PFU]/ml or 0.26 PFU/assay) than the latter (3.12×10^2 PFU/ml or 1.04 PFU/assay). In the seeding experiment, the developed acid adsorption–alkaline elution gave high sensitivity of rotavirus detection (125 PFU/g of oyster). From August 2005 to February 2006, 120 oyster samples (*Crassostrea belcheri*) were collected from local markets and oyster farms, concentrated, and tested for naturally occurring rotaviruses. Four oyster samples were group A rotavirus-positive. Based on phylogenetic analysis of rotavirus DNA sequences in those positive samples, the oyster samples contained the sequences associated with human rotavirus G9 (two samples), G3 (one sample), and G1 (one sample). The present study demonstrates the successful application of developed virus concentration method and RT-nested PCR for the detection of rotaviruses in naturally contaminated oyster samples. The method might be used as a tool for evaluating the presence of enteric viruses in shellfish for monitoring and control of public health.

Keywords: Virus concentration method; Rotavirus; Oyster; RT-nested PCR

1. Introduction

Enteric virus contamination in shellfish associated with outbreaks is a global public health concern (Koopmans and Duizer, 2004). Bivalve shellfish are filter-feeders and can concentrate any pathogenic microorganisms in sewage-containing water. Consumption of raw or slightly cooked shellfish such as oysters, cockles and mussels can cause gastrointestinal infection in humans. Food-borne outbreaks of enteric viruses have been reported in association with shellfish (Sanchez et al., 2002; Le Guyader et al., 2006; Shieh et al., 2007). Moreover, the viruses are resistant to physical and chemical treatment, and the depuration process may not remove viruses effectively (Croci et al., 1999; Kingsley and Richards, 2003; Loisy et al., 2005). Shellfish are contaminated with enteric viruses including norovirus or Norwalk-like virus (Boxman et al., 2006), hepatitis A virus (Croci et al., 2007), adenovirus (Formiga-Cruz et al., 2005) and rotavirus (Le Guyader et al., 2000).

Rotavirus is one of the important viruses present in water and shellfish. The virus mainly causes acute gastroenteritis in children and asymptomatic illness in adults. In Thailand, rotavirus is the most important etiologic agent causing acute diarrhea in infants and children less than five years old. Rotavirus causes 30–50% of all hospital admissions for acute diarrhea (Maneekarn and Ushijima, 2000; Jiraphongsa et al, 2005). Rotavirus is a member of family *Reoviridae* with segmented double-stranded RNA genome, surrounded by a

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0168-1605/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2007.11.071

triple-layered capsid. The virus is transmitted easily from person-to-person, or indirectly via food, water, or fomites contaminated with virus-containing feces (Butz et al., 1993; Soule et al., 1999; Gallimore et al., 2005; van Zyl et al., 2006). Although rotavirus mainly infects young children, the adults with rotavirus infection can spread the virus through direct or indirect transmission to children and environment. Food- or waterborne outbreaks caused by rotavirus are uncommon, however, the findings of rotavirus present in various sources of water in Thailand may imply a health risk to humans (Kittigul et al., 2000, 2005). Furthermore, genetic analysis of rotavirus in the environment is helpful for epidemiological study and surveillance of acute gastrointestinal disease. However, detection and identification of viruses in water and shellfish is problematic because of the low density of contamination, inefficient recovery of viruses during the concentration process and the presence of natural inhibitors to detection by polymerase chain reaction (PCR). The methods commonly used to concentrate viruses from whole shellfish include direct alkaline elution (De Medici et al., 2001), and acid adsorption–neutral elution (Mullendore et al, 2001).

An efficient and sensitive method for concentrating rotavirus and detection by RT-nested PCR from water samples has been established in our laboratory. The virus concentration method was based on acid adsorption–alkaline elution and reconcentration using speedVac centrifugation (Kittigul et al, 2005). In the present study, this method was modified for improving the detection limit of rotavirus in oyster samples and compared with direct alkaline elution and acid adsorption–neutral elution. In a field trial, the developed method was used to assess the rotavirus contamination in raw oysters collected from local markets and oyster farms in Thailand.

2. Materials and methods

2.1. Virus and oyster samples

Rotavirus seeded experimentally in oyster samples was inactivated bovine rotavirus (Calf rotavirus strain 3209176) and contained approximately 10^5 plaque forming units (PFU)/ml, as determined by cell culture before inactivation.

One hundred and twenty oyster samples (*Crassostrea belcheri*) were collected from various local markets (60 samples) in Bangkok and ten oyster farms (60 samples) in Surat Thani Province, located at the south of Thailand, from August 2005 to February 2006. All oyster samples were transported to the laboratory in chilled containers. On arrival, the oysters were washed, scrubbed, and the shells opened with a sterile shucking knife. The liquor or mantle fluid was drained into a discard container. The oyster meat, except the adductor muscle that was left attached to the shell, was collected, cut into small pieces, and trimmed to 25 g for analysis of rotavirus.

2.2. Oyster processing methods to concentrate rotavirus

Three different concentration methods were used to determine the detection limit of rotavirus in oyster samples. Twenty five grams of oyster meat were inoculated with known concentrations of rotavirus and left to stand for 60 min at room temperature. Direct alkaline elution was performed as described by De Medici et al. (2001) with some modifications. Briefly, seven volumes of chilled, sterilized, distilled water were added to the oyster flesh and homogenized using a blender (Hamilton Beach, Southern Pines, NC). The virus was eluted from oyster tissues with 10% tryptose phosphate broth (TPB) containing 0.05 M glycine, pH 9.0 and re-eluted with 0.5 M arginine–0.15 M sodium chloride (NaCl), pH 7.5. The pooled eluates were precipitated twice by adding polyethylene glycol (PEG 8000) and NaCl to a final concentration of 12.5% PEG–0.3 M NaCl. After dissolving the pellet, the virus was extracted with chloroform to a final concentration of 30% and re-extracted with 0.5 volume of arginine–NaCl, pH 7.5. The aqueous phase was collected and kept at −80 °C prior to assay.

The procedure of acid adsorption–neutral elution was based on the method essentially described by Mullendore et al. (2001) with some modifications. Briefly, after oyster homogenization, the conductivity of homogenates was measured and reduced to less than 2000 μS/cm. The virus was adsorbed to the homogenates at pH 4.8 and eluted from oyster tissues with 0.75 M glycine–0.15 M NaCl, pH 7.6 followed by re-elution with 0.5 M threonine–0.15 M NaCl, pH 7.5. Then, the virus was first precipitated by adding 8% PEG–0.3 M NaCl and further purified by extraction with chloroform and re-extraction with threonine-NaCl, pH 7.5. The virus was precipitated again using PEG–NaCl. After centrifugation, the pellet was stored at −80 °C until use.

The acid adsorption–alkaline elution method developed in this study was modified from the virus concentration methods as described by Kittigul et al. (2005) and Mullendore et al. (2001). Seven volumes of chilled, sterilized distilled water were added to 25 g of oyster flesh and homogenized at high speed twice. The conductivity of the homogenate was reduced to less than 2000 μS/cm by adding sterilized distilled water. The homogenate of oyster was adjusted to pH 4.8 with 1 N HCl, shaken for 15 min, and centrifuged at 2000 ×g for 20 min at 4 °C. The supernatant was discarded and the pellet was suspended in 25 ml of 2.9% TPB containing 6% glycine, pH 9.0 for elution of the virus, shaken for 15 min, and centrifuged at 10,000 ×g for 15 min at 4 °C. The supernatant (S1) was collected and the pellet was re-eluted with 25 ml of 0.5 M arginine–0.15 M NaCl, pH 7.5. The suspension was shaken for 15 min and centrifuged. The supernatant (S2) was collected, combined with S1 and adjusted to pH 7.2 with 1 N HCl. The virus in the supernatant was precipitated by adding 12.5% PEG 8000 and 0.3 M NaCl. The mixture was refrigerated overnight at 4 °C. The pellet was dissolved in 15 ml of 0.05 M phosphate-buffered saline (PBS), pH 7.5 and precipitated again with PEG–NaCl. The mixture was stirred for 2 h at 4 °C and then centrifuged at 10,000 ×g for 10 min. The pellet was dissolved in 5 ml of PBS. The virus was extracted with chloroform at a final concentration of 30%. After centrifugation at 3000 ×g for 10 min, the top layer of the aqueous phase was collected (S3). The pellet at the interface between the solvent and the aqueous

phase was re-extracted with 0.5 volume of arginine–NaCl, pH 7.5. After centrifugation, the top layer (S4) was collected and combined with S3. The sample was reconcentrated using speedVac centrifugation to reduce the volume of the concentrate to approximate 1 ml and stored at −80 °C until nucleic acid extraction.

In seeding experiments, rotavirus was inoculated in the oyster tissues and processed by the virus concentration methods. The supernatant or pellet discarded in the concentration process was collected at each step of the acid adsorption–alkaline elution method to study virus loss. The virus was extracted with chloroform and the aqueous phase was collected and reduced in volume using speedVac centrifugation prior to RNA extraction.

2.3. Viral RNA extraction from oyster samples

Viral RNA from oyster samples was extracted using RNeasy® mini kit (Qiagen AG, Basel, Switzerland). In brief, 200 µl of the concentrated oysters was lysed and RNA was purified on the silica-based column according to the manufacturer's protocol. RNA bound to the membrane in the column was eluted in 60 µl of warm RNase-free water.

2.4. RT-nested PCR

2.4.1. One-Step RT-PCR system

Two microliters of extracted RNA was heated at 94 °C for 4 min and placed on ice for at least 10 min. RNA was examined by SuperScript™ One-Step RT-PCR system with Platinum® *Taq* DNA polymerase (Invitrogen, Life Technologies, Carlsbad, CA). One-Step RT-PCR was performed with 50 µl reaction volume. The extracted RNA sample (2 µl) was added to RT-PCR mixture (48 µl) consisting of 1× Reaction Mix (a buffer containing 0.2 mM of each dNTP, 2 mM $MgSO_4$), SuperScript™ III RT/Platinum® *Taq* Mix, 0.25 µM primer RV1, 0.25 µM primer RV2 (Gilgen et al., 1997) and nuclease-free water. The RT and PCR were carried out with following steps: RT at 41 °C for 60 min; PCR cycle 1–25, 94 °C for 2 min, 94 °C for 30 s, 55 °C for 30 sec, 72 °C for 60 s; final extension, 72 °C for 3 min.

2.4.2. RT followed by PCR

Two microliters of extracted RNA was heated at 94 °C for 4 min and placed on ice for at least 10 min. RNA was examined by RT followed by PCR (Promega, Madison, WI) according to the method previously described by Kittigul et al. (2005). In 20 µl reaction volume, the extracted RNA sample (2 µl) was added to RT mixture (18 µl) consisting of 10 mM Tris–HCl (pH 9.0 at 25 °C), 50 mM KCl, 0.1% Triton® X-100, 5 mM $MgCl_2$, 1 mM each of dNTP, 20 U of Recombinant Rnasin® (Ribonuclease Inhibitor), 15 U of AMV reverse transcriptase, 1.25 µM RV1, and nuclease-free water. After incubation at 41 °C for 60 min, the sample was heated at 99 °C for 5 min, and immediately cooled at 4 °C for 5 min.

First-strand cDNA diluted 1:5 (10 µl) was added to 40 µl of PCR mixture. The final concentrations were: 0.2 mM of each dNTP, 2 mM of $MgCl_2$, 1× PCR buffer (10 mM Tris–HCl, pH 9.0 at 25 °C, 50 mM KCl, 0.1% Triton® X-100), 0.25 µM of primer RV1, 0.25 µM of primer RV2, 2.5 U of *Taq* DNA polymerase. All were mixed with nuclease-free water. The PCR cycling conditions were as follows: 60 s at 94 °C followed by 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, and the final extension step at 72 °C for 3 min.

For nested PCR, 1 µl of the first amplification reaction from One-Step RT-PCR system or RT followed by PCR was further amplified under the same conditions of amplification as for the first PCR except for changing the primer pair to RV3 and RV4 and their concentrations to 0.5 µM and the concentration of $MgCl_2$ to 3.5 mM (Gilgen et al., 1997). PCR products were analysed by electrophoresis on 1.5% agarose gels and ethidium bromide staining. A DNA fragment of 346-bp was considered the rotavirus DNA. The sample concentrates were inoculated with various concentrations of rotavirus and used to test the sensitivity of RT-nested PCR in order to study the presence of PCR inhibitors in concentrated oyster samples.

2.5. Sequence and phylogenetic analyses of rotavirus-positive oyster samples

Amplified products (346-bp) were sequenced at the Bioservice Unit of National Science and Technology Development Agency, Bangkok, using the same forward (RV3) primer. The nucleotide sequences of VP7 gene were compared with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using BLAST (Basic Local Alignment Search Tool) server (Altschul et al., 1990). Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 3.1 (Kumar et al., 2004).

2.6. Nucleotide sequence accession numbers

The nucleotide sequence of VP7 of rotavirus-positive oyster samples; THOYS019, THOYS020, THOYS068, and THOY S108 were deposited in GenBank under the accession numbers EF687852, EF687853, EF687854, and EF687855, respectively.

2.7. Bacteriological analysis of rotavirus-positive oyster samples

The values of most probable number (MPN) fecal coliforms and *E. coli* in rotavirus-positive oyster samples were determined using multiple fermentation tube method in a five tube series of five dilutions ranged from 10^{-1} to 10^{-5} according to the Bacteriological Analytical Manual of the Food and Drug Administration (Hitchins et al., 1998). Fecal coliform density was determined using lactose fermentation with gas production. Numbers of gassing tubes were recorded for calculation of MPN fecal coliforms by means of MPN table. The presence of *E. coli* was examined by culture on MacConkey agar. The suspected colonies were picked up, and identified by TSI (Triple sugar iron) and IMViC (Indole, MR, VP, Citrate) tests. Acid slant, acid butt, and gas producing without hydrogen

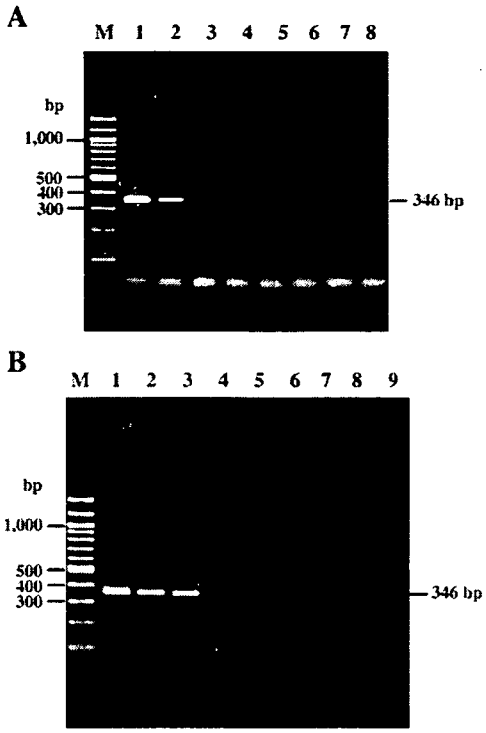

Fig. 1. Sensitivity and specificity of RT-nested PCR for detection of rotavirus in phosphate-buffered saline (A) one-step RT-PCR and nested PCR. Lane: M, DNA marker (100-bp DNA Ladder); 1, rotavirus, 3.12×10^2; 2, 78.12; 3, 19.53; 4, 4.88 PFU/ml; 5, poliovirus (1.25×10^5 $TCID_{50}$/ml); 6, hepatitis A virus (7.20×10^2 EI.U./ml); 7 and 8 negative controls for RT-PCR and nested PCR, respectively. (B) RT followed by PCR and nested PCR. Lane: M, DNA marker (100-bp DNA Ladder); 1, rotavirus, 5×10^3; 2, 1.25×10^3; 3, 3.12×10^2; 4, 78.12 PFU/ml; 5, poliovirus (1.25×10^5 $TCID_{50}$/ml); 6, hepatitis A virus (7.20×10^2 EI.U./ml); 7–9, RT, PCR, and nested PCR negative controls, respectively. Gel electrophoresis of the RT-nested PCR products of rotavirus showed 346-bp band.

sulfide indicated that organism was presumably *E. coli*. The suspected colony was identified as *E. coli* when the results of IMViC were ++−−, respectively. MPN *E. coli* was interpreted from numbers of gassing tubes that contained *E. coli* by means of MPN table.

3. Results

3.1. Sensitivity and specificity of RT-nested PCR

The rotavirus detection sensitivity was initially determined using SuperScript™ One-Step RT-PCR system and compared with RT followed by PCR. The PCR products from both assays were re-amplified using nested PCR. The assays were performed with various concentrations of the rotavirus stock in 0.05 M PBS. It was found that One-Step RT-PCR system could detect rotavirus at the lowest concentration of 78.12 PFU/ml or 0.26 PFU/assay; whereas, RT followed by PCR gave the lowest concentration of rotavirus at 3.12×10^2 PFU/ml or 1.04 PFU/assay. The specificity of RT-nested PCR was examined with primers RV1, RV2, RV3, and RV4 employing different enteric viruses such as poliovirus type 1 ($\geq 1.25\times10^5$ 50% tissue culture infective dose [$TCID_{50}$]/ml) or formaldehyde-inactivated hepatitis A virus vaccine strain HM175 ($\geq 7.20\times10^2$ enzyme-linked immunosorbent assay Units [EI.U.]/ml). The RT-nested PCR amplified the DNA of rotavirus but not for poliovirus or hepatitis A virus in both PCR procedures (Fig. 1A and B). Therefore, the sensitive One-Step RT-PCR was chosen for the subsequent studies.

To determine the sensitivity of RT-nested PCR for rotavirus detection in oysters concentrated by direct alkaline elution, acid adsorption–neutral elution, and acid adsorption–alkaline elution, various concentrations of the rotavirus stock were prepared in concentrated oysters and assayed for DNA amplification. The acid adsorption–neutral elution and acid adsorption–alkaline elution concentration methods provided the same sensitivity (78.12 PFU/ml of concentrated oysters) as found for rotavirus in 0.05 M PBS but the rotavirus detection limit in oysters concentrated by direct alkaline elution method was higher concentration (1.25×10^3 PFU/ml) than that in PBS. This result showed the presence of PCR inhibitors in the oysters concentrated by the direct alkaline elution.

3.2. Detection limit of RT-nested PCR in seeding experiments

Three different methods for concentrating rotavirus from oysters were compared for detection of experimentally contaminated rotavirus. Various concentrations of the rotavirus stock were inoculated in oyster samples, concentrated, and determined for viral RNA using RT-nested PCR. The acid adsorption–alkaline elution gave the highest sensitivity and could detect rotavirus at the lowest concentration of 3.12×10^3 PFU/25 g or 125 PFU/g of oyster. The acid adsorption–neutral elution and the direct alkaline elution were able to detect rotavirus at 1.25×10^4 PFU/25 g and 2.5×10^4 PFU/25 g, respectively (Table 1).

The residual rotavirus was determined in the supernatant or pellet that was discarded in each step of virus concentration. Using the acid adsorption–alkaline elution method, the rotavirus loss was observed in the pellet of oyster tissue after

Table 1
Detection limits of three concentration methods for rotavirus seeded in oyster samples and determined using RT-nested PCR

Rotavirus (PFU/25 g [a])	Acid adsorption–alkaline elution	Acid adsorption–neutral elution	Direct alkaline elution
5×10^4	4+ [b]	3+	2+
2.50×10^4	4+	3+	2+
1.25×10^4	3+	2+	−
6.25×10^3	2+	−	−
3.12×10^3	1+	−	−
1.56×10^3	− [c]	nd	nd

nd, not done.

[a] Twenty five grams of oyster tissues were inoculated with various concentrations of rotavirus, homogenized, and concentrated by acid adsorption–alkaline elution, acid adsorption–neutral elution, or direct alkaline elution method.

[b] 1+, 2+, 3+, 4+, degree of intensity of DNA bands from weak to strong positive results.

[c] −, negative result.

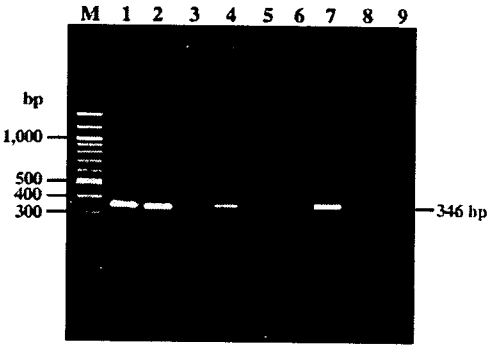

Fig. 2. Detection of residual rotavirus in supernatant or pellet discarded from the concentration process of the acid adsorption–alkaline elution method by RT-nested PCR. Lane: M, DNA marker (100-bp DNA Ladder); 1, rotavirus-positive control (3.12×10^2 PFU/ml); 2, concentrated oysters seeded with rotavirus 5×10^4 PFU/25 g; 3, supernatant at acid adsorption step; 4, pellet after re-elution step; 5, supernatant at first PEG precipitation step; 6, supernatant at second PEG precipitation step; 7, pellet after re-extraction step; 8, negative control for RT-PCR; 9, negative control for nested PCR. Gel electrophoresis of the RT-nested PCR products of rotavirus showed 346-bp band.

re-elution (faint band) and re-extraction (intense band) steps, as shown in Fig. 2.

3.3. Examination of rotavirus in oyster samples

A total of 120 raw oyster samples collected from local markets and oyster farms were concentrated by the acid adsorption–alkaline elution method. Rotavirus RNA in the concentrated oyster samples was determined using One-Step RT-PCR followed by nested PCR. Four oyster samples (3.33%) were positive for rotavirus (Fig. 3): 5% (3/60 samples) collected from three oyster farms from the south of Thailand, and 1.67% (1/60 samples) collected from one local market in Bangkok. The amplicons of these rotavirus-positive samples were sequenced and the phylogenetic analysis revealed that the genetic

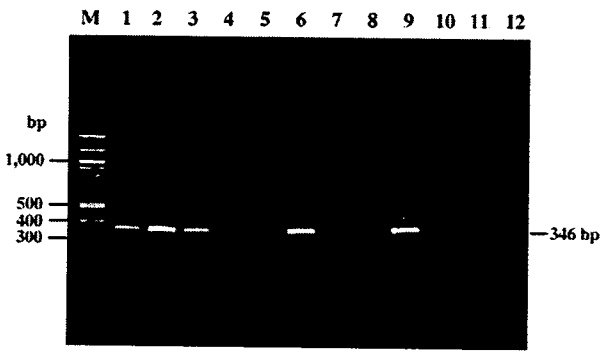

Fig. 3. Detection of naturally occurring rotavirus in oyster samples collected from local markets and oyster farms. Twenty five grams oyster meat was concentrated as described for the acid adsorption–alkaline elution method and rotavirus RNA was detected using RT-nested PCR. Lane: M, DNA marker (100-bp DNA Ladder); 1, rotavirus-positive control (3.12×10^2 PFU/ml); 2–10, concentrates from oyster samples; 11, negative control for RT-PCR; 12, negative control for nested PCR. Gel electrophoresis of the RT-nested PCR products of rotavirus showed 346-bp band in lanes 2, 3, 6, and 9 of oyster samples code THOYS 019, 020, 068, and 108, respectively.

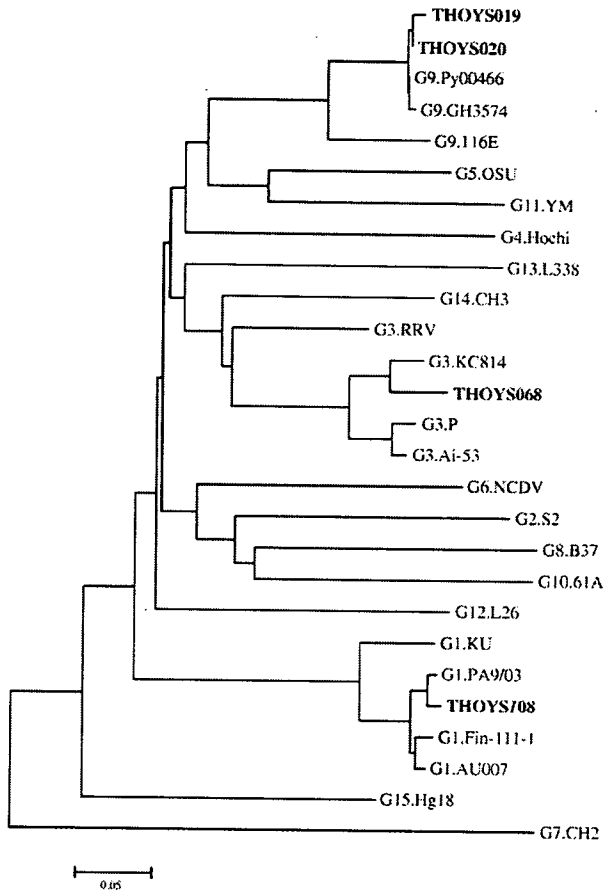

Fig. 4. Phylogenetic tree of the VP7 nucleotide sequences. The tree displays the relationships among rotaviruses (THOYS019, THOYS020, THOYS068, and THOYS108) found in oyster samples and of the 15 known G genotypes. The GenBank accession numbers for strains include: G1; KU (D16343), PA9/03 (DQ377597), Fin-111-1 (Z80278), AU007 (AB081799), G2; S2 (M11164), G3; KC814 (AJ311739), P (AB118024), Ai-53 (D86269), RRV (AF295303), G4; Hochi (AB012078), G5; OSU (X04613), G6; NCDV (M12394), G7; CH2 (X56784), G8; B37 (J04334), G9; Py00466 (DQ015689), GH3574 (AY211068), 116E (L14072), G10; 61A (X53403), G11; YM (M23194), G12; L26 (M58290), G13; L338 (D13549), G14; CH3 (D25229), G15; Hg18 (AF237666), The tree was generated based on the neighbor-joining method using program MEGA 3.1.

sequences were associated with human rotavirus G9 (two samples), G3 (one sample), and G1 (one sample), as shown in Fig. 4. These oyster samples were collected from September to

Table 2
Characteristics of the oyster samples where rotavirus was detected

Oyster sample	Date of collection	Source of collection	Rotavirus genotype	Fecal coliforms MPN/g [a]	*E. coli* MPN/g [a]
THOYS019	19 Sep 05	Oyster farm A	G9	230	230
THOYS020	19 Sep 05	Oyster farm B	G9	210	210
THOYS068	7 Nov 05	Oyster farm C	G3	35	35
THOYS108	12 Dec 05	Local market	G1	93	3.6

[a] The oyster samples were examined for fecal coliforms and *E. coli* by multiple fermentation tube method. The acceptable level of fecal coliforms present in raw shellfish was <20 MPN/g.

December, 2005, the annual peak of rotavirus outbreaks. All four oyster samples contained both bacterial indicators for fecal contamination (fecal coliforms and *E. coli*) of MPN/g in higher levels than the standard level of raw shellfish (<20 MPN/g) proposed in Thailand, as shown in Table 2.

4. Discussion

Outbreaks of viral gastroenteritis have been associated with the consumption of sewage-contaminated shellfish (Lees, 2000). Two major areas of methodological developments are needed for detection of viruses in shellfish: the development of efficient methods to extract and concentrate viruses and the refinement of RT-PCR amplification methods to increase sensitivity and specificity of detecting viruses in shellfish.

The present study attempted to develop a virus concentration method using rotavirus as a model and to detect the virus in oyster samples collected from local markets and oyster farms in Thailand. RT-nested PCR was used for detection of rotavirus from oysters because of its high sensitivity. Rotavirus RNA was determined by two RT-PCR procedures: SuperScript™ One-Step RT-PCR system was more practical because of its simplicity, rapidity and lower risk of cross-contamination than RT followed by PCR. Moreover, the sensitivity of One-Step RT-PCR system was four-fold higher than that of the RT followed by PCR. A previous study showed that the SuperScript™ One-Step RT-PCR System could amplify 0.2–1 PFU/μl of hepatitis A virus and that it provided the highest sensitivity among different commercial RT-PCR kits (Ribao et al., 2004).

Direct alkaline elution, acid adsorption–neutral elution, and acid adsorption–alkaline elution were evaluated for concentration of rotavirus from oyster tissues. The acid adsorption–alkaline elution provided the lowest detection limit, about 8-fold and 4-fold lower, compared to direct alkaline elution and acid adsorption–neutral elution, respectively. The procedure of direct alkaline elution was simple and rapid but less effective in removing inhibitors. This finding was consistent with the study of Sunen et al. (2004). The acid adsorption–alkaline elution and acid adsorption–neutral elution seem to have a greater ability to remove PCR inhibitors, possibly due to the two-step process used. Nevertheless, the procedure of acid adsorption–neutral elution produced a pellet in the final step which was difficult to dissolve prior to RNA extraction. Meanwhile, the acid adsorption–alkaline elution method has several advantages. First, 2.9% TPB containing 6% glycine, pH 9.0, used for elution of virus, is the most effective eluent, as reported previously in a study on concentrating rotavirus from water samples (Kittigul et al., 2001). Second, arginine used in re-elution and re-extraction steps instead of threonine was 7 times cheaper and gave comparable sensitivity to threonine, data not shown. Third, reconcentration of the eluates using speedVac centrifugation gave high recovery of rotavirus (Kittigul et al., 2001). The evaporation combined with centrifugation simultaneously reduced the volume of the eluate and concentrated each sample 25 fold, meaning that 25 g of oyster meat will reduce to about 1 ml of concentrate. The final concentrates in soluble forms were easily extracted for RNA prior to RT-nested PCR.

A number of virus particles were still adsorbed to oyster solids after re-elution or re-extraction with 0.5 M arginine–0.15 M NaCl, pH 7.5. Virus loss at the step of elution and solvent extraction from oyster solids was previously mentioned by Mullendore et al. (2001), who studied the concentration method for hepatitis A virus. The study of concentration of norovirus from oysters also showed the crucial step of virus elution (Schultz et al., 2007). Therefore, the elution and extraction of virus from oyster tissues are important steps to allow the successful detection of enteric viruses.

Of note, the high sensitivity and simplicity of the acid adsorption–alkaline elution method and One-Step RT-PCR system followed by nested PCR make them suitable for application to examination of rotavirus in environmental oyster samples. In Thailand, the most important edible oysters are representative of the genus *Crassostrea*. The oysters are often eaten raw or only slightly cooked by humans. Naturally occurring rotaviruses were found in four (3.33%) out of 120 oyster samples. Three oyster samples with rotavirus contamination in oyster farms might occur during production or harvesting. The oysters were probably contaminated from sewage pollution at the source of production. The one oyster sample from the local market contaminated with rotavirus might have been contaminated during production, transportation or storage.

Computer aided DNA sequence analysis for comparison of rotavirus-positive oyster samples with data banks enabled classification of human rotavirus G9 (two samples), G3 (one sample), and G1 (one sample). Although a low number of rotavirus-positive samples was found, it is likely that human rotavirus G9 is predominant in the environment, as reported for water samples (Kittigul et al., 2005). The emergence of serotype G9 human rotavirus has been reported worldwide, including in Thailand (Khamrin et al., 2006) and might originate through genetic reassortment between human and animal rotaviruses (Gratacap-Cavallier et al., 2000; Ramachandran et al., 2000). The presence of rotavirus in natural oysters might present a potential health risk of bivalves for the consumer and support the need for appropriate controls of the viral contamination of oysters. Among the rotavirus-positive oyster samples, fecal coliforms and *E. coli* were also found in higher level of MPN/g than the standard level. The presence of these bacterial indicators confirmed the rotavirus-positive oyster samples were contaminated with fecal materials both at production source and during transportation or storage.

The present study is the first report of naturally occurring rotavirus contamination in oysters from Thailand and demonstrated the effectiveness of the developed method for concentrating rotavirus and highlighted RT-nested PCR for detecting the VP7 gene of rotavirus in environmental oyster samples. Improvement in sanitary quality of oysters is one preventive measure that can reduce or prevent outbreaks related to raw consumption. Shellfish should be tested for the presence of health significant enteric viruses to guarantee the virological quality of oyster products for human consumption or exportation.

Acknowledgements

This work was supported by research grant from Thai Government Budget through Mahidol University, fiscal year 2004–2005. We thank Mr. Manup and Mrs. Kanya Janphuchung for their assistance in collection of oyster samples from oyster farms and Mr. Peter Hall, The Language Center, Faculty of Graduate Studies, Mahidol University for editorial assistance.

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Microbiol. Immunol., 51(2), 177–184, 2007

Genotyping and Quantitation of Noroviruses in Oysters from Two Distinct Sea Areas in Japan

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Received June 20, 2006; in revised form, October 10, 2006. Accepted October 31, 2006

Abstract: **Norovirus (NV) is a causative agent of acute gastroenteritis in humans, and shellfishes including oysters act as major vehicles of the virus. To investigate the genetic characteristics of NVs, we collected 1,512 oysters for raw consumption between October 2002 and March 2005 from two distinct areas (area A: the Sanriku Sea area; area B: the Setouchi Sea area). We detected the capsid gene and subjected it to phylogenetic analysis. By further quantification of the copy number of the genome by using real-time PCR, the NV capcid gene was detected in approximately 5% of the oysters, and they showed wide diversity. Two percent of the oysters from area B showed relatively large number of NVs, i.e., over 100 copies of capsid gene/oyster, whereas this was not observed in area A. Most of the detected NVs from oysters and humans were genetically related when the capsid region was compared. These results suggested that NVs obtained from humans and those obtained from oysters showed a potential relationship to each other and that some populations of Japanese oysters accumulated a relatively large number of NVs.**

Key words: **Oyster, Norovirus, Phylogenetic analysis, Real-time PCR**

Introduction

Norovirus (NV) is a member of the *Caliciviridae* family (1, 11) and a major causative pathogen of acute nonbacterial gastroenteritis worldwide (4, 6, 8, 11). NV causes fecal-oral infection and is highly infectious (4). According to the data from 2002 to 2004 in Japan, NV is the most common viral agent of food poisoning, and this virus accounted for approximately 30% to 45% of all food poisoning cases (http://www.mhlw.go.jp/topics/syokuchu). The prevalence of NV in other developed countries including the U.S.A. and France is even higher (2, 5). In 1994, Wang et al. showed that NVs isolated from humans are classified into two major genogroups—genogroup I (GI; Norwalk-type viruses) and genogroup II (GII; Snow mountain-type viruses) (26). Each genogroup is further subdivided into many genotypes; thus NVs exhibit wide genetic diversity (10, 12). It is suggested that NVs expelled from patients with gastroenteritis are condensed in shellfishes such as oysters and clams (3, 22, 25). The Japanese and other Asians consume large amounts of raw shellfish, and Europeans and North Americans are also eating increasing amounts of raw shellfish. Raw consumption of shellfishes causes large outbreaks of food poisoning and infectious gastroenteritis (14, 16, 23). Previous epidemiological studies showed a linkage between gastroenteritis due to NV and the oyster-harvesting season (http://idsc.nih.go.jp/iasr) (8). In several studies, NVs were detected in approximately 5% to 20% of oysters and clams (7, 15, 19). Thus, these shellfishes are one of the sources of NV infections (4, 18). Person-to-person infection is another common mode of NV infection (4, 18). In our previous study, we used reverse transcription polymerase chain reaction (RT-PCR) and showed that the NV capsid gene was detected in approximately 10% of the oysters for raw consumption (20). Furthermore, most of the oysters had relatively large amounts

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Abbreviations: GI, genogroup I; GII, genogroup II; N-J, neighbor-joining; NV, norovirus; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

of NV viral genomes with more than 100 copies of the capsid gene (20). However, the molecular study on NVs in oysters is not well known in Japan. In this study, we performed genetic analysis of NVs obtained from 1,512 Japanese oysters for raw consumption which were harvested from two distinct sea areas (Sanriku Sea and Setouchi Sea areas) from October 2002 to March 2005.

Materials and Methods

Samples and preparation of viral suspension. A total of 1,512 Japanese oysters (*Crassostrea gigas*) were collected from the fish distributors that manage harvesting in the Sanriku Sea (483 oysters; Area A) and Setouchi Sea (1,029 oysters; Area B). The distance between these areas is approximately 1,000 km. The Sanriku and Setouchi Seas are located in the northeastern and western parts of the Honshu Island, respectively. The number of samples and the months during which the oysters were harvested in these areas are listed in Table 1. All of the oysters harvested had been approved for raw consumption according to the Food Sanitation Law Enforcement Regulations (in which the standard plate count of bacteria in 1 g of an oyster should be <50,000 with <230 coliforms). The fresh oysters were shucked, and their stomachs and digestive tracts were removed by dissection and then weighed and homogenized in 10 mM phosphate-buffered saline, pH 7.4 without magnesium or calcium to prepare a 10% suspension. The suspension received 0.1 ml antifoam B (Sigma, St. Louis, Mo., U.S.A.) and then was homogenized twice at a 30 sec interval at the maximum speed by using an Omni-mixer (OCI Instruments, Waterbury, Conn., U.S.A.). Six milliliters of chloroform:butanol (1:1 vol) was added to the homogenate. The mixture was then homogenized for an additional 30 sec and 170 μl Cat-Floc T (Calgon, Elwood, Pa., U.S.A.) was added to the homogenate (14). In addition, to monitor for efficiency of the RNA extraction, we added echovirus type 9 to the homogenate samples. After the homogenate samples were centrifuged at 3,000×*g* for 30 min at 4 C, their supernatants were layered onto 1 ml of 30% sucrose solution and ultracentrifuged at 154,000×*g* for 3 hr at 4 C. Subsequently, the pellet was resuspended in 138 μl of double distilled water (DDW) and stored at −80 C until use.

RNA extraction, RT-PCR, sequencing, and real-time PCR. Viral RNA was extracted with the QIAamp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) from 138 μl of the viral suspension. Two microliters of echovirus type 9 (Hill strain; corresponding to approxi-

Table 1. Sample numbers and positive rate of capsid gene of noroviruses in oysters in this study

Month/year	Area A		Area B	
	Sample No.	Positive for RT-PCR (%)	Sample no.	Positive for RT-PCR (%)
Oct/2002	12		6	
Nov/2002	27	1 (3.7)	39	
Dec/2002	45	1 (2.2)	138	6 (4.3)
Jan/2003	48	6 (12.5)	93	12 (12.9)
Feb/2003	39		36	2 (5.6)
Mar/2003	18		12	
Subtotal	189	8 (4.2)	324	20 (6.2)
Oct/2003	15		12	
Nov/2003	18		45	
Dec/2003	81	17 (21)	147	7 (4.8)
Jan/2004	60	7 (11.7)	126	1 (0.8)
Feb/2004	45		78	2 (2.6)
Mar/2004	9		21	1 (4.8)
Subtotal	228	24 (10.5)	429	11 (2.6)
Oct/2004	12		12	
Nov/2004	6	1 (16.7)	51	1 (2)
Dec/2004	12		57	
Jan/2005	9		84	3 (3.6)
Feb/2005	15		48	7 (14.6)
Mar/2005	12		24	
Subtotal	66	1 (1.5)	276	11 (4)
Total	483	33 (6.8)	1,029	42 (4.1)
		75/1,512 (5.0)		

mately 10^4 viral genes) was used to test the adequacy of RNA extraction using the QIAamp Viral RNA Mini kit, and samples was finally suspended in 30 μl of DNase- or RNase-free water. In order to prevent carryover contamination by NV cDNA and to reduce the nonspecific amplicon, the RNA solution was treated with 2 U of RNase-free DNase I (TaKaRa, Tokyo) for 30 min at 37 C followed by inactivation of the enzyme at 75 C for 5 min. RT-PCR was performed in 15 μl of DNase I-treated RNA solution and 15 μl of the RT solution that contained 1 mM dNTP mixture, 10 mM dithiothreitol, 0.75 μg random hexamers (TaKaRa), 33 U RNase inhibitor (TaKaRa), 300 U reverse transcriptase (Superscript II, RNaseH(−); Invitrogen, San Diego, Calif., U.S.A.) and 4.5 μl Superscript II buffer. The RT mixture was incubated at 42 C for 75 min and subsequently at 99 C for 5 min. Five microliters of cDNA was added to 45 μl of the PCR mixture containing 5 μl of 10× Ex *Taq* buffer; 2.5 mM $MgCl_2$ and 20 μM of dATP, dGTP, dTTP and dCTP. The PCR was carried out using 2.5 U of TaKaRa Ex *Taq* (TaKaRa) with GI, antisense primer (G1-SKR): 5'-CCA ACC CAR CCA TTR TAC A-3' and sense primer (COG1F): 5'-CGY TGG ATG CGN TTY CAT GA-3'; GII, antisense primer (G2-SKR): 5'-CCR CCN GCA TRH CCR TTR TAC AT-3' and sense primer (COG2F): 5'-CAR GAR BCN ATG TTY AGR TGG ATG AG-3' (13). For the Alphatron-type strains, the antisense primer (G2AL-SKR) 5'-CCA CCA GCA TAT GAA TTG TAC AT-3' and the sense primer (ALPF) 5'-TTT GAG TCC ATG TAC AAG TGG ATG CG-3' were used.

The amplification was performed under the following conditions: initial denaturation at 94 C for 3 min, 40 cycles of denaturation at 94 C for 1 min, annealing at 50 C for 1 min, extension at 72 C for 2 min and final extension at 72 C for 15 min. Seminested PCR was performed under identical conditions by using the following primers: GI antisense primer: G1-SKR and sense primer (G1-SKF): 5'-CTG CCC GAA TTY GTA AAT GA-3'; GII antisense primer: G2-SKR and sense primer (G2-SKF): 5'-CNT GGG AGG GCG ATC GCA A-3', and Alphatron-type antisense primer: G2AL-SKR and sense primer: ALPF. PCR was performed for 35 cycles at 94 C for 3 min followed by a 15-min incubation at 72 C. Each amplicon was analyzed on 1.5% agarose gels. The products were visualized by 0.5 μg per ml of ethidium bromide staining. The amplicons were purified using the QIAquick PCR purification kit (Qiagen) and the nucleotide sequence was determined by an automated DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, Calif., U.S.A.) by using the Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

We also quantified the NV capsid genes by real-time PCR as described previously (9). The real-time PCR mixture contained 5 μl of cDNA, 2 μl of the LightCycler master mix (Roche, Penzberg, Germany), 0.7 mM of each primer and fluorogenic probes (probes for GI, 2 pmol of RING1(a)-TP and 2 pmol of RING1(b)-TP; probe for GII, 4 pmol of RING2-TP). The fluorogenic probes used for real-time PCR were as follows: 5'-AGA TYG CGA TCY CCT GTC CA-3' (RING1(a)-TP) and 5'-AGA TCG CGG TCT CCT GTC CA-3' (RING1(b)-TP) and 5'-TGG GAG GGC GAT CGC AAT CT-3' (RING2-TP) (9). The amplification was performed by using a LightCycler (Roche). The following PCR protocol was employed: 10 min at 95 C followed by 50 cycles at 95 C for 10 sec, 60 C for 25 sec and 40 C for 30 sec. The data were corrected using internal standards as described previously (9).

Phylogenetic analysis. The capsid sequences were compared with those of the strains detected in gastroenteritis patients living near oyster farms and the reference strains from GenBank. The strains and accession numbers of these sequences have been provided in the legend for Fig. 1. Phylogenetic analysis was performed as described previously (12). In brief, all of the NV capsid region sequences (nt 244 to 313) were aligned using Clustal W (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). A phylogenetic tree was constructed by the neighbor-joining (N-J) technique, Kimura's two-parameter method, by using the Tree Explorer software (ver. 2.12). The reliability of the tree was estimated using 1,000 bootstrap replications.

Statistical analysis. Statistical analysis was performed using Fisher's exact test. A P value of <0.05 was considered significant.

Results

Detection of NV Capsid Gene and Determination of Copy Number in Oysters

We detected the NV capsid gene from a total of 1,512 oysters that were for raw consumption and harvested from the Sanriku (483 oysters) and Setouchi Seas (1,029 oysters). Detailed data regarding these oysters have been listed in Table 1. In total, for 33 of the 483 oysters from area A (6.8%) and 42 of the 1,029 oysters from area B (4.1%), the NV capsid genome was detected during the entire investigation periods. During the 2003–2004 harvesting season, a higher number of NVs was detected in the oysters from area A than in those from area B ($P<0.05$). However, during the 2002–2003 and the 2004–2005 harvesting seasons, the detection of NVs in oysters from area B was higher than that from area A ($P<0.05$). In both areas, most of

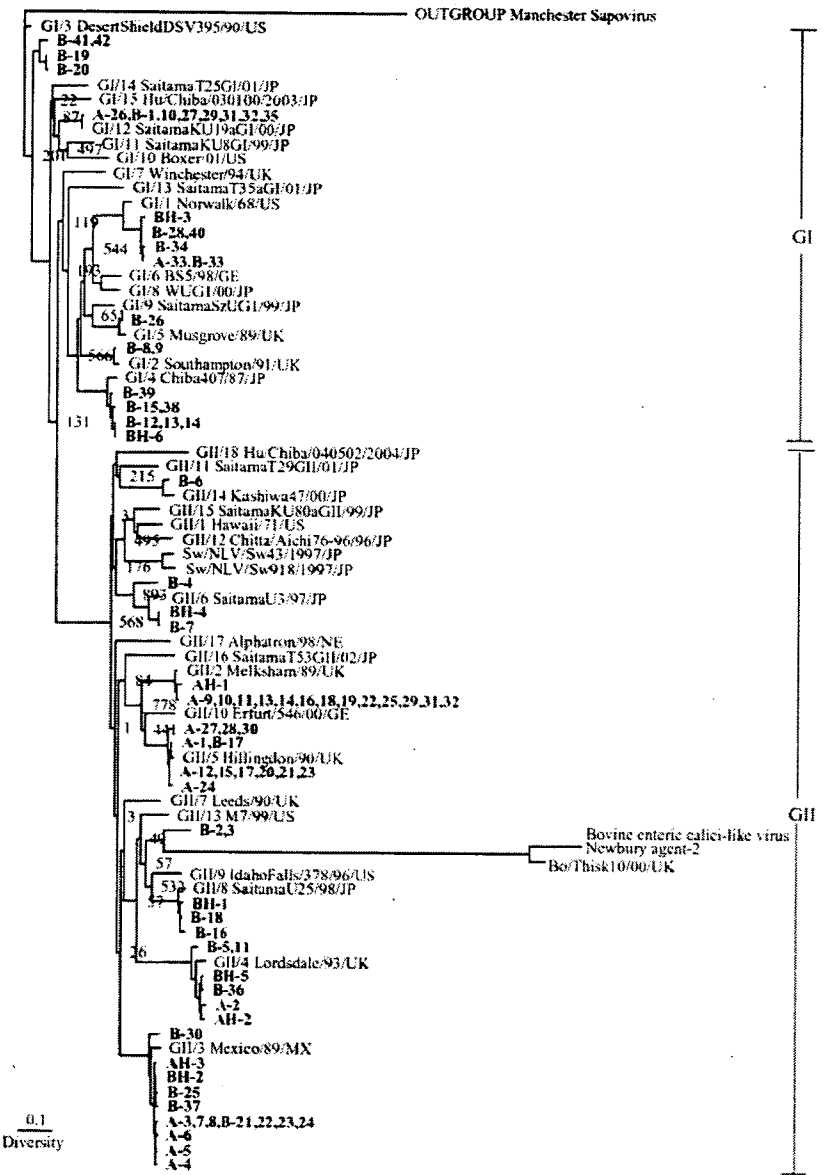

Fig. 1. Phylogenetic tree constructed on the basis of the partial sequences of the Norovirus (NV) capsid gene. The distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining (N-J) method. The numbers at each branch indicate the bootstrap values for the clusters supported by that branch. As an outgroup, the sapovirus strain Manchester: X86560 was used. A and B indicate the harvest areas, and positive samples in this study are designated in bold face. AH and BH refer to human NV strains detected in gastroenteritis patients living near areas A and B. GI: genogroup I; GII: genogroup II. The GenBank accession numbers of the reference strains are as follows: GI/1 Norwalk/68/US (M87661), GI/2 Southampton/91/UK (L07418), GI/3 DesertShieldDSV395/90/US (U04469), GI/4 Chiba407/87/JP (AB042808), GI/5 Musgrove/89/UK (AJ277614), GI/6 BS5/98/GE (AF093797), GI/7 Winchester/94/UK (AJ277609), GI/8 WUG1/00/JP (AB081723), GI/9 SaitamaSzUG1/99/JP (AB039774), GI/10 Boxer/01/US (AF538679), GI/11 SaitamaKU8GI/99/JP (AB058547), GI/12 SaitamaKU19aGI/00/JP (AB058525), GI/13 SaitamaT35aGI/01/JP (AB112132), GI/14 SaitamaT25GI/01/JP (AB112100), GI/15 Hu/Chiba/030100/2003/JP (AJ865494), GII/1 Hawaii/71/US (U07611), GII/2 Melksham/89/UK (X81879), GII/3 Mexico/89/MX (U22498), GII/4 Lordsdale/93/UK (X86557), GII/5 Hillingdon/90/UK (AJ277607), GII/6 SaitamaU3/97/JP (AB039776), GII/7 Leeds/90/UK (AJ277608), GII/8 SaitamaU25/98/JP (AB067543), GII/9 Idaho Falls/378/96/US (AY054299), GII/10 Erfurt/546/00/GE (AF427118), GII/11 SaitamaT29GII/01/JP (AB112221), GII/12 Chitta/Aichi76-96/96/JP (AB032758), GII/13 M7/99/US (AY130761), GII/14 Kashiwa47/00/JP (AB078334), GII/15 SaitamaKU80aGII/99/JP (AB058582), GII/16 SaitamaT53GII/02/JP (AB112260), GII/17 Alphatron/98/NE (AF195847), GII/18 Hu/Chiba/040502/2004/JP (AJ865586), AH-1 (AB262166), AH-2 (AB262167), AH-3 (AB262168), BH-1 (AB262169), BH-2 (AB262170), BH-3 (AB262171), BH-4 (AB262172), BH-5 (AB262173), BH-6 (AB262174), Bovine enteric calici-like virus Newbury agent-2 (AF097917), Bo/Thisk10/00/UK (AY126468), and OUTGROUP Manchester Sapovirus (X86560).

Table 2. Genotypes and copy numbers of NVs in Japanese oysters

Month/year	Area	Amplicons[a]	Genogroup/genotype	NV genome copy number	GenBank accession No.
Nov/2002	A	A-1	II/5	ND[b]	AB262091
Dec/2002	A	A-2	II/4	ND	AB262092
	B	B-1	I/12	5.0×10^{2}	AB262093
	B	B-2	II/untypable	9.1×10^{2}	AB262094
	B	B-3	II/untypable	8.3×10^{2}	AB262095
	B	B-4	II/6	3.8×10^{2}	AB262096
	B	B-5	II/4	2.1×10^{2}	AB262097
	B	B-6	II/14	5.8×10^{2}	AB262098
Jan/2003	A	A-3	II/3	ND	AB262099
	A	A-4	II/3	ND	AB262100
	A	A-5	II/3	ND	AB262101
	A	A-6	II/3	ND	AB262102
	A	A-7	II/3	ND	AB262103
	A	A-8	II/3	ND	AB262104
	B	B-7	II/6	1.7×10^{3}	AB262105
	B	B-8	I/2	1.4×10^{2}	AB262106
	B	B-9	I/2	2.0×10^{2}	AB262107
	B	B-10	I/12	1.8×10^{3}	AB262108
	B	B-11	II/4	1.8×10^{3}	AB262109
	B	B-12	I/4	4.1×10^{2}	AB262110
	B	B-13	I/4	ND	AB262111
	B	B-14	I/4	ND	AB262112
	B	B-15	I/4	ND	AB262113
	B	B-16	II/8	ND	AB262114
	B	B-17	II/5	ND	AB262115
	B	B-18	II/8	ND	AB262116
Feb/2003	B	B-19	I/3	4.8×10^{2}	AB262117
	B	B-20	I/3	ND	AB262118
Dec/2003	A	A-9	II/2	ND	AB262119
	A	A-10	II/2	ND	AB262120
	A	A-11	II/2	ND	AB262121
	A	A-12	II/5	ND	AB262122
	A	A-13	II/2	ND	AB262123
	A	A-14	II/2	ND	AB262124
	A	A-15	II/5	ND	AB262125
	A	A-16	II/2	ND	AB262126
	A	A-17	II/5	ND	AB262127
	A	A-18	II/2	ND	AB262128
	A	A-19	II/2	ND	AB262129
	A	A-20	II/5	ND	AB262130
	A	A-21	II/5	ND	AB262131
	A	A-22	II/2	ND	AB262132
	A	A-23	II/5	ND	AB262133
	A	A-24	II/5	ND	AB262134
	A	A-25	II/2	ND	AB262135
	B	B-21	II/3	1.4×10^{2}	AB262136
	B	B-22	II/3	4.0×10^{2}	AB262137
	B	B-23	II/3	9.6×10^{2}	AB262138
	B	B-24	II/3	1.0×10^{3}	AB262139
	B	B-25	II/3	9.9×10^{2}	AB262140
	B	B-26	I/5	ND	AB262141
	B	B-27	I/12	ND	AB262142
Jan/2004	A	A-26	I/12	ND	AB262143
	A	A-27	II/5	ND	AB262144
	A	A-28	II/5	ND	AB262145
	A	A-29	II/2	ND	AB262146
	A	A-30	II/5	ND	AB262147
	A	A-31	II/2	ND	AB262148
	A	A-32	II/2	ND	AB262149
	B	B-28	I/1	ND	AB262150
Feb/2004	B	B-29	I/12	ND	AB262151
	B	B-30	II/3	ND	AB262152
Mar/2004	B	B-31	I/12	ND	AB262153
Nov/2004	A	A-33	I/1	ND	AB262154
	B	B-32	I/12	ND	AB262155
Jan/2005	B	B-33	I/1	ND	AB262156
	B	B-34	I/1	ND	AB262157
	B	B-35	I/12	ND	AB262158
Feb/2005	B	B-36	II/4	9.9×10^{2}	AB262159
	B	B-37	II/3	4.1×10^{2}	AB262160
	B	B-38	I/4	3.1×10^{2}	AB262161
	B	B-39	I/4	ND	AB262162
	B	B-40	I/1	ND	AB262163
	B	B-41	I/3	ND	AB262164
	B	B-42	I/3	ND	AB262165

[a] Amplicon; A and B refer to the areas of harvest.
[b] ND; not detected (<100 copies/oyster).

the NVs were detected during the December to January investigation periods. We also quantitated the copy number of the NV capsid gene in all oysters by real-time PCR (Table 2). The sensitivity (limit of detection) for this method is more than 100 copies of the NV genomes/oyster (20). Interestingly, the number of oysters from area A, which harbored the NVs genome, was significantly greater than that from area B. Oysters harvested from area B had a relatively large number of capsid genomes with more than 100 copies/oyster, while those harvested from area A had less than 100 copies of the NV genome (Table 2).

Phylogenetic Analysis of NVs from Oysters

We sequenced all the amplicons of the NV capsid genes in oysters by using the direct sequencing method. In the direct sequencing method, if the shellfishes have accumulated several kinds of viruses, there are cases where the peaks of the nucleotide sequences in the chromatogram overlap and determination of the sequence is impossible. In this study, the main fluorescence peak of each nucleotide sequence was observed without overlapping in all the sequence data (data not shown). The capsid gene amplicon was considered to have been amplified from homogeneous NVs in the samples. Based on these sequences, we performed a phylogenetic analysis of the NV capsid gene by using the N-J method (Fig. 1). The genetic distance between the clusters of GI and GII in the phylogenetic tree was observed to be 0.35 and 0.27, respectively. Of 75 amplicons, 26 were classified as GI and the remaining 49 as GII. The GI amplicons were further classified into 6 genotypes and those of GII into 8 genotypes (Fig. 1). In GI, there were 8, 6, and 5 amplicons in the GI/12 (SaitamaKU19a-type), GI/4 (Chiba407-type) and GI/1 (Norwalk-type), respectively. In GII, there were 13 amplicons each in the GII/3 (Mexico-type) and GII/2 (Melksham-type), and 12 amplicons in the GII/5 (Hillingdon-type). We also characterized the geographical and seasonal features of the genotypes. Throughout the investigation period, 12 amplicons in the oysters from area A were classified under GII/2 (Melksham-type). In area B, 6 amplicons were classified under GI/4 (Chiba407-type). In this study, many NV genotypes were detected in oysters, but only 5 genotypes, GI/1, GI/12, GII/3, GII/4 (Lordsdale-type) and GII/5 were common to both areas.

Very recently, all the GI and GII genogroups were classified into 15 and 18 genotypes, respectively (21). This classification method was based on the range of genetic distance (10). However, our 2 amplicons, i.e., B-2 and B-3, were not included in any genotype because the genetic distances exceeded the range for a single cluster, suggesting that these strains may be new NV genotypes (Fig. 1). In addition, the sequences of some amplicons matched each other completely, although most of these were detected in different seasons and different areas. The sequences of some amplicons detected in oysters and humans showed high similarity. Moreover, the sequences from oysters (cf. B-6 in Fig. 1) and animals (cf. Sw/NLV/Sw43/1997/JP and Sw/NLV/Sw918/1997/JP in Fig. 1) were closely related, indicating that the NV capsid genes in oysters exhibit wide genetic diversity but some of them were genetically related to swine NVs (24).

Discussion

In this study, we demonstrated that the NV capsid gene was detected in approximately 5% of the Japanese oysters for raw consumption. Of the oysters from area B, 50% carried relatively large amounts of the NV genomes which were not observed in oysters from area A. In addition, the capsid gene exhibited wide genetic diversity in both genogroups, GI and GII. The nucleotide sequences of the capsid gene of some strains from both areas matched completely, suggesting that some strains of oysters were genetically related to a considerable extent although the oysters were harvested far apart.

Recent studies in some countries showed that approximately 0% to 20% of oysters contained NVs and these viruses are associated with gastroenteritis in humans (16, 17, 19). For example, Le Guyader et al. showed that the polymerase gene of NVs was detected in approximately 20% of French oysters and showed wide genetic diversity (16). Myrmel et al. demonstrated approximately 7% of the shellfishes from the Norwegian coast contained the NV gene (19). In addition, we demonstrated that 10% of Japanese oysters contain the NV capsid genomes and show wide genetic diversity, although these oysters were harvested in areas different from those used in this study (20). On the other hand, Lodder-Verschoor et al. demonstrated that NV genes were not detected in Dutch oysters although the enterovirus genes were detected in these oysters, suggesting that NV does not always contaminate oysters (17). The genetic characteristics of NVs contaminating shellfishes are poorly understood. Therefore, we conducted a molecular typing study on NVs in oysters obtained from two distinct sea areas in Japan, in order to make an accurate comparison of the prevalence patterns of NVs in Japanese oysters. The present data indicates that the NVs detected in Japanese oysters from different sea areas showed wide genetic diversity. This is consistent with the results of earlier reports (16, 17, 19).

Recent data suggested that the infectivity of NVs is relatively strong and less than 100 particles of NVs can easily cause gastroenteritis in humans (4). Our previous data showed that approximately 5% of the oysters for raw consumption from two distinct areas in the Setouchi Sea contained more than 100 copies of the NV genomes in each oyster (20). In contrast, we found that only 1.4% of the oysters had more than 100 copies of NVs in this study. In addition, the oysters from area A had less than 100 copies of NVs. During our investigation periods, 34,000–37,000 tons of oysters were harvested per year (http://www.maff.go.jp/www/info/bun08.html). More than 70% of the oysters were harvested from these two areas. We detected NVs in approximately 5% of the oysters for raw consumption; however, this rate did not directly reflect the possibilities of NV infection. In order to prevent food poisoning caused by microorganisms present in raw oysters, the Japanese government regulates the oyster industry via the Enforcement Regulation of the Food Sanitation Law. However, this regulation mainly focuses on bacteriology. To our knowledge, there is no universal risk assessment of viruses, such as NVs or the hepatitis A virus, which are contained in shellfishes. Ensuring the virological safety of oysters would have an enormous effect. If we can control outbreaks of oyster-related gastroenteritis, the number of patients with gastroenteritis may be reduced. Accurate risk assessment of raw oysters with regard to NVs infections and regulation based on viral sanitation is needed.

It is suggested that various genotypes of NVs in oysters are associated with the outbreaks of gastroenteritis in humans (25). Therefore, it is important to clarify the genetic characteristics of NVs detected from both shellfishes and humans, to gain a better understanding of the epidemiology of NV infection (10). Our data indicated that most GI and GII strains detected in oysters and humans were genetically related to a considerable extent (Fig. 1).

Interestingly, some NVs that are usually detected in swine are also genetically related to the NVs in oysters. Therefore, we could not exclude the possibility that some NV strains detected in oysters were derived from other animals such as swine. In conclusion, further molecular studies of NVs and quantitative real-time PCR methods are needed for a better understanding of the NV infection, which may provide more accurate assessment of the risk factors for shellfish-associated diseases.

We thank Drs. Mitsuaki Oseto (Ehime Prefectural Institute of Public Health and Environmental Science), Toshiyuki Mikami (Aomori Prefectural Institute of Public Health and Environment), Shinji Fukuda (Hiroshima Prefectural Institute of Public Health and Environment), Mamoru Noda (Hiroshima City Institute of Public Health), Michiyo Shinohara (Saitama Prefectural Institute of Public Health), Yo Ueki (Miyagi Prefectural Institute of Public Health and Environment), Nobuhiro Iritani (Osaka City Institute of Public Health and Environmental Sciences), and Miho Akiyama (National Institute of Public Health) for their constructive discussions. This work was supported by Research on Food and Chemical Safety for Health, Labour and Welfare Programs from the Ministry of Health, Labour and Welfare, Japan.

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