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ORIGINAL PAPER

Development of a Practical Method to Detect Noroviruses Contamination in Composite Meals

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Abstract Various methods to detect foodborne viruses including norovirus (NoV) in contaminated food have been developed. However, a practical method suitable for routine examination that can be applied for the detection of NoVs in oily, fatty, or emulsive food has not been established. In this study, we developed a new extraction and concentration method for detecting NoVs in contaminated composite meals. We spiked NoV-GI.4 or -GII.4 stool suspension into potato salad and stir-fried noodles. The food samples were suspended in homogenizing buffer and centrifuged to obtain a food emulsion. Then, anti-NoV-GI.4 or anti-NoV-GII.4 rabbit serum raised against recombinant virus-like particles or commercially available human gamma globulin and *Staphylococcus aureus* fixed with formalin as a source of protein A were added to the food emulsion. NoV-IgG-protein A-containing bacterial complexes were collected by centrifugation, and viral RNA was extracted. The detection limits of NoV RNA were 10–35 copies/g food for spiked NoVs in potato salad and stir-fried noodles. Human gamma globulin could also concentrate other NoV genotypes as well as other foodborne viruses, including sapovirus, hepatitis A virus, and adenovirus. This newly developed method can be used as to identify NoV contamination in composite foods and is also possibly applicable to other foodborne viruses.

Keywords Norovirus · Sapovirus · Hepatitis A virus · Adenovirus · Food · Real-time PCR

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Introduction

Noroviruses (NoVs) are major causes of food poisoning and food-related outbreaks of gastroenteritis. Many protocols based on different principles have been reported for the detection of NoV contamination in food samples (Stals et al. 2012). Currently, NoV detection from food relies on PCR techniques, and it is necessary to concentrate the virus from a large volume of food emulsion into a small volume of suspension to extract viral RNA. Polyethylene glycol (PEG) concentration methods have been developed (Schwab et al. 2000; Leggit and Jaykus 2000; Sair et al. 2002; Baert et al. 2008; Kim et al. 2008; Cheong et al. 2009a; Stals et al. 2011a) and applied to shellfish (Mullendore et al. 2001; Loisy et al. 2005) and smooth-surfaced food samples, such as raw vegetables, fruits, and ham (Kim et al. 2008; Cheong et al. 2009a; Sherer et al. 2010). However, these PEG concentration methods are not effective for composite meals because of difficulty in recovering NoVs from a turbid food emulsion. Recovery methods for virus particles by ultracentrifugation (Rutjes et al. 2006), ultrafiltration (Butot et al. 2007; Cheong et al. 2009b), and positively charged membrane filtration (Borchardt et al. 2003; Morales-Rayas et al. 2010) are restricted to use with water samples or cleared supernatants derived from food emulsions. Virus-specific recovery methods using immunomagnetic beads have been reported as effective for turbid emulsions (Bidawid et al. 2000; Kobayashi et al. 2004; Park et al. 2008; Suffredini et al. 2011). As a

common reagent to adsorb an immune complex, *Staphylococcus aureus* (*S. aureus*) fixed with formalin (Kessler 1975) has been utilized widely in many fields (Goding 1978; Langone 1982). We devised an application involving immunoprecipitation using *S. aureus* to recover virus-captured gamma globulin from a food emulsion. In this study, this versatile NoV extraction and concentration method from various foods, termed the "PANtrap method," has been developed in combination with rabbit anti-NoV virus-like particle antisera or commercially available human gamma globulin. Our data also showed a broad reactivity of human gamma globulin against various NoV genotypes as well as the selection of other foodborne gastroenteritis-associated viruses.

Materials and Methods

Viral Specimens, Primers, and Probes

NoV-, sapovirus (SaV)-, or adenovirus type 41 (AdV41)-positive stool specimens were collected from patients with nonbacterial gastroenteritis as part of the National Infectious Agents Surveillance Program in Japan. The genotypes of the NoVs were determined by capsid sequence-based phylogenetic analysis (Kroneman et al. 2013). Genotype and GenBank accession numbers of the NoVs used in this study are as follows: GI.3, AB685382; GI.4, AB685383; GI.5, AB685384; GI.6, AB448733; GII.2, AB685706; GII.3, AB685707; GII.4, AB293424; GII.5, AB448707; GII.6, AB685708; GII.12, AB685709; GII.13, AB819901; GII.14, AB685710; and GII.22, AB083780. The genotypes of SaVs were also determined by capsid gene-based phylogenetic analysis (Oka et al. 2012, 2015). Genotype and GenBank accession numbers of the SaVs used in this study are as follows: GI.1, AB685711; GII.3, AB685712; GIV.1, AB685713; and GV.1, AB685714. The AdV41 genotype used in this study (AB685715) was confirmed by nucleotide sequencing of the hexon gene (Inagawa et al. 1996). An inactivated vaccine, Aimmugen (Astellas Pharma Inc., Tokyo, Japan), was used as the hepatitis A virus (HAV) sample. These viral specimens were suspended in phosphate-buffered saline (PBS) and centrifuged at 20,000×*g* for 10 min. The supernatants were filtered using a 0.25-μm syringe cartridge filter and stored at −80 °C until use. All primers and probes used in this study are shown in Tables 1 and 2.

Standard DNAs and Determination of Viral Copy Numbers

Standard DNA copies of NoV (Kegeyama et al. 2003) and SaV (Oka et al. 2006) were provided by the Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan. Partial DNA fragments containing amplification regions were synthesized by Thermo Fisher Scientific GmbH (Dreieich, Germany) to use as standards for AdV41 (AB685715) and HAV (AB258387). To generate standard curves for quantitative determinations and to evaluate virus recovery rates and detection limits, serial tenfold dilutions of these standard plasmids or DNA fragments were prepared. Viral copy numbers in the reaction tubes were determined by interpolation of the standard curve, followed by conversion into viral copy numbers in stool and food samples.

Virus Recovery from Artificially Contaminated Foods by the PANtrap Method

The food samples used in this study were purchased from a local market. For the recovery test, 10 g of each food sample was spiked with NoV-GI.4 or NoV-GII.4. The protocol of the PANtrap method is shown in Fig. 1. Artificially contaminated food samples were mixed with 50 ml of homogenizing buffer (0.1 M Tris–HCl, 0.5 M NaCl, 0.1 % Tween20, pH 8.4) in a stomacher bag (Sanispec test bag, AS ONE Co., Osaka, Japan) with a large-meshed filter compartment and sonicated in an ultrasonic laboratory washer (AU-301C, TOKYO RIKAKIKAI Co., Ltd., Tokyo, Japan) for 15 min to obtain a food emulsion. The fluid that had passed through the filter was transferred to a 50-ml polypropylene test tube, and α-amylase powder (from *Bacillus subtilis*, Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to give a final concentration of 2.5 mg/ml. After centrifugation at 1870×*g* in a swing rotor for 30 min, the turbid supernatant was transferred to a new 50-ml test tube. Rabbit antiserum against NoV-GI.4 or NoV-GII.4 prepared by immunization with virus-like particles (Hansman et al. 2006) were provided by the Department of Virology II, National Institute of Infectious Diseases. Five microliters of the rabbit antiserum or 0.15 ml of "Gammagard", 5 % gamma globulin (Baxter Japan Ltd., Tokyo, Japan), was added to make NoV-IgG complex. Subsequently, PANSORBIN® (Merck KGaA., Darmstadt, Germany), *S. aureus* fixed with formalin, was added at amounts of 0.3 ml for rabbit antiserum and 1.0 ml for gamma globulin. After incubation at 37 °C for 15 min, NoV-IgG complexes adsorbed by PANSORBIN® were precipitated by centrifugation at 1870×*g* for 20 min. The pellet was resuspended in 0.25 ml AVL buffer (part of the QIAamp® Viral RNA Mini Kit, QIAGEN, Tokyo, Japan) and transferred to a 1.5-ml microcentrifuge tube. Nucleic acids were extracted from the pellet using 0.75 ml TRIzol® LS Reagent (Life Technologies Japan Ltd., Tokyo, Japan) and 0.2 ml chloroform. After centrifugation at 20,000×*g* for 15 min, the aqueous solution was transferred to a new 1.5-ml microcentrifuge tube, and 0.8 volumes of ethanol were added. This mixture

Table 1 Nucleotide sequences of primers and probes used for foodborne virus detection by real-time PCR and conventional RT-PCR

Virus	Name	Sequence (5′ → 3′)[a]	Position and polarity	Reference
NoV-GI	COG1F	CGYTGGATGCGNTTYCATGA	5291 → 5310[d]	Kageyama et al. 2003
	COG1R	CTTAGACGCCATCATCATTYAC	5351 ← 5375[d]	
	RING1-TP(a)	FAM-AGATYGCGATCYCCTGTCCA-TAMRA[b]	5321 ← 5340[d]	
	RING1-TP(b)	FAM-AGATCGCGGTCTCCTGTCCA-TAMRA[b]	5321 ← 5340[d]	
	G1SKF	CTGCCCGAATTYGTAAATGA	5342 → 5361[d]	Kojima et al. 2002
	G1SKR	CCAACCCARCCATTRTACA	5653 ← 5671[d]	
NoV-GII	COG2F	CARGARBCNATGTTYAGRTGGATGAG	5003 → 5028[e]	Kageyama et al. 2003
	COG2R	TCGACGCCATCTTCATTCACA	5080 ← 5100[e]	
	RING2-TP	FAM-TGGGAGGGCGATCGCAATCT-TAMRA[b]	5048 → 5067[e]	
	G2SKF	CNTGGGAGGGCGATCGCAA	5046 → 5064[f]	Kojima et al. 2002
	G2SKR	CCRCCNGCATRHCCRTTRTACAT	5367 ← 5389[f]	
SaV	SaV124F	GAYCASGCTCTCGCYACCTAC	5078 → 5098 [g]	Oka et al. 2006
	SaV1F	TTGGCCCTCGCCACCTAC	700 → 717 [h]	
	SaV5F	TTTGAACAAGCTGTGGCATGCTAC	5112 → 5135[i]	
	SaV1245R	CCCTCCATYTCAAACACTA	5163 ← 5181 [g]	
	SaV124TP	FAM-CCRCCTATRAACCA-MGB-NFQ[c]	5105 ← 5118 [g]	
	SaV5TP	FAM-TGCCACCAATGTACCA-MGB-NFQ[c]	5142 ← 5157[i]	
HAV	HAV + 449	AGGGTAACAGCGGCGGATAT	450 → 469[j]	Furuta et al. 2003
	HAV-557	ACAGCCCTGACARTCAATYCACT	536 ← 558[j]	
	HAV + 482-P-FAM	FAM-AGACAAAAACCATTCAACRCCGRAGGAC-TAMRA[b]	483 → 510[j]	
AdV41	Adeno.fwd	TTCCAGCATAATAACTCWGGCTTTG	2383 → 2407 [k]	Logan et al. 2006
	Adeno.rev	AATTTTTTCTGWGTCAGGCTTGG	2491 ← 2513 [k]	
	Adeno.probe	FAM-CCWTACCCCCTTATTGG-MGB-NFQ[c]	2461 → 2477 [k]	

[a] Mixed bases in degenerate primers and probes are as follows: Y = C or T, R = A or G, S = G or C, W = A or T, M = A or C, B = c, G or T, H = A, C or T, N = any base

[b] Probes were labeled with 6-carboxy fluorescein (FAM) at the 5′ end and with 6-carboxy-tetramethylrhodamine (TAMRA) at the 3′ end

[c] Probes were labeled with FAM at the 5′ end and with minor groove binder (MGB)-non-fluorescent quencher (NFQ) at the 3′ end

[d] Corresponding nucleotide position of Norwalk/68 virus (M87661)

[e] Corresponding nucleotide position of Camberwell virus (AF145896)

[f] Corresponding nucleotide position of Loadsdale virus (X86557)

[g] Corresponding nucleotide position of GII Mc10 strain (AY237420)

[h] Corresponding nucleotide position of GI Parkville strain (U73124)

[i] Corresponding nucleotide position of GV NK24 strain (AY646856)

[j] Corresponding nucleotide position of HM-175 strain (M14707)

[k] Corresponding nucleotide position of adenovirus type 41 hexon gene (AB610527)

was applied to a QIAamp® Viral RNA Mini Kit spin column to collect 50–60 µl of concentrated, cleared solution containing viral nucleic acid.

Virus Recovery from Artificially Contaminated Food by the PEG Concentration Method

The procedure for obtaining the supernatant from a food sample emulsion was the same as that just before the addition of antibody and PANSORBIN® in the PANtrap method described above. PEG6000 and NaCl were added to 50 ml of supernatant to final concentrations of 8 % and 0.5 M, respectively. After overnight incubation at 4 °C, the precipitate was collected by centrifugation at 9500×*g* for 20 min. The pellet was resuspended in 100 µl distilled water and applied to a QIAamp® Viral RNA Mini Kit for the extraction of RNA.

Table 2 Nucleotide sequences of reverse transcription primers used for NoV-GI and NoV-GII

Virus	Name[a]	Sequence (5′ → 3′)	Position and polarity
NoV-GI	PANR-G1a	GTBCKMACATCAGCAATCA[b]	5800 ← 5818[c]
	PANR-G1b	GGKTCAAGSRYCCTAACATCWGCAATGA	5800 ← 5827[c]
NoV-GII	PANR-G2a	TCYARWKKYCTWACATCTAYAATYAYRTGGGGGAACAT	5502 ← 5539[d]
	PANR-G2b	ARDGTCCTAACATCWATAATYAYATGAGGGAACAT	5502 ← 5536[d]
	PANR-G2c	CTSACATCCACMAYYACRTGCGGRCACAT	5502 ← 5530[d]

[a] Reverse transcription primer "PANR-G1" consists of "PANR-G1a" and "PANR-G1b" (mixed at 1:1). "PANR-G2" consists of "PANR-G2a," "PANR-G2b," and "PANR-G2c" (mixed at 3:1:1)

[b] Underlined nucleotides were synthesized with LNA

[c] Corresponding nucleotide position of Norwalk/68 virus (M87661)

[d] Corresponding nucleotide position of Camberwell virus (AF145896)

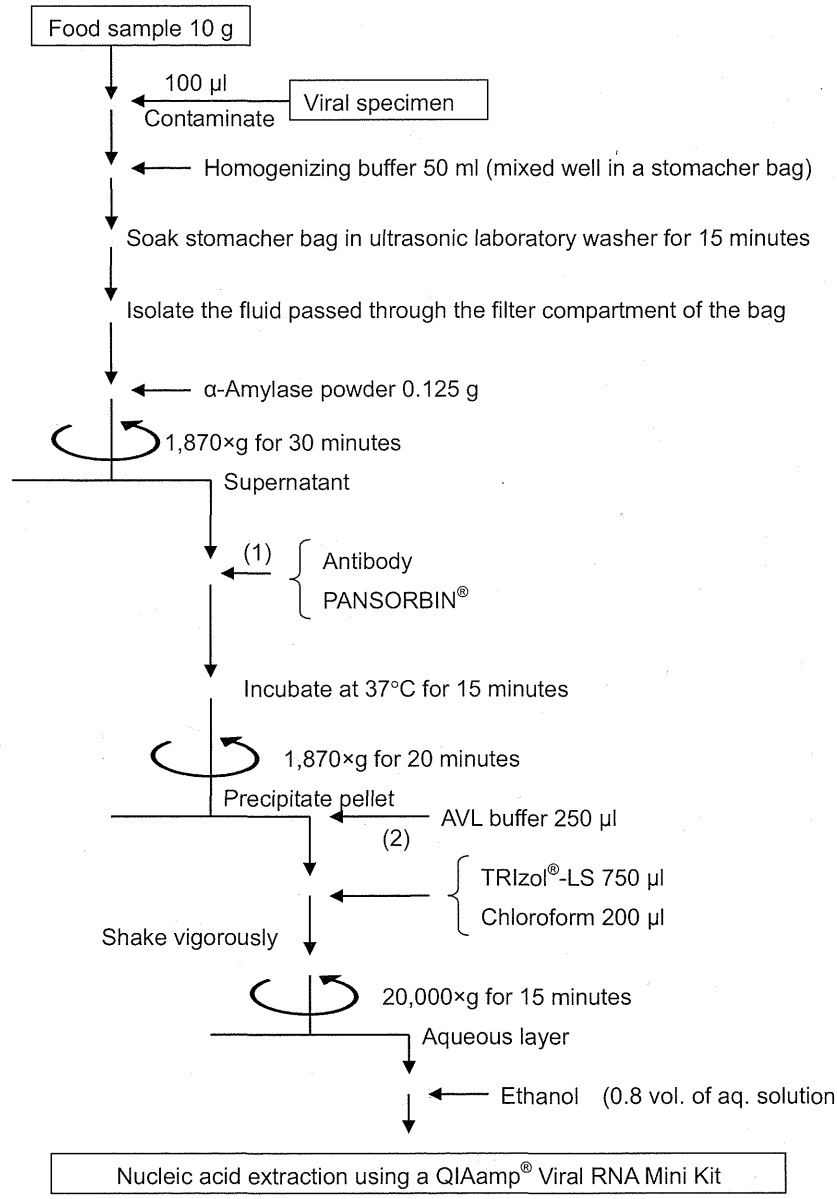

Fig. 1 General protocol of the PANtrap method. (*1*) In order to construct the virus-IgG-*S. aureus* (protein A) complex, 5 µl virus-specific rabbit antiserum and 0.3 ml PANSORBIN® or 0.15 ml gamma globulin and 1.0 ml PANSORBIN® were added. (*2*) The pellet was resuspended in AVL buffer

Reverse Transcription Reaction

Deoxyribonuclease I (DNase I) and α-amylase treatment was carried out to remove interfering substances before reverse transcription in a 15.5-μl reaction mixture including 9.25 μl nucleic acid solution, 4 μl 5 × RT buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$, 50 mM dithiothreitol, pH 8.3), 1 unit DNase I (RT grade, Nippon Gene Co. Ltd., Tokyo, Japan), 10 units ribonuclease inhibitor (Nippon Gene Co. Ltd.), and 7 units α-amylase "Ultrapure" (Nippon Gene Co. Ltd.) and incubated at 37 °C for 10 min, after which the enzymes were inactivated at 65 °C for 5 min. After snap cooling on ice, 25 pmol NoV-GI-specific reverse primer COG1R or NoV-GII-specific reverse primer COG2R, 20 nmole each deoxyribonucleotide triphosphate (dNTP), 2 μl RT-enhancer "RTmate" (Nippon Gene Co. Ltd.), and 100 units "ReverTra Ace®," reverse transcriptase (Toyobo Co. Ltd., Osaka, Japan), were added, and the reaction volume was increased to 20 μl with distilled water. After incubation at 42 °C for 30 min, synthesized cDNA was used for real-time PCR.

Real-Time PCR Amplification for Evaluation of Recovery rate

The 20-μl amplification reaction mixture was composed of "LightCycler® FastStart DNA Master^{PLUS} HybProbe" (Roche Diagnostics GmbH, Mannheim, Germany) containing 0.5 μM forward primer COG1F or COG2F, 0.5 μM reverse primer COG2R or COG2R, 0.1 μM dual-labeled probe RING1-TP(a) and RING1-TP(b) or RING2-TP, and 5 μl cDNA reaction mixture. Real-time PCR was performed using a LightCycler® 320S (Roche Diagnostics GmbH) with the following thermal cycling conditions: 10 min at 95 °C followed by 45 cycles of 10 s at 95 °C and 30 s at 56 °C.

Semi-nested RT-PCR Amplification for Evaluation of the Detection Limit

Each 10 g food sample of potato salad or stir-fried noodles was inoculated with different amounts of NoV-GI.4 or NoV-GII.4, from 3.5 × 10^4 to 3 copies/g of food. NoVs were recovered by the PANtrap method, followed by RNA extraction. cDNA was synthesized using the conditions described above except for using reverse transcription primer "PANR-G1" or "PANR-G2" (Table 2). In "PANR-G1," a locked nucleic acid (LNA) was used to improve the Tm value (Latorra et al. 2003), which was synthesized by Gene Design Inc. (Osaka, Japan). For first conventional RT-PCR, the 25-μl reaction mixture was composed of 1 × Taq buffer (part of the Taq DNA polymerase kit), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM forward primer COG1F or COG2F, 0.5 μM reverse primer G1SKR or G2SKR, 0.5 units Taq DNA polymerase (Greiner Bio-One Co. Ltd., Tokyo, Japan), and 5 μl cDNA. The amplification reaction was performed using a PC320 Thermal Cycler (Astec Co., Fukuoka, Japan) with following the program: 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C. Five microliters of the resulting PCR products, including NoV fragments of 381 bp (GI.4) or 387 bps (GII.4), was re-amplified by real-time PCR using the conditions described above.

Application of the PANtrap Method to Recover Various Foodborne Viruses

Approximately, 1 × 10^5 copies of each foodborne virus suspended in 50 mL homogenizing buffer without food samples were recovered by the PANtrap method using gamma globulin and rabbit antiserum against each virus. For the detection of AdV41, 5 μl of nucleic acid fraction extracted by the PANtrap method was amplified directly by real-time PCR (Logan et al. 2006). Reverse transcription was carried out for other RNA viruses using the conditions described above with a virus-specific reverse primer as follows: NoV-GI, COG1R; NoV-GII, COG2R; SaV, SaV1245R; and HAV, HAV-557. Real-time PCR was performed in accordance with the conditions reported previously for NoV (Kageyama et al. 2003), SaV (Oka et al. 2006), and HAV (Furuta et al. 2003).

Results

Development of the PANtrap Method and Evaluation of NoV Recovery Rates from Artificially Contaminated Food Samples

The principle of the PANtrap method is to recover viruses in the form of virus-IgG-*S. aureus* (PANSORBIN®) complex. We examined optimal conditions to make this immune complex, including pH and NaCl concentration of the homogenizing buffer, concentration of the antibodies and PANSORBIN®, and incubation time. We also examined the use of an ultrasonic laboratory washer to release virus particles from the surface of the food matrix and the use of α-amylase to digest carbohydrate as an interfering substance. As shown in Fig. 1, we designed a protocol to collect virus using centrifugation from 50 ml of turbid food emulsion using ordinary laboratory equipment and without a long incubation process. Using a commercial extraction kit, viral nucleic acid was obtained from a PANSORBIN® pellet in an amount sufficient for subsequent reverse transcription and real-time PCR.

Table 3 Recovery tests of NoV-GI.4 and NoV-GII.4 from spiked potato salad and stir-fried noodles

Food[a]	Added antibody	Recovered NoV RNA (copies)	Recovery rate (%)
Potato salad spiked with NoV-GI.4[a]	Anti-NoV-GI.4 rabbit serum	$2.32 \times 10^6 \pm 3.14 \times 10^5$	58.0 ± 7.9
	Gamma globulin	$2.22 \times 10^6 \pm 6.56 \times 10^5$	55.5 ± 16.4
	No antibody	$7.79 \times 10^3 \pm 1.29 \times 10^3$	0.22 ± 0.05
Stir-fried noodles spiked with NoV-GI.4[a]	Anti-NoV-GI.4 rabbit serum	$2.87 \times 10^6 \pm 5.09 \times 10^5$	71.8 ± 12.7
	Gamma globulin	$1.35 \times 10^6 \pm 1.68 \times 10^5$	33.8 ± 4.2
	No antibody	$9.64 \times 10^3 \pm 2.09 \times 10^3$	0.44 ± 0.11
Potato salad spiked with NoV-GII.4[b]	Anti-NoV-GII.4 rabbit serum	$6.40 \times 10^5 \pm 8.84 \times 10^4$	78.2 ± 10.8
	Gamma globulin	$1.99 \times 10^5 \pm 2.90 \times 10^4$	24.3 ± 3.5
	No antibody	$2.66 \times 10^3 \pm 8.23 \times 10^2$	0.33 ± 0.08
Stir-fried noodles spiked with NoV-GII.4[b]	Anti-NoV-GII.4 rabbit serum	$6.67 \times 10^5 \pm 8.38 \times 10^4$	81.5 ± 10.2
	Gamma globulin	$2.09 \times 10^5 \pm 1.88 \times 10^4$	25.6 ± 2.3
	No antibody	$9.42 \times 10^3 \pm 2.55 \times 10^3$	1.15 ± 0.25

[a] Each 10 g food sample was spiked with 4.00×10^6 copies of NoV-GI.4

[b] Each 10 g food sample was spiked with 8.18×10^5 copies of NoV-GII.4. Viral RNA was extracted by the PANtrap method with anti-NoV rabbit serum or gamma globulin. cDNA was synthesized from extracted RNA using primer COG1R or COG2R. Real-time PCR was carried out with a primer/probe set of COG1F/COG1R/RING1-TPa/RING1-TPb or COG2F/COG2R/RING2-TP, using a LightCycler® 320S. Recovered viral RNA and recovery rates are shown as a mean of three trials with standard deviation

Evaluation of Recovery Rates and Detection Limit of the PANtrap Method

The recovery rate using anti-NoV-GI.4 or NoV-GII.4 rabbit serum from potato salad, representing a carbohydrate-rich food, was 58.0 ± 7.9 and 78.2 ± 10.8 %, respectively. Similar results were obtained from the stir-fried noodles, representing a fat-rich food: 71.8 ± 12.7 and 81.5 ± 10.2 %, respectively (Table 3). We also attempted to recover NoVs using commercially available human gamma globulin instead of virus-specific antiserum for widespread use in many laboratories. The recovery rates of spiked NoV-GI.4 or NoV-GII.4 from potato salad were 55.5 ± 16.4 and 24.3 ± 3.5 %, respectively, and those from stir-fried noodles were 33.8 ± 4.2 and 25.6 ± 2.3 %, respectively (Table 3), with higher recovery rate than those without antibody (P value <0.01). Although NoV recovery rates with human gamma globulin were lower (<3.5-fold) than those with the virus-specific antisera (P values <0.05) except NoV-GI.4 in potato salad (P value = 0.85), human gamma globulin can be obtained easily without any restrictions. We estimated the detection limit of NoV-GI.4 and NoV-GII.4 in the PANtrap method using rabbit antiserum and gamma globulin by semi-nested RT-PCR. To confirm amplification of the DNA fragment derived from NoV, a second PCR was performed using the same procedure as used for real-time PCR. The sample was determined to be "positive" when the amplification curve was raised. As shown in Table 4, we could detect both viruses in contaminated foods including 10–35 copies of NoV/g of food.

Application of the PANtrap Method to Various Food Samples and Foodborne Viruses

We applied the established protocol of the PANtrap method to other food samples (Table 5) and foodborne viruses (Table 6). The recovery rates of NoV-GII.4 from various food samples using the PANtrap method with anti-NoV-GII.4 rabbit serum were higher than those of the conventional PEG concentration method. The relative ratio of recovery rate by the PANtrap method to that by the PEG concentration method was up to 1000-fold (Table 5). Then, we attempted to estimate the versatility of human gamma globulin for concentrating other NoV genotypes and SaV, HAV, and AdV41. The broad reactivity of commercially available human gamma globulin to concentrate various NoV genotypes and other foodborne viruses was demonstrated (Table 6). Thirteen NoV genotypes as well as four SaV genogroups, HAV, and AdV41 could be recovered in the ranges of 2.7–55.5 % for NoV, 8.0–35.3 % for SaV, 13.7 % for HAV, and 38.4 % for AdV41. Although the recovery rates using human gamma globulin were lower (<5-fold) than those of rabbit antisera against NoV VLPs, these data demonstrated that human gamma globulin could recover various foodborne viruses.

Discussion

The aim of this study was to develop a general method for detecting foodborne viruses, especially NoV, in contaminated foods. In this study, we showed that the PANtrap

Table 4 Detection of NoV-GI.4 and NoV-GII.4 in spiked food samples with different contamination levels using semi-nested RT-PCR

Food[a]	Antibody	Contamination level (copies/g)						
		3.5×10^4	3.5×10^3	3.5×10^2	1.0×10^2	3.5×10	1.0×10	3
Potato salad spiked with NoV-GI.4	Anti-NoV-GI.4 rabbit serum	3/3[b]	3/3	3/3	3/3	3/3	0/3[b]	0/3
	Gamma globulin	3/3	3/3	3/3	3/3	3/3	0/3	0/3
Stir-fried noodles spiked with NoV-GI.4	Anti-NoV-GI.4 rabbit serum	3/3	3/3	3/3	3/3	3/3	0/3	0/3
	Gamma globulin	3/3	3/3	3/3	3/3	3/3	0/3	0/3
Potato salad spiked with NoV-GII.4	Anti-NoV-GII.4 rabbit serum	3/3	3/3	3/3	3/3	3/3	1/3	0/3
	Gamma globulin	3/3	3/3	3/3	3/3	3/3	0/3	0/3
Stir-fried noodles spiked with NoV-GII.4	Anti-NoV-GII.4 rabbit serum	3/3	3/3	3/3	3/3	3/3	2/3	0/3
	Gamma globulin	3/3	3/3	3/3	3/3	3/3	0/3	0/3

[a] Food samples were spiked with different amounts of NoV-GI.4 and NoV-GII.4

[b] After the first PCR using primers COG1F and G1SKR or COG2F and G2SKR, products of 381 and 387 bps were re-amplified by real-time PCR. Detection was determined by amplification curve raising. The experiments were repeated at three times. The numerator indicates the number of raised amplification curves (positive reactions/performed reactions)

Table 5 Relative evaluation of the PANtrap method with the PEG concentration method for recovery rate of NoV-GII.4 from various foods

Food[a]	Recovery rate with the PANtrap method (%)[b]	Recovery rate with the PEG concentration method (%)	Relative ratio (PANtrap/PEG)
Burdock salad	19.7	0.0197	1000
Fried lotus root	69.0	0.0820	841
Chicken boiled with vegetables	18.3	0.451	40.6
Spaghetti Napolitana	35.2	2.11	16.7
Sliced raw tuna	85.9	25.4	3.38
Mushroom mixed with mashed tofu	43.7	21.1	2.07

[a] Food samples were spiked with NoV-GII.4 at about 10^6 copies/g

[b] NoVs were recovered with the PANtrap method using anti-rabbit serum against NoV-GII.4

method is effective for recovering virus from food emulsions. The PANtrap method consists of three processes, as shown in Fig. 1. Initially, the contaminated food was mixed with homogenizing buffer to prepare the food emulsion, followed by filtration and mild centrifugation to remove large debris. Carbohydrate is the major substance that interferes with the detection of viral genomes by PCR. Therefore, it is necessary to digest carbohydrate using α-amylase at an early stage for the successful preparation of a nucleic acid extract. In the second process, an antibody that specifically reacts with NoV and PANSORBIN® was added to the supernatant obtained from the food emulsion. In the incubation, formation of a virus-IgG-PANSORBIN® complex and digestion of carbohydrate proceeded at the same time. The viruses adsorbed on the surface of PANSORBIN® could be recovered by mild centrifugation. Although the supernatant from the food was turbid in most cases, there was no problem in discarding the remaining fluid by decanting. Finally, the PANSORBIN® pellet with adsorbed virus was resuspended in a small volume of AVL buffer, followed by extraction with phenol/chloroform. The aqueous layer after centrifugation was processed using a commercial extraction kit to obtain a nucleic acid solution. Viral nucleic acid was detected by PCR, as reported previously (Furuta et al. 2003; Kojima et al. 2002; Kageyama et al. 2003; Logan et al. 2006; Oka et al. 2006). For RNA viruses (i.e., NoV, SaV, and HAV), the use of a virus-specific primers instead of a random primer or oligo(dT) primer for reverse transcription is necessary, because the extracted solution contained a large amount of RNA derived from *S. aureus*. In this study, we used reverse primers in real-time PCR protocols (i.e., COG1R, COG2R, SaV1245R, and HAV-557) as virus-specific primers to evaluate the recovery rates, in order to minimize the factor of reverse transcription efficiency and RNA degradation. For semi-nested RT-PCR, we designed NoV-specific reverse transcription primers (Table 2) which locate downstream from the PCR reverse primers (G1SKR and G2SKR) as the reference for NoV strains (Kageyama et al. 2003). We took account of sequence analysis of the semi-nested PCR products in the future. It was difficult to obtain the DNA fragments suitable for sequence analysis, when reverse transcription was performed with G1SKR or G2SKR. Using PANR-G1 or PANR-G2, distinct

Table 6 Reactivity of antibodies against foodborne viruses based on recovery rate

Virus[a]	Added antibody and recovery (%)	
	Gamma globulin	Rabbit anti-serum
NoV		
GI.3	12.6	50.4
GI.4	12.7	14.3
GI.5	7.0	N.T.
GI.6	2.7	13.0
GII.2	45.1	93.4
GII.3	12.4	14.8
GII.4	45.7	77.3
GII.5	22.4	36.4
GII.6	11.9	18.4
GII.12	43.0	65.8
GII.13	17.1	N.T.
GII.14	55.5	N.T.
GII.22	9.4	N.T.
SaV		
GI.1	8.0	N.T.
GII.3	30.2	N.T.
GIV.1	16.9	N.T.
GV.1	35.3	N.T.
HAV	13.7	N.T.
AdV41	38.4	N.T.

[a] About 10^5 copies of each foodborne virus suspended in 50 ml homogenizing buffer were recovered by the PANtrap method

N.T. not tested

amplification fragments were observed by agarose gel electrophoresis (data not shown). DNase I and α-amylase treatment was also effective for reducing non-specific amplification and loss of RNA caused by adhesion to the remaining carbohydrate.

We evaluated the protocol of the PANtrap method from the perspectives of recovery rates and detection limits. We used potato salad and stir-fried noodles as representative carbohydrate-rich and fat-rich foods, respectively. NoV-GI.4 or NoV-GII.4 could be recovered using anti-NoV-GI.4, anti-NoV-GII.4 rabbit serum, or human gamma globulin. In this study, the optimum amounts of antibody were optimized to be 5 μl rabbit antiserum or 0.15 ml 5 % gamma globulin agent. Overabundance of antibody reduced the recovery rate in excess of the capacity of PANSORBIN® (data not shown). As shown in Table 3, the recovery rates of NoV-GI.4 and NoV-GII.4 from artificially contaminated food were reproducible and sufficient for practical use. On the other hand, most viral food poisoning outbreaks are probably caused by low-level contamination with a virus (Teunis et al. 2008). We estimated the detection limits of NoV-GI.4 and NoV-GII.4 in potato salad and stir-fried noodles. To enhance the sensitivity, we attempted semi-nested RT-PCR, in which real-time PCR was used as the second PCR instead of confirmation by Southern blotting hybridization. In both foods, we could detect 10–35 copies of NoV-GI.4 and NoV-GII.4/g of food using a virus-specific rabbit antiserum and gamma globulin (Table 4). There have been several investigations of foods contaminated with low levels of NoV other than fruits, vegetables, and shellfish. NoV was detected in hamburger using RT-PCR followed by Southern blotting hybridization after direct RNA extraction from a food emulsion (Sair et al. 2002). Likewise, NoV was detected using direct RNA extraction from an emulsion of penne salad followed by semi-nested RT-PCR (Baert et al. 2008). The sensitivities in these studies were similar to our results; however, the PANtrap method has the advantage of rapid and simple processing with standard diagnostic laboratory equipment. Remaining problems include which internal control should be used to monitor extraction efficiency. Although bacteriophage MS2 is used commonly for this purpose (Dreier et al. 2006; Mäde et al. 2013), it is necessary to use another virus for which an IgG is present in many people for the PANtrap method. As one of the candidate viruses, echoviruses seem to be promising because their IgG is contained in human gamma globulin, in addition there is little possibility of this being a cause of food poisoning.

The PEG concentration method has been used as a de-facto standard in many cases. Analysis of the PEG concentration method for NoV in a previous study showed recovery rates of 23 % from lettuce, 7 % from raspberries, and 24 % from ham (Sherer et al. 2010). We attempted to apply the established protocol of the PANtrap method to various food samples (Table 5). The recovery rates by the PEG concentration method from meals mixed with mayonnaise (burdock salad), tomato sauce (spaghetti napolitana), or oily dressing (fried lotus root and chicken boiled with vegetables) were not sufficient for viral examination (Table 5). Moreover, it was often difficult to process the very large amount of precipitate unrelated to virus particles during the incubation with PEG. Although direct extraction of NoV RNA from food emulsion has been attempted for composite meals (Stals et al. 2011b), time-consuming, labor-intensive processes were necessary. We obtained a steady recovery rate over 15 % using the PANtrap method independent of food type. Although the relative ratio reached 1000-fold in comparison with the recovery rate by the PEG concentration method (Table 5), it is necessary to carry out more-detailed examinations to evaluate the PANtrap method for many kinds of food.

At the beginning of this study, we developed the PANtrap method using NoV-specific rabbit antisera raised against representative genotypes from two major genogroups (i.e., GI.4 and GII.4) in accordance with our central premise. However, different antigenicities among

different genogroups and genotypes of NoVs have been reported (Hansman et al. 2006), and antisera panels reactive to diverse NoV genogroups and genotypes are available. Usually, the contaminating virus is not known in outbreaks. We attempted to use a commercially available gamma globulin instead of virus-specific antisera, because commercially available pooled human gamma globulin collected from numerous adult donors seems to contain antibodies against many viruses including foodborne viruses. We firstly confirmed the ability to recover spiked NoV-GI.4 and NoV-GII.4 from a food emulsion using gamma globulin (Table 3). We also evaluated the reactivity of the gamma globulin against different viruses on the basis of recovery rate from a diluted suspension (Table 6). We could recover 13 genotypes of NoV using gamma globulin as well as four genogroups of SaV, HAV, and AdV41. Although the recovery rate using NoV-specific antiserum was higher, gamma globulin has some advantages such as no limitation of antibody supply and might have been expected use for many foodborne viruses for such diagnostic purpose when the causative contaminating virus is unknown.

Recently, a method for recovery of NoV using porcine gastric mucin-conjugated magnetic beads has been reported (Tian et al. 2008; Morton et al. 2009). The principle is based on the interaction between NoV and histo-blood group antigens (Huang et al. 2005). Although the protocol seems to be applicable for turbid food emulsions, their availability for various NoV genotypes and other foodborne viruses is unknown.

In conclusion, we have developed a method for detecting various NoVs in contaminated foods including composite meals. The protocol described in this study has advantages compared with other methods. As shown in Fig. 1, the protocol does not involve time-consuming processes such as overnight incubation or expensive apparatus (e.g., ultracentrifuge). In addition, all reagents in the protocol are available commercially. Even if the food emulsion is turbid, virus can be recovered more effectively than widely used PEG concentration method independent of food type. Using commercially available gamma globulin, various genogroups and genotypes of NoVs can be recovered. We also demonstrated that human gamma globulin could concentrate other foodborne viruses (e.g., SaV, HAV, and AdV). Thus, the PANtrap method in combination with virus-specific sera, gamma globulin, or convalescent-phase serum will be a valuable asset for further studies in food safety.

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Detection and Genetic Characterization of Human Enteric Viruses in Oyster-Associated Gastroenteritis Outbreaks Between 2001 and 2012 in Osaka City, Japan

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Enteric viruses are an important cause of viral food-borne disease. Shellfish, especially oysters, are well recognized as a source of food-borne diseases, and oyster-associated gastroenteritis outbreaks have on occasion become international occurrences. In this study, 286 fecal specimens from 88 oyster-associated gastroenteritis outbreaks were examined for the presence of 10 human enteric viruses using antigenic or genetic detection methods in order to determine the prevalence of these infections. All virus-positive patients were over 18 years old. The most common enteric virus in outbreaks (96.6%) and fecal specimens (68.9%) was norovirus (NoV), indicating a high prevalence of NoV infection associated with the consumption of raw or under-cooked oysters. Five other enteric viruses, aichiviruses, astroviruses, sapoviruses, enteroviruses (EVs), and rotavirus A, were detected in 30.7% of outbreaks. EV strains were characterized into three rare genotypes, coxsackievirus (CV) A1, A19, and EV76. No reports of CVA19 or EV76 have been made since 1981 in the Infectious Agents Surveillance Report by the National Infectious Diseases Surveillance Center, Japan. Their detection suggested that rare types of EVs are circulating in human populations inconspicuously and one of their transmission modes could be the consumption of contaminated oysters. Rapid identification of pathogens is important for the development of means for control and prevention. The results of the present study will be useful to establish an efficient approach for the identification of viral pathogens in oyster-associated gastroenteritis in adults. ***J. Med. Virol. 86:2019–2025, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: aichivirus; astrovirus; enterovirus; norovirus; sapovirus

INTRODUCTION

Foods are well established as vehicles for the transmission of infectious pathogens. Food production and trade have become increasingly globalized, and pathogens may spread worldwide via contaminated foods.

In recent years, viruses have been recognized as an important cause of infectious food-borne diseases, and enteric viruses are considered as a major pathogen of viral food-borne gastroenteritis [Koopmans et al., 2002]. Foods are contaminated generally through sewage-contaminated water or by infected food handlers [Seymour and Appleton, 2001; Koopmans and Duizer, 2004]. Shellfish concentrate enteric viruses when grown in sewage-contaminated water because of their filter-feeding activities. Consumption of raw or under-cooked shellfish can lead to infection and illness caused by enteric viruses [Christensen et al., 1998; Le Guyader et al., 2008; Alfano-Sobsey et al., 2012]. In particular, oysters are well recognized to be a source of norovirus (NoV) infection [Iritani et al.,

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2000; Shieh et al., 2000]. Gastroenteritis outbreaks associated with NoV infection after oyster consumption have occurred in many countries [Iritani et al., 2000; Shieh et al., 2000; Prato et al., 2004; Webby et al., 2007; Le Guyader et al., 2008], and these have on occasion become large, international occurrences [Christensen et al., 1998; Le Guyader et al., 2006; Westrell et al., 2010]. The detection of other human enteric viruses in oysters has also been reported [Le Guyader et al., 2000, 2008; Ueki et al., 2010; Benabbes et al., 2013]. However, data to clarify the etiologic importance of enteric viruses, other than NoV, is insufficient in oyster-associated outbreaks because of the small number of reports [Le Guyader et al., 2008; Nakagawa-Okamoto et al., 2009]. Although previous studies [Iritani et al., 2000, 2002, 2010] have also shown the etiologic importance of NoV infection associated with the consumption of oysters, investigations of other enteric viruses were not performed.

In this study, fecal specimens from oyster-associated gastroenteritis outbreaks were examined for the presence of 10 human enteric viruses, NoV, rotavirus A (RVA), rotavirus C (RVC), enteric adenovirus (types 40 and 41) (EAdV), sapovirus (SaV), astrovirus (AstV), enterovirus (EV), aichivirus (AiV), human parechovirus (HPeV), and human bocavirus (HBoV), in order to determine the prevalence of these infections.

MATERIALS AND METHODS

Oyster-Associated Gastroenteritis Outbreaks

During the period from January 2001 to March 2012, 286 fecal specimens were collected from patients in 88 oyster-associated outbreaks with acute non-bacterial gastroenteritis in Osaka City, Japan. Seventy-seven of these 88 outbreaks have been reported in previous papers [Iritani et al., 2010, 2012] and 11 outbreaks were reported newly in this study. Most of these outbreaks occurred during December to March (92.0%). A gastroenteritis outbreak was defined as two or more patients with diarrhea and/or vomiting who are linked by place and time [Iritani et al., 2008]. In oyster-associated gastroenteritis outbreaks, oysters were the common food for all patients, and the infection was related to the consumption of oysters, as described previously [Iritani et al., 2010]. The age distribution of patients was as follows, $\geq$18 years (n = 278), 15 years (n = 1), and unknown (n = 7).

Viral RNA/DNA Extraction and Reverse Transcription

Viral RNA and DNA were extracted from 10% stool suspensions using a QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. As reported previously [Kaida et al., 2010], this kit is useful for the simultaneous extraction of RNA and DNA. Reverse transcription (RT) was performed using random hexamers, as described previously [Iritani et al., 2010].

Detection of Enteric Viruses

NoVs were detected using RT-PCR [Iritani et al., 2000] or real-time RT-PCR [Kageyama et al., 2003]. SaVs [Oka et al., 2006] and EVs [Kaida et al., under review] were detected using real-time RT-PCR, and HBoVs were detected using real-time PCR [Kantola et al., 2010]. AstVs [Sakon et al., 2000], AiVs [Yamashita et al., 2000], HPeVs [Ito et al., 2004], and RVC [Kuzuya et al., 1996] were detected using RT-PCR. RVA and EAdVs were detected using Rotaclone and Adenoclone-Type 40/41 enzyme immunoassay kits, respectively (Meridian Bioscience, Cincinnati, OH).

In 286 fecal specimens from 88 outbreaks, 164 fecal specimens from 45 outbreaks during January 2001 to April 2004 were tested for EVs, HBoVs, HPeVs, and RVC, and all specimens were tested for six other viruses.

Genetic Characterization of Detected Viruses

NoV, SaV, EV, and AiV strains detected by RT-PCR or real-time RT-PCR were characterized further. NoV genotyping was done in accordance with a previous report [Iritani et al., 2010]. For SaV genotyping, the partial capsid gene was amplified using primer pairs for the first PCR, SaV124F, SaV1F, SaV5F [Oka et al., 2006], R13, and R14 [Okada et al., 2006], and for the second PCR, SaV124F, SaV1F, SaV5F, and R2 [Okada et al., 2006]. SaV genotyping based on the capsid region was performed as described by Oka et al. [2012]. For EV genotyping, the partial VP1 gene was amplified using the primer pair AN88 and AN89 [Nix et al., 2006]. EV genotyping based on the VP1 region was performed using an EV genotyping tool (version 0.1; National Institute of Public Health and the Environment [RIVM], the Netherlands [http://www.rivm.nl/mpf/enterovirus/typingtool#]) [Kroneman et al., 2011]. For genetic characterization of AiV, direct sequencing of amplicons from RT-PCR-positive samples and homology searches using these sequences were performed with the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/).

The near-full-length genome sequence of an EV strain, OC01017025e, was determined. Briefly, cDNA was synthesized using specific primers, and four fragments were amplified: nucleotides (nts) 360–2,892, 2,718–4,453, 4,084–4,844, and 4,429–7,410 with poly A corresponding to the CVA19 strain, NIH-8663 (GenBank accession no. AF081308). Identities between strains were calculated using BioEdit (ver. 7.053) [Hall, 1999].

Phylogenetic Analysis

Nucleotide and amino acid (aa) sequence alignment was done using BioEdit (ver. 7.053) or Clustal X (ver. 2.1) [Thompson et al., 1997]. A phylogenetic tree with 1,000 bootstrap replications was constructed using the neighbor-joining method. The genetic distances were calculated according to the Kimura two-parameter method [Kimura, 1980].

Nucleotide Sequences and Accession Numbers

The nucleotide sequences used in the phylogenetic analysis were deposited in DDBJ with the following accession nos: AB828286 (OC01017015e strain), AB828287 (OC01017019e strain), AB828288 (OC01017022e strain), AB828289 (OC01017024e strain), AB828290 (OC01017025e strain), and AB828291–AB828318.

RESULTS

Human enteric viruses were detected in 211 fecal specimens from 86 outbreaks, which included 60 outbreaks (69.8%) with at least a single type of virus and 26 outbreaks (30.2%) with two or more different types of virus (Table I). All virus-positive patients were >18 years old. The most common enteric virus was NoV, which was present in 197 specimens from 85 outbreaks (Table II), and both NoV and another enteric virus were confirmed in 26 outbreaks. These NoV strains were classified into at least 28 genotypes, which comprised 12 GI (GI.1–7, WUG1, SzUG1, KU8GI, KU19aGI, T25GI), 15 GII (GII.1–9, GII.12–14, GII.16, GII.17, T53GII), and one GIV genotypes. Two or more genotypes were detected in 38 outbreaks (44.7%). The most common genotype was GII.4, which was detected in 21 outbreaks (24.7%), followed by GII.3 (18.8%), GII.5 (16.5%), and GII.2 (14.1%). The most common GI genotypes were GI.4 (10.6%) and GI.7 (10.6%).

Among the other enteric viruses, excluding NoVs, five viruses were detected; the most common virus was AiV, which was detected with 28 strains from 19 outbreaks, followed by AstV (8 strains from 5 outbreaks), SaV (7 strains from 7 outbreaks), EV (5 strains from 1 outbreak), and RVA (1 strain) (Table II). An outbreak with a single type of virus other than NoV was only observed in one outbreak (OC08005), which consisted of SaV. Fecal specimens with a single type of virus comprised 174 cases of NoV, 9 of AiV, 2 of SaV, 1 of AstV, and 1 of EV.

TABLE I. Detection of Human Enteric Viruses in Oyster-Associated Gastroenteritis Outbreaks

	Number of specimens (%)	Number of outbreaks (%)
Virus-positive	211	86
Single detection	180 (85.3)	60 (69.8)
Co-detection	31 (14.7)	26 (30.2)
Virus-negative	75	2
Total	286	88

TABLE II. Description of Human Enteric Viruses Detected in Oyster-Associated Gastroenteritis Outbreaks

Virus	Number of specimens (%), n = 286	Number of outbreaks (%), n = 88
NoV	197 (68.9)	85[a] (96.6)
AiV	28 (9.8)	19 (21.6)
AstV	8 (2.8)	5 (5.7)
SaV	7 (2.4)	7 (8.0)
RVA	1 (0.3)	1 (1.1)
EV	5 (3.0[b])	1 (2.2[b])

[a]Two to four viruses including NoV were co-detected in 26 outbreaks.
[b]Forty-five outbreaks and 164 specimens tested.

Of the 28 AiV strains, 1 strain was characterized into genotype B and the others were characterized into genotype A (Fig. 1). The AiV genotype A strains were closely related to each other (≥97.2% nt identity) on the 367-nt sequences between the C terminus of 3C and the N terminus of 3D. Of the seven SaV strains, six strains were characterized into four genotypes (GI.2, GI.5, GII.2, and GIV) and one strain was not characterized because of a lack of amplification of the capsid region by RT-PCR. Of the five EV strains, two strains (OC01017015e and OC01017019e) were characterized as coxsackievirus (CV) A1 (86.2–86.5% nt and 94.4% aa identities with Tompkins stain, GenBank accession no. AF081293), and one strain (OC01017024e) was characterized as EV76 (90.3% nt and 100% aa identities with EV76 strain, GenBank accession no. AY697458) on the basis of the partial nt sequences of the VP1 region (Fig. 2). The other two EV strains (OC01017022e and OC01017025e) were similar each other (99.1% nt identity) and were related to CVA19 (71.0–71.6% nt and 83.3–84.2% aa identities with NIH-8663 strain) based on the partial nt sequences of the VP1 region. However, two other genetic analysis tools showed that these two EV strains were unassigned types in human enterovirus C (HEV-C) by an EV genotyping tool of RIVM and had high identities (91.1–92.0% nt identity) with HEV-C, strain F-1575/N.Nov/RU/2008 (GenBank accession no. JN588564) using BLAST. For the clarification of the genotype, a near-full-length genome sequence of strain OC01017025e was determined, which was shown to have 82.0% nt identity with CVA19, strain NIH-8663. The identities of individual viral proteins between strains OC01017025e and NIH-8663 were determined for proteins VP4 (80.6% nt, 94.2% aa), VP2 (76.7% nt, 90.0% aa), VP3 (75.4% nt, 90.4% aa), VP1 (76.1% nt, 86.8% aa), 2A (82.7% nt, 97.3% aa), 2B (78.6% nt, 97.9% aa), 2C (83.9% nt, 99.6% aa), 3A (83.3% nt, 97.7% aa), 3B (89.3% nt, 100% aa), 3C (84.5% nt, 97.8% aa), and 3D (88.2% nt, 98.9% aa). The 5′ and 3′ untranslated regions were not