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Outbreaks of Norwalk-like virus-associated gastroenteritis traced to shellfish: coexistence of two genotypes in one specimen

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SUMMARY

We determined the nucleotide sequences of Norwalk-like viruses in 10 PCR products from stool or oyster specimens obtained from four outbreaks of gastroenteritis in which shellfish was suspected as the cause in Shizuoka prefecture in Japan between 1987–94. The sequences were determined from nucleotide positions 4561–4852 (292 bp) in the polymerase region. Two types of sequences were detected. One (genotype 1) had 87% sequence homology with the prototype Norwalk virus, and the other (genotype 2) had 59% sequence homology. The sequences from isolates belonging to the same genotype were almost the same regardless of the year of isolation. Because sequences of 2 genotypes were detected in 2 of the 4 outbreaks, nested PCR was performed with genotype-specific primers to detect the presence of 2 genotypes in the same specimen. In 5 of 10 specimens, PCR bands were detected with both genotype-specific primers, indicating the coexistence of 2 genotypes in 1 specimen. We also detected two genotypes of Norwalk-like virus in an oyster from a sample implicated in one of the outbreaks which may provide direct evidence of oysters as the cause of the gastroenteritis.

INTRODUCTION

Norwalk virus is a small round-structured virus (SRSV) discovered in faecal specimens from patients involved in an outbreak of gastroenteritis in Norwalk, Ohio, in 1968 [1, 2]. SRSVs are important aetiological agents of acute, non-bacterial gastroenteritis and have been implicated in numerous food and waterborne outbreaks [3]. However, because of the lack of adequate laboratory techniques for culturing SRSVs, the detection of Norwalk-like viruses in faecal specimens depend mainly on the recognition of viruses by electron microscopy (EM). Heterogeneity of the antigenic properties of SRSVs isolated from various regions of the world has been demonstrated by Western blotting [4], immune electron microscopy [5–7], and ELISA [8–10]. The prototype strains with different antigenic properties are called Norwalk virus [2], Hawaii agent [11], Snow Mountain agent [12], and Taunton agent [13].

Recent cloning and sequencing of the Norwalk virus genome [14, 15] has led to its classification in the family *Caliciviridae* [16, 17] and to the development of a reverse transcription (RT)–PCR method that detects Norwalk-like virus in faecal specimens [18, 19]. The molecular characterization of SRSVs has been further advanced by recent sequence information about an

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additional Norwalk-like virus strain, the Southampton agent, isolated in 1991 [20]. Considerable heterogeneity among the nucleotide sequences in the Norwalk-like viruses was found. On the other hand, the nucleotide sequences of the PCR products obtained from specimens isolated from the same outbreak were usually very similar [21]. Recently, Norwalk-like viruses were genotyped by using sets of primers based on the nucleotide sequence diversity in the polymerase gene of SRSVs. The results suggested that the SRSVs antigenically related to Norwalk virus, Hawaii agent, and Snow Mountain agent can be classified into two genotypes [22, 23].

We used the RT–PCR method to detect the Norwalk-like virus gene in faecal specimens from patients involved in outbreaks of acute gastroenteritis in Japan which have been suspected to be caused by shellfish and further applied this method to the detection of the Norwalk-like virus gene in oysters.

MATERIALS AND METHODS

Specimens for RT–PCR

Specimens of faeces and vomit from patients with acute non-bacterial gastroenteritis were collected during outbreaks in Shizuoka prefecture in Japan between 1987–94, and stored at −20 °C until used. Uneaten oysters were also stored at −20 °C.

Electron microscopy

A 10% suspension of specimens of faeces, vomit, or ground oyster midgut gland in phosphate-buffered saline (PBS) containing 0·05% NaN_3 was prepared and extracted with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane, and after low speed centrifugation at 4000 g for 30 min at 4 °C, the supernatant was centrifuged at 190000 g for 150 min at 4 °C through a 30% sucrose cushion in a SW50·1 rotor (Beckman). The pellets were suspended with a few drops of distilled water, stained with 2% phosphotungstic acid pH 7·2, placed on a 400-mesh Formvar-carbon coated grid, and examined with an electron microscope.

Extraction of viral RNA

Viral RNA for RT–PCR tests was extracted from specimens by the cetyltrimethylammonium bromide method described by Jiang and colleagues [19]. Briefly, a 10% stool, vomit, or ground oyster midgut gland suspension in PBS was prepared and extracted with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane. After clarification by low speed centrifugation, virus in the supernatant was concentrated by precipitation with 24% polyethylene glycol 6000 overnight at 4 °C and then the preparation was centrifuged at 13000 g for 20 min at 4 °C. The pellets were suspended and digested with proteinase K, and the viral RNA was purified by phenol-chloroform-isoamylalcohol extraction in the presence of cetyltrimetylammonium bromide followed by extraction with chloroform. Viral RNA was then precipitated with ethanol, and the pelleted viral RNA was suspended in a final volume of 20 μl of water and used directly for RT–PCR.

Oligonucleotide primers

Two sets of primers in the polymerase region reported by Wang and colleagues [23] were used. They were primer 35 (antisense, 4944, 5′ > CTTGTTGGTTTGAGGCCATAT < 3′) (NV-35) and primer 36 (sense, 4475, 5′ > ATAAAAGTTGGCATGAACA < 3′) (NV-36); primer 81 (antisense, 4872, 5′ > *ACA*ATCTCATCATCACCATA < 3′) (NV-81), and primer 82 (sense, 4543, 5′ > *TCATTTT*GATGCAGATTA < 3′) (NV-82). NV-81 and NV-82 had modified sequences compared to the original primers 81 and 82, and are shown in italics. Information about the primer sequences was obtained from Dr Y. Hayashi at the Tokyo Metropolitan Research Laboratory of Public Health. We prepared two additional sense primers 1AV and 2GN, corresponding to nucleotide positions 4702–4720, which had nucleotide sequences of 5′ > CAAGATCACCATTAGTGAA < 3′ and 5′ > TGTTATAAGGGTCAAAGAG < 3′, respectively.

RT–PCR

Purified viral RNA (5 μl) was reverse transcribed for 1 h at 42 °C with 10 units of avian myeloblastosis virus reverse transcriptase in 25 μl of RT buffer containing 10 mM-Tris-HCl (pH 8·3), 1·5 mM-$MgCl_2$, 50 mM-KCl, 1 mM 2-mercaptoethanol, 0·7 mM each dNTP, 0·5 μM-NV-35 primer, and 40 units of RNasin. After heating at 94 °C for 5 min, PCR (40 cycles) was

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Table 1. *Results of diagnostic tests for Norwalk-like virus from five outbreaks of gastroenteritis*

Case	Sample number	Date of outbreak	Suspected source of causative agent	EM*	PCR with primer pairs		Material for PCR
					NV36–NV35	NV82–NV81	
A	1	April 1987	Short-necked clam	ND	+	+	Stool
	2			ND	−	−	Stool
	3			ND	−	−	Stool
	4			ND	−	−	Stool
	5			ND	−	−	Stool
B	6	November 1988	Oyster	−	−	−	Stool
	7			−	−	−	Stool
	8			+	−	−	Stool
	9			−	−	−	Stool
	10			+	−	−	Stool
C	11	February 1989	Oyster	+	+	+	Stool
	12			−	+	+	Stool
D	13	March 1989	Oyster	+	−	+	Stool
	14			−	−	−	Stool
	15			+	+	+	Stool
	16			−	+	+	Stool
	17			+	−	+	Stool
	18			−	+	+	Stool
E	19	May 1994	Oyster	+	−	+	Stool
	20			−	−	+	Stool
	21			+	−	−	Stool
	22			−	−	+	Stool
	23			+	−	−	Vomit
	24			−	+	+	Oyster
	25			−	−	−	Oyster

* Stool was examined for SRSV. ND; not done.

performed by adding 70 μl of PCR buffer containing 10 mM-Tris-HCl (pH 8·3), 1·5 mM-$MgCl_2$, 50 mM-KCl, 1 mM 2-mercaptoethanol, 0·2 μM NV-36 primer, and 5 units of Taq DNA polymerase (TaKaRa Taq, Takara Shuzo Co. Shiga, Japan) to the RT reaction mixture. Nested PCR was performed with 0·5 μM each of primers NV-82, 1AV, or 2GN and NV-81. The first PCR product of double distilled water with all the necessary components for PCR was used as a negative control for the nested PCR. The PCR products were analysed by 1·5% agarose gel electrophoresis.

Cloning and sequencing of PCR products

We cloned the RT–PCR product into the pT7Blue T-vector (Novagen, Madison, WI) and then sequenced the cloned cDNA. The pT7Blue T-vector (50 ng ≡ 0·03 pmol) was ligated with 0·2 pmol (50 ng of a 300 bp fragment) of amplified product in a 10 μl volume containing 1 μl of × 10 ligase buffer (200 mM-Tris-HCl pH 7·6, 50 mM-$MgCl_2$), 5 mM-DTT, 0·5 mM-ATP, and 0·5 μl of T4 DNA ligase (2–3 Weiss units). Ligation was carried out at 16 °C overnight. Transformation was performed by using JM109 competent cells. Plasmid DNA was extracted by the alkaline lysate method, condensed by precipitation with polyethylene glycol 6000 and then sequenced with a DNA autosequencer (Shimadzu Co. Kyoto, Japan) according to manufacturer's protocol. Sequencing was done with a Taq cycle sequencing kit using M4 or RV-M dye-labelled primer (Takara Shuzo).

RESULTS

Diagnostic tests for Norwalk-like virus

Specimens from 5 outbreaks of acute gastroenteritis suspected of being caused by shellfish were examined for the presence of SRSVs or Norwalk-like virus gene by EM or RT–PCR. Positive PCR products were detected in faecal specimens from 4 of the 5 outbreaks examined with primer pairs NV36-NV35 (first PCR product) or NV82-NV81 (nested PCR product) (Table

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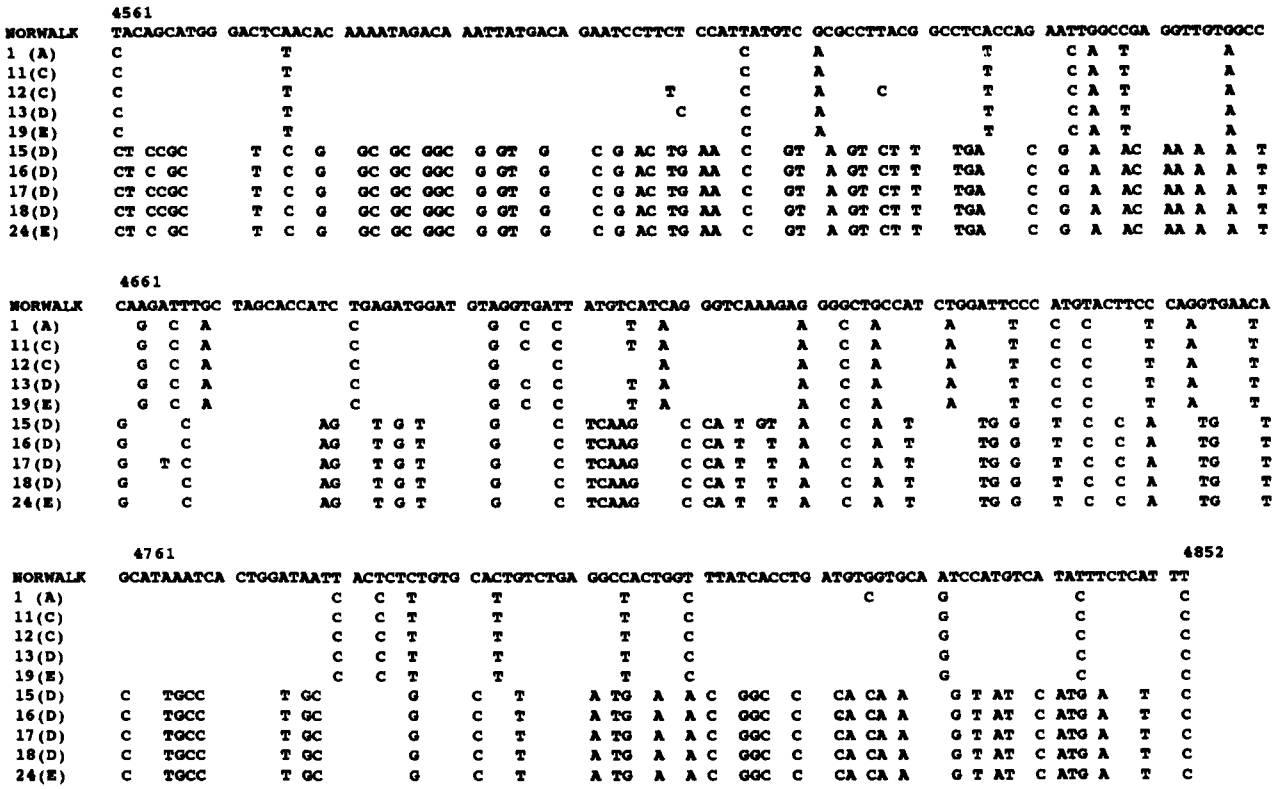

Fig. 1. Norwalk-like virus nucleotide sequences of the PCR products from 10 specimens in the RNA polymerase region (4561–4852). The case letter is shown in parentheses. Only nucleotides which were different from those of the Norwalk virus reference strain [14] are shown.

1). EM examination was not done for case A which occurred in 1987. In case B which occurred in 1988, SRSVs were found in two stool specimens by EM, but the Norwalk-like virus gene was not detected in any specimens by RT–PCR. This indicated the difficulty of finding universal primers for the SRSV viruses. In cases C–E, RT–PCR detected the presence of the Norwalk-like virus more efficiently than EM. The Norwalk-like virus gene was detected in an oyster by RT–PCR associated with case E.

Sequence analysis of the PCR products

The nucleotide sequences between 4561 and 4852 (292 bp) were determined with nested PCR products after they were incorporated into pT7Blue T-vector. The sequences of the PCR products were clearly separable into two groups (Fig. 1). The sequences obtained from specimen 1 in case A, 11 and 12 in case C, 13 in case D, and 19 in case E formed one group (group 1; genotype 1) and those of specimens 15–18 in case D, and 24 in case E formed another group (group 2; genotype 2). The homologies between the prototype Norwalk virus gene and the nucleotide sequences of group 1 and group 2 in the region were 87% and 59%, respectively. Thus, two different genotypes of Norwalk-like virus coexisted in the same outbreaks. The PCR products of specimens 20 and 22 were faint and their nucleotide sequences could not be determined. The nucleotide sequences belonging to the same group were almost the same (0–4 base differences), while those belonging to different groups were very different (121–125 base differences).

Detection of two genotypes of Norwalk-like virus gene in a PCR product from an oyster

The Norwalk-like virus gene was detected by RT–PCR in an oyster (specimen 24) which was suspected of causing an outbreak of acute gastroenteritis in 1994 (case E). The nucleotide sequence of the PCR product from the oyster belonged to genotype 2 and differed from genotype 1 sequence detected in the PCR product of specimen 19, which was obtained from a stool in the same outbreak. To determine whether the genotype 1 sequence coexisted in the PCR product

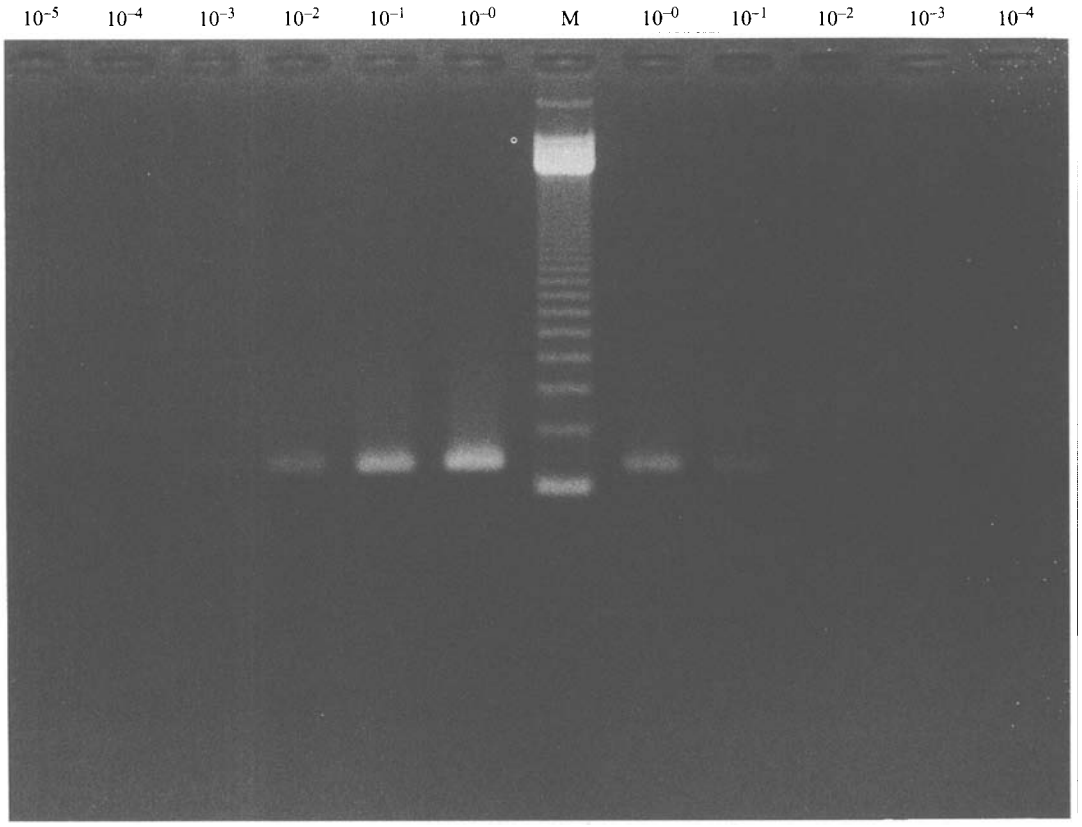

Fig. 2. Detection of two genotypes of Norwalk-like virus gene in a PCR product from an oyster. The first PCR product from an oyster [24–1] was serially diluted and examined by nested PCR as described in the text. Left, genotype 1-specific 2GN-NV81; right, genotype 2-specific 1AV-NV81. M indicates molecular size marker (123 bp ladder).

obtained from the oyster, a dilution experiment was done. First PCR product 24–1 was serially diluted 10-fold and nested PCR was performed with each dilution by using the primer pairs of genotype 1-specific 2GN or genotype 2-specific 1AV, and NV81 (Fig. 2). Unlike the nucleotide sequence analysis of 24 which showed the genotype 2 sequence, the PCR bands were detected until the 10^{-3} dilution by primer pair 2GN-NV81 and until the 10^{-1} dilution by primer pair 1AV-NV81. These results suggested that two genotypes coexisted in PCR product 24-1.

Identification of two genotypes of Norwalk-like virus gene in a PCR product from the oyster

To identify the sequences of two genotypes in the PCR product obtained from the oyster in case E, we cloned the PCR products in bacteria. An experiment to group the PCR products of 28 clones was performed with the primer pairs 2GN or 1AG, and NV81. Part of the results are shown in Figure 3. Thirteen DNAs produced PCR bands with the genotype 1-specific primer pair, 10 produced PCR bands with the genotype 2-specific primer pair, 5 produced a band 369–492 bp in size that was detected by both primer pairs. Detailed analysis of this band was not done yet. Sequencing was done with one DNA belonging to each of the two genotypes, and the genotype-specific sequences were confirmed (data not shown). The results showed that the PCR product obtained from the oyster in case E contained both genotype 1 and genotype 2 sequences.

Detection of two genotype-specific nucleotide sequences in the PCR specimens obtained from stools

Because the sequences of 2 genotypes were identified in 2 of the 4 outbreaks and in an oyster, each PCR specimen was examined for the presence of 2 genotypes by using genotype-specific primers. Nested PCR using genotype-specific primers and NV81 was performed with the first or nested PCR products of stool specimens. PCR products were detected in specimens 1, 11 and 13 by genotype 1-specific primer

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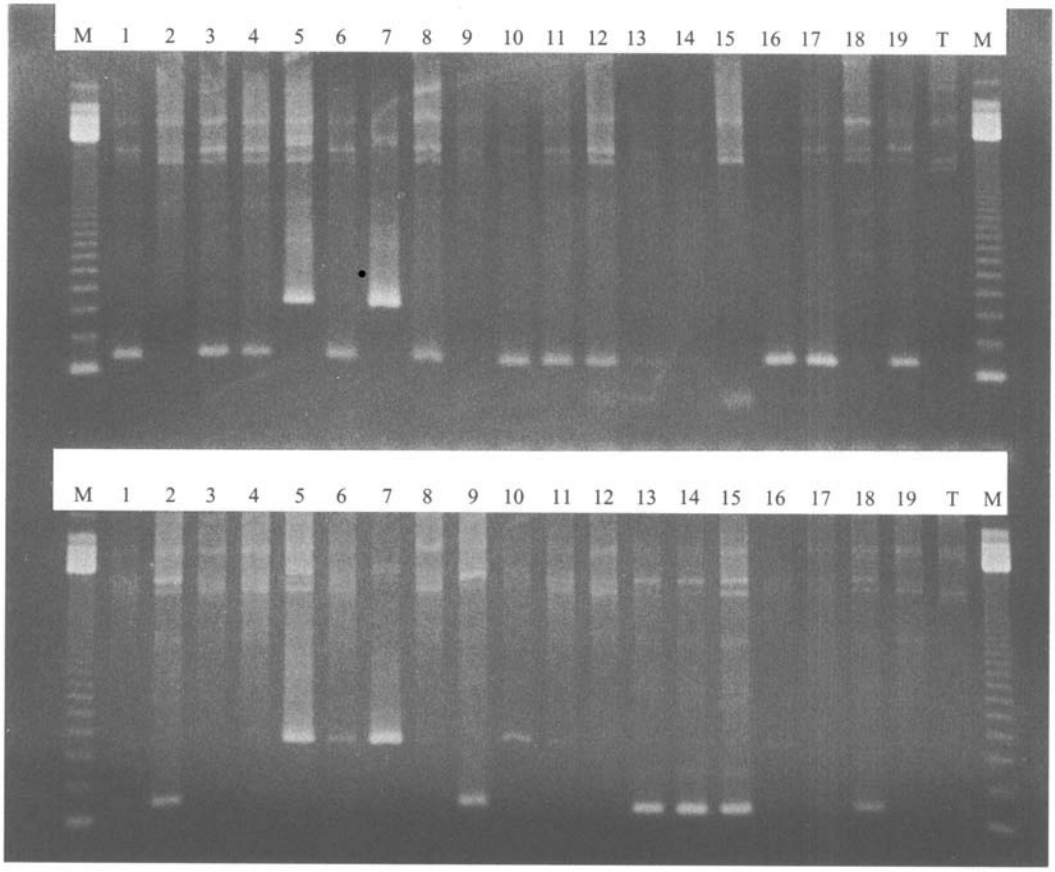

Fig. 3. Identification of two genotypes of Norwalk-like virus gene in the PCR product from the oyster. Nested PCR product from an oyster [24–2] was ligated with the pT7Blue T-vector. With the DNAs obtained from 28 clones, PCR was performed with the primer pairs 2GN-NV81 (top) or 1AG-NV81 (bottom). Results with 19 clones are shown. T indicates control pT7Blue T-vector DNA. M indicates molecular size marker (123 bp ladder).

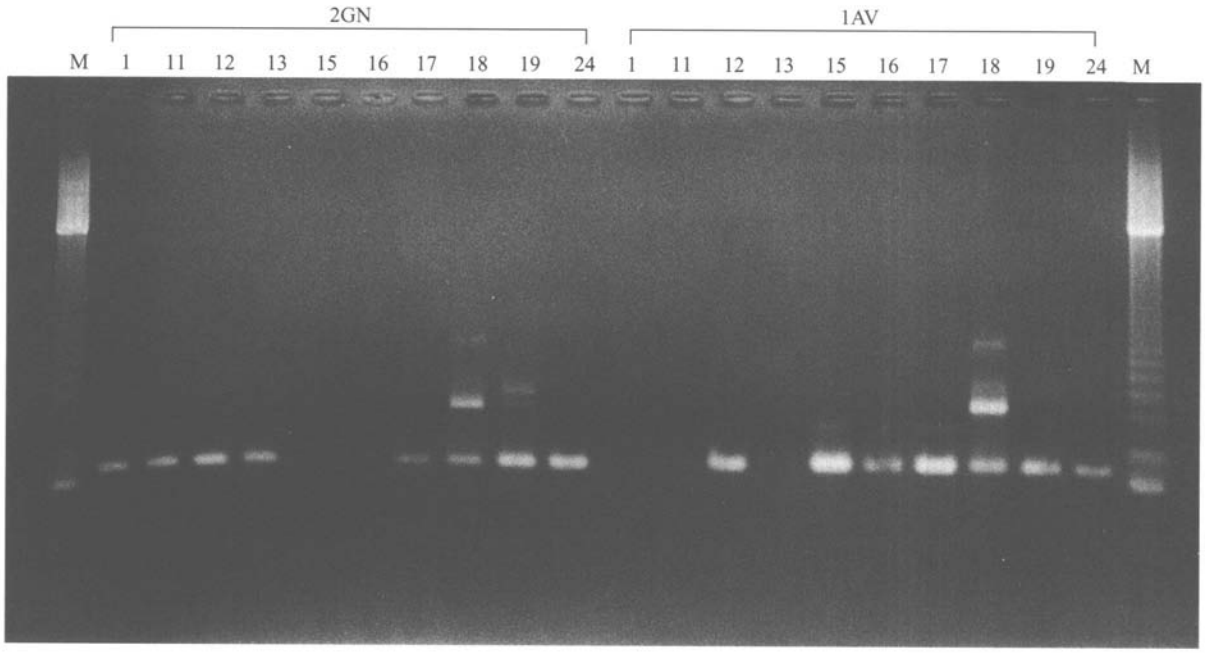

Fig. 4. Detection of two genotype-specific nucleotide sequences of Norwalk-like virus gene in the PCR specimens obtained from stools. Nested PCR was performed with the first or nested PCR products by using 2GN-NV81 (left) and 1AV-NV81 (right) primer pairs. M indicates molecular size marker (123 bp ladder).

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Table 2. *Comparison of nucleotide sequence homology**

	Homology (%)									
	Norwalk virus	Japan	Cruise 1992A	Nursing home 1986A†	Oysters 1978	Cruise 1990A	Cruise 1986B‡	Southampton	1 1987	15 1989
Norwalk virus	100	86	82	76	77	73	64	74	84	61
1 1987	84	97	78	76	77	77	65	74	100	59
15 1989	61	59	64	66	61	66	80	67	59	100

* Nucleotide sequences between 4693 and 4837 (145 bp) were compared. Data were taken from Moe et al. 1994 [21].
† Nucleotide sequences between 4693 and 4835 (143 bp) were compared.
‡ Nucleotide sequences between 4693 and 4833 (142 bp) were compared.

pairs, 15 and 16 by genotype 2-specific primer pairs, and 12, 17, 18, 19 and 24 by both primer pairs (Fig. 4).

DISCUSSION

Raw oysters have been implicated as one of the common sources of the aetiologic agent of SRSV-related gastroenteritis. Oysters filter seawater and may concentrate SRSVs in the midgut gland. A procedure for detecting the nucleic acid of enteric viruses in oysters by the PCR has been developed [24]. However, direct evidence to prove the presence of SRSV nucleic acid in oysters has not been reported. Here we present evidence that an oyster caused an outbreak of SRSV-associated gastroenteritis. This outbreak occurred in children who ate raw oysters which they picked up at a rocky beach. Oysters were suspected of causing the outbreak and were stored at −20 °C. The recent development of an RT–PCR method that detects the Norwalk-like virus gene in faecal specimens [19, 20] let us to examine our samples for the presence of SRSVs. Sequence analysis of the PCR products obtained from nine stool specimens of the patients and one oyster, using the primer pairs which amplified nucleotide positions between 4561 and 4852 in the polymerase region of the Norwalk virus gene, showed that two genotypes existed in the PCR products. The nucleotide sequences obtained from specimens 1, 11–13 and 19 were quite similar and formed group 1 (genotype 1), while those of specimens 15–18, and 24 were also very similar and formed group 2 (genotype 2). When nested PCR was performed with genotype-specific primers, specimens 1, 11, 13 reacted with the genotype 1-specific primer, and specimens 15 and 16 reacted with the genotype 2-specific primer. However, other specimens (12, 17–19 and 24) reacted with both genotype-specific primers, indicating the coexistence of two genotypes in one specimen. Specimen 24, one of the later specimens, was from an oyster. When the PCR product of specimen 24 was cloned into the pT7Blue T-vector, the sequences of two genotypes were detected. The sequences of the two genotypes were also detected in the stool specimen of the patient who ate the oyster (specimen 19). Therefore, the oyster was thought to be the source of the causative agent of acute gastroenteritis in case E. It is worthy noting that the nucleotide sequence in the polymerase region obtained in 1987 (specimen 1) and 1994 (specimen 19) in our study were almost the same. The homologies between the prototype Norwalk virus gene and the nucleotide sequences of group 1 and group 2 in the region between 4561 and 4852 (292 bp) were 87% and 59%, respectively. When these sequences in the region between 4693–4837 (145 bp) were compared with sequences reported by Moe and colleagues [21] (Table 2), the nucleotide sequences of group 1 were closest to that of Japanese virus SA-1283 isolated in 1984 (97% homology) [7], while those of group 2 were closest to that of Cruise 1986B (80% homology). The coincidence that the nucleotide sequences of group 1 of our study isolated between 1987 and 1994 were closest to that of the Japanese virus isolated in 1984, at 97% homology, suggested that the prevalent Norwalk-like virus differs with the country or place. The nucleotide sequences of group 2 had homology to OTH-25/89/J (a Japanese virus) at 99% in the region between 4693 and 4837 indicated that group 2 genes belonged to Snow Mountain agent genotype (genotype 2) [23]. The sequence homologies in the same region of the PCR products obtained from outbreaks associated with Norwalk-like virus infection to the prototype Norwalk virus reported by Moe and colleagues [21], ranged from 64–86%. However, the PCR products of the specimens isolated at the same time from three outbreaks (Cruise 1992; Nursing Home 1986; Cruise

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1990) had identical sequences. On the other hand, two separate strains (A and B) from Cruise 1986 shared only 66% sequence identity, results similar to those we obtained in this study. They suggested that the reason for the two different sequences was either that more than one strain was involved in the outbreak or that one of the patients had a sporadic case of illness caused by another strain. In our case it was clear that in some specimens from the patients and the oyster, two different strains of SRSV coexisted.

Molecular approach using RT–PCR method also can be used to the epidemiological investigation of common source of SRSVs from different outbreaks [25].

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