Multiple Viral Infections and Genomic Divergence among Noroviruses during an Outbreak of Acute Gastroenteritis

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An epidemic outbreak of both norovirus (NV) and astrovirus (ASV) occurred on a research ship surveying Tokyo Bay, causing acute gastroenteritis in 26 of its 37 crew members. The presence of viral pathogens in fecal specimens was analyzed, and noroviruses were identified by reverse transcription-PCR in 18 (48.6%) of these specimens. In addition, astroviruses were identified in 14 (37.8%) of the fecal samples from the affected crew members, and multiple viral infections of both NV and ASV were observed in 6 cases. The genogrouping of the NV-positive samples was then examined by dot blot hybridization, and it was determined that all of the isolates were from genogroup II (GII). No bacterial pathogens were subsequently isolated from fecal specimens. Furthermore, a variety of NV strains were identified by sequencing and single-stranded conformational polymorphism (SSCP) analyses of PCR products from the fecal samples. One recombinant NV isolate, Minato/14, was identified as a recombinant NV strain of GII/6 and GII/1. The other NV isolates from this outbreak were classified into three NV genotypes (GII/1 [Minato/10], GII/4 [Minato/33], and GII/5 [Minato/6]). Furthermore, ASVs in positive samples were determined to belong to serotypes 1 and 2 by sequencing analysis. Our findings thus indicate that coinfections with NV and ASV, including a number of NV genotypes, persisted during an outbreak of gastroenteritis in a closed environment.

Several viral strains have been associated with the development of acute gastroenteritis in humans. Norovirus (NV), rotavirus (RV), and astrovirus (ASV) are the most common causes of sporadic cases of this disease and have been responsible for a number of outbreaks of nonbacterial gastroenteritis in a variety of settings, including hospitals, day care centers, nursing homes, and schools (1, 13, 17, 30). The detection of NV infections has previously been limited, because NV cannot be propagated in culture, but detection methods based on molecular biology have recently been developed and are now in use during epidemiological studies (19, 23, 28). NV can be divided into two distinct genogroups, genogroup I (GI) and genogroup II (GII), each of which can be divided into several clusters. A recent study indicated that NV GI and GII strains consist of at least 14 and 17 genotypes, respectively (23). Representatives of the NV GI genotypes include GI/1 (Norwalk/68/US), GI/2 (Southampton/91/UK), GI/3 (Desert Shield/90/SA), and GI/4 (Valetta/95/MA). The GII genotypes of NV include GII/1 (Hawaii/71/US), GII/2 (Melksham/94/UK), GII/3 (Toronto/TV24/93/UK), GII/4 (Bristol/93/UK), GII/5 (Hillingdon/90/UK), and GII/6 (Seacroft/90/UK). It has also been shown in previous reports that NV genes consist of diverse sequences, and multiple genotypes have now been identified worldwide (4, 9, 11, 14).

The acquisition of DNA sequences is therefore now a fundamental component of most phylogenetic and molecular ep-

idemiologic studies. Single-stranded conformational polymorphism (SSCP) analysis is one of the sensitive techniques that is employed for assaying such sequence diversity and can reduce the amount of direct sequencing that is necessary for these evaluations. With regard to the origins of newly identified NV genotypes, Jiang et al. have previously proposed that RNA recombination occurs at the open reading frame 1 (ORF1)-ORF2 junction of the virus (21). Additionally, Dingle et al. have described key mutations in the NV genome that contribute to genetic diversity (8).

It has been proposed that the association of ASV with gastroenteritis has been underestimated because infection with this virus generally results in a milder and far less contagious form of this disease (7, 16, 27, 29). However, several examples of viral gastroenteritis in children below the age of 5 years have been observed as mixed infections of RV with NV, RV with ASV, NV with ASV, and RV with other viral pathogens (5, 27, 34). Moreover, because of the availability of molecular diagnostics in recent years, the coexistence of multiple viral pathogens has now been confirmed in a number of cases of gastroenteritis, and a variety of NV genotypes have also now been detected from epidemic outbreaks of this disease (23, 36, 38).

In our current report, we describe an occurrence of coinfection of NV and ASV in an acute gastroenteritis outbreak on a research ship. Genogrouping of the NV-positive samples was performed by a dot blot hybridization method, and all of the isolates were found to be of the GII genogroup. One recombinant NV isolate, Minato/14, was identified as a recombinant NV strain of GII/6 and GII/1. In addition, nine NV isolates were classified into three NV genotypes (GII/1 [Minato/10],

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TABLE 1. Primary and nested primer sets used for detection of norovirus and astrovirus by PCR

Virus	Primer set	Name	Polarity ^a	Sequence ^b	Positions ^c
Norovirus	Primary	Yuri22F	S	ATG AAT GAG GAT GGA CCC AT	4232–4251
		G2SKR	AS	CCR CCN GCA TRH CCR TTR TAC AT	5366–5389
	Nested	LV82	S	ACT ATG ATG CTG ACT ACT C	4284–4302
		SN82	S	GYC AYT WTG ATG CAG ATT A	4282–4299
		G2F1R	AS	AGA TTG CGA TCG CCC TCC	5049–5067
	Nested	G2F1	S	GTG GGA GGG CGA TCG CAA TCT	5047–5067
		G2R1	AS	TGC ATA ACC ATT GTA CAT TCT	5364–5384
Astrovirus	Primary	AST5	S	TCA CCA ATG GAA GGC GGC TT	3508–3527
		AST-END	AS	TCC TAC TCG GCG TGG CCG C	6675–6693
	Nested	Mon244	S	GGT GTC ACA GGA CCA AAA CC	4544–4563
		Mon245	AS	TTA GTG AGC CAC CAG CCA TC	4937–4955

^a Abbreviations: S, sense; AS, antisense.

^b Mixed base codes conform to standard IUB/IUPAC nomenclature: R (A or G), Y (C or T), W (A or T), and N (A, C, G, or T).

^c The primer target location is given for a representative strain for each virus as follows: norovirus, Lordsdale/93/UK (X86557); astrovirus, Oxford/type 1 (L23513).

GII/4 [Minato/33], and GII/5 [Minato/3, 4, 6, 8, 9, 13, and 31]) in this outbreak. The contribution of these NV genotypes to this localized epidemic may provide valuable information concerning both the mode of NV transmission and the mechanisms underlying the development of genetic diversity in these viruses.

MATERIALS AND METHODS

Case study of an outbreak of acute gastroenteritis. An outbreak of acute gastroenteritis occurred on a research ship that departed on 7 October 1999 with a crew of 37 on a planned 21-day voyage to conduct a survey of Tokyo Bay. Twenty-six of these crew members subsequently developed acute gastroenteritis, which began at approximately 0900 on October 22 and continued for another 4 days. Prior to the outbreak, two of the crew were recorded as showing symptoms of this disease on 13 and 15 October, respectively. We designate these as preceding cases below. The research ship returned early to Tokyo Bay port on 28 October 1999, and we subsequently began an investigation to determine the cause of this outbreak and began to collect fecal specimens from each of the crew. We also collected from the ship menu cards (issued from 12 to 18 October), samples of foodstuffs which had not been used on board after 26 October, and water samples (tap water, cooler, sink). We collated and summarized our clinical findings based on the ship's logbook and on the information recorded on a questionnaire given to the crew by a medical doctor.

Bacteriologic examinations. Bacterial cultures from fecal, food, and environmental water samples were examined for pathogenic food-borne bacteria, including *Salmonella* spp., *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus*. The processing methods for these samples and the subsequent bacteriologic examinations were adopted from the protocols of the *Standard Methods of Analysis in Food Safety Regulation* (19a).

Virological examination of fecal samples: RNA extraction, RT-PCR, and dot blot hybridization. Only fecal specimens were subjected to virological examination. RNA extraction from these specimens and subsequent reverse transcription (RT) and nested PCR were carried out as described previously (35). Briefly, a 10% solution of fecal matter in 10 ml of phosphate-buffered saline was subjected to low-speed centrifugation at $1,500 \times g$ for 15 min at 4°C. The supernatant was then recentrifuged at $10,000 \times g$ for 30 min at 4°C, and the resultant supernatant was concentrated by pelleting at $100,000 \times g$ for 180 min at 4°C. Total RNA was extracted from 150 µl of the concentrated fecal suspension in distilled water using the cetyltrimethylammonium bromide (CTAB) extraction method (20). The primers used for nested PCR are listed in Table 1. To correct for possible false positives caused by contamination, a negative (no-template) control was included in each PCR amplification. The sizes of the amplified DNA fragments were confirmed by electrophoresis on 2.0% agarose gels. Four microliters of the resulting RT-PCR products was then transferred to Hybond N+ membranes

(Amersham Pharmacia Biotech, Tokyo, Japan) and cross-linked by UV radiation. GI and GII probes were prepared as described previously (35) and labeled using the random primer method with fluorescein-conjugated nucleotides. Following hybridization, the blots were visualized with the ECL system (Amersham Pharmacia Biotech).

Silver-stained SSCP analysis. For SSCP analysis, 4-µl aliquots of each PCR product were mixed with 4 µl of loading buffer containing 95% formamide, 0.025% (wt/vol) bromophenol blue, and 0.025% (wt/vol) xylene cyanol. This mixture was denatured at 95°C for 5 min and then cooled on ice, and 6-µl aliquots were loaded onto a precast GeneGel SSCP gel (Amersham Pharmacia Biotech). Electrophoresis was performed using the temperature-controlled GenePhor electrophoresis system (Amersham Pharmacia Biotech) under the conditions recommended by the manufacturer. The gels were stained using the PhastGel DNA silver staining kit (Amersham Pharmacia Biotech).

Sequencing and phylogenetic analysis. The RT-PCR-positive samples were further characterized by sequencing and phylogenetic analysis using the ABI Prism BigDye Terminator cycle-sequencing ready reaction kit (Applied Biosystems, Tokyo, Japan) on an ABI 310 automated sequencer (Applied Biosystems, Tokyo, Japan). The results were then aligned to sequences obtained from the GenBank database. Phylogenetic analyses were performed using GENETYX (ver. 11.0; Genetix, Tokyo, Japan), and a phylogenetic tree was constructed by the unweighted-pair group method using average linkages (UPGMA). The BLAST search program was used to find the sequences in GenBank most closely related to the sequence obtained.

Nucleotide sequence accession numbers. Sequence data from this study have been deposited in the EMBL/GenBank data libraries. The genome sequences of four Minato strains isolated in this study were deposited in the DNA Data Bank of Japan (DDBJ). The accession numbers are AB233471 to AB233474 (Minato/6, Minato/10, Minato/33, and Minato14, respectively). The GenBank accession numbers for reference strains are U07611 (Hawaii/71/US), X81879 (Melksham/89/UK), U02030 (Toronto/TV24/93/CA), X76716 (Bristol/93/UK), AB112315 (SaitamaT49/01/JP), AB039777 (SaitamaU4/00/JP), AB067543 (Saitama U25/98/JP), AY038599 (VA97207/97/US), AY237415 (Mc37/JP), AB009876 (Yuri/52/99/JP), and AY772730 (Neustrelitz/260/00/GE).

RESULTS

Epidemiological investigations. (i) The outbreak. The incidence rate during the outbreak under study, which began on 22 October, was estimated to be 70% (26/37 crew members). The disease characteristics among the 26 affected crew members were determined both from clinical findings and by the use of a questionnaire given to the crew by a medical doctor. As shown in Table 2, these symptoms were categorized as non-bloody diarrhea (85%), nausea (58%), vomiting (35%), ab-

TABLE 2. Clinical features and results of laboratory testing for norovirus and astrovirus

Crew member no. ^a	Time of onset (h)	Result of RT-PCR		No. of times		Symptom		
		Norovirus	Astrovirus	Diarrhea	Vomiting	Nausea	Chills	Abdominal pain
3	0	+	+	10	2	+	+	+
4	3	+	—	15	0	+	—	+
5	10	+	+	6	0	—	+	+
6	12	+	+	5	0	—	—	—
7	13	+	—	8	3	+	+	+
8	14.5	+	—	2	2	+	—	—
9	21	+	+	3	0	+	—	+
10	21	+	+	30	1	+	+	—
11	22	—	+	2	0	+	+	—
12	23	+	—	3	0	—	+	+
13	24	+	—	1	0	—	—	—
14	26.5	+	—	0	1	—	+	—
15	27	+	—	2	0	—	+	—
16	27	—	—	0	0	+	+	—
17	27	+	—	0	4	+	+	+
18	28	—	—	10	0	—	+	—
19	30.7	—	—	30	0	—	+	+
20	31	—	—	10	0	+	—	—
21	31	—	+	0	1	—	+	—
22	35	—	+	3	3	—	—	+
23	38	—	+	3	4	+	—	—
24	49	+	—	2	0	+	—	—
25	52	+	—	20	0	+	—	—
26	54	—	—	5	0	+	+	—
27	54	—	+	7	0	+	—	—
28	71	—	—	2	0	—	+	—
1	Preceding (–213)	—	—	(10)	(0)	(+)	(+)	(–)
2	Preceding (–195)	—	—	(5)	(2)	(+)	(–)	(+)
29	Asymptomatic	—	+	0	0	—	—	—
30	Asymptomatic	—	+	0	0	—	—	—
31	Asymptomatic	+	—	0	0	—	—	—
32	Asymptomatic	+	—	0	0	—	—	—
33	Asymptomatic	+	+	0	0	—	—	—
34	Asymptomatic	—	+	0	0	—	—	—
35	Asymptomatic	—	—	0	0	—	—	—
36	Asymptomatic	—	—	0	0	—	—	—
37	Asymptomatic	—	—	0	0	—	—	—

^a The symptomatic crew members were divided into two clusters. Cluster 1 included crew member no. 3 to 13, and cluster 2 included crew member no. 14 to 28.

dominal pain (31%), headache (27%), fever (38%), and chills (58%). All of the affected individuals recovered within several days. An epidemic curve was then constructed according to the time of the onset of symptoms during the outbreak (Fig. 1); it

FIG. 1. Number of cases of acute gastroenteritis among 37 crew members according to the designated date of onset. The number of cases is given above each bar.

demonstrates a single peak. For the purpose of this study, we designated the onset of the first case (0900 on 22 October) as the zero time point. The ages of the affected crew members ranged from 24 to 56 years, with an average of 42.5 years.

(ii) **Food consumption and preceding cases.** According to the menu cards supplied on board the research ship, each of the crew members consumed oysters and littleneck clams that had been prepared on 12, 13, and 15 October 1999. Two of these individuals became ill on 13 and 15 October (preceding cases), respectively, with symptoms of nonbloody diarrhea (2/2), nausea (2/2), vomiting (1/2), abdominal pain (1/2), headache (1/2), and chills (1/2). However, although these were the same symptoms that were characterized later for victims of the outbreak, it is still uncertain whether there is any association between the subsequent gastroenteritis cases and these two preceding cases.

We collected from the ship menu cards (issued from 12 to 18 October), samples of foodstuffs that had not been used on board after 26 October, and water samples (tap water, cooler,

FIG. 2. Phylogenetic relationships between the outbreak strains and reference strains of NV based on analyses of 3'-ORF1 (A) and 5'-ORF2 (B). Nucleotide sequences were aligned using GENETYX, ver. 11.0, and a phylogenetic tree was constructed by UPGMA. Branch lengths are proportional to the calculated genetic distances (scale shown).

sink). According to the menu card, a littleneck clam soup was prepared for supper on 12 and 15 October, and a side dish of oysters had been cooked from frozen stocks for the evening meal on 14 October. The possible involvement of the seafood consumed on board the ship as a source of the infection is also uncertain, since we have not been able to ascertain the exact extent to which these foods were consumed by the crew members. The food ingredients and environmental water samples were not subjected to viral testing due to insufficient quantity but were examined only by bacteriological tests.

Laboratory investigations. (i) Examination of fecal specimens from the crew members. The fecal specimens from each of the crew members were found to be negative for bacterial pathogens. NV strains were detected, however, by RT-PCR in 15 of the 26 specimens from symptomatic crew members (58%) and in 3 of the 11 specimens from asymptomatic individuals (27%). In total, 18 of the 37 specimens (48.6%) analyzed were positive for NV. ASV strains were detected in 10 symptomatic (38.5%) and 4 asymptomatic (36%) cases. A total of 14 individuals (37.8%) were therefore positive for ASV. Moreover, both NV and ASV were detected in 5 (19%) of the symptomatic crew members and in 1 (9%) asymptomatic individual.

The results of our RT-PCR analyses, the onset times, the number of incidences of diarrhea and vomiting, and some symptoms are shown in Table 2. Five individuals were identified as having coinfections of NV and ASV. These five patients had contracted symptoms within 24 h of disease onset and are designated as cluster 1. Either NV or ASV, but not both, were identified in the specimens from individuals who became ill

after this initial 24-h period, and we refer to these cases as cluster 2. The positive percentages of cluster 1 cases for NV and ASV were 91% and 54.5%, respectively. In contrast, these figures for cluster 2 were 33% and 27%, respectively. Moreover, our finding that coinfections of NV and ASV are assigned only to cluster 1 revealed that the infection source contained both NV and ASV. Hence it is possible that the individuals in cluster 1 were exposed to higher levels of both viruses. It is noteworthy also that a specimen from 1 case among the 11 asymptomatic crew members also showed multiple NV and ASV infections.

(ii) Examination of foodstuffs, water specimens, and the preceding cases. Neither NV nor ASV was detected in the fecal specimens from the crew members with the two preceding cases. We suspect, however, that the sampling of the preceding cases occurred too late to positively detect viral pathogens. Two food samples (chili pepper in soy sauce and mysids in soy sauce) contained *S. aureus*, and one water sample (tap water) contained *E. coli*, although the fecal specimens from each of the crew members were found to be negative for bacterial pathogens. The source of the infection cannot therefore be specified.

(iii) Sequence analysis of the 3'-ORF1 and 5'-ORF2 regions of noroviruses isolated from affected crew members. Possible variations in the genomes of the NV strains that were isolated from the affected crew members were investigated by sequence analysis. As shown in Fig. 2A, the nucleotide sequences of the 3'-ORF1 (3' terminus of ORF1) regions were determined for eight NV isolates and subsequently aligned with those of 11 reference strains. The sequences from six isolates (Minato/3, Mi-

TABLE 3. Comparison of nucleotide and amino acid sequence identities of the 3'-ORF1 and 5'-ORF2 regions

GII/genotype	Strain (accession no.)	% Nucleotide (amino acid) sequence identity ^a							
		Minato/6 (AB233471)		Minato/10 (AB233472)		Minato/14 (AB233474)		Minato/33 (AB233473)	
		3'-ORF1	5'-ORF2	3'-ORF1	5'-ORF2	3'-ORF1	5'-ORF2	3'-ORF1	5'-ORF2
GII/1	Hawaii (U07611)	72 (84)	79 (89)	77 (92)	ND	69 (79)	84 (97)	ND	75 (78)
GII/2	Melksham (X81879)	72 (85)	76 (86)	78 (92)	ND	69 (80)	77 (89)	ND	76 (79)
GII/3	Toronto (U02030)	71 (83)	79 (86)	76 (91)	ND	70 (80)	76 (88)	ND	75 (77)
GII/4	Bristol (X76716)	73 (84)	75 (76)	78 (91)	ND	70 (79)	72 (79)	ND	86 (91)
GII/5	SaitamaT49 (AB112315)	89 (98)	79 (83)	74 (85)	ND	71 (78)	78 (89)	ND	75 (78)
GII/6	SaitamaU4 (AB039777)	72 (78)	76 (82)	69 (78)	ND	99 (99)	75 (86)	ND	75 (79)
GII/8	SaitamaU25 (AB067543)	70 (76)	78 (83)	73 (81)	ND	78 (93)	74 (86)	ND	76 (76)
GII/9	VA97207 (AY038599)	71 (78)	73 (81)	69 (79)	ND	93 (98)	74 (84)	ND	77 (77)
GII/10	Mc37 (AY237415)	72 (83)	81 (87)	77 (91)	ND	69 (79)	79 (90)	ND	75 (79)
≡ GII/5	Yuri52 (AB009876)	98 (100)	ND	69 (78)	ND	71 (78)	ND	ND	ND
≡ GII/1	Neustrelitz (AY772730)	73 (84)	80 (87)	96 (98)	ND	70 (80)	95 (100)	ND	75 (78)

^a ND, not done.

nato/4, Minato/6, Minato/9, Minato/13, and Minato/31) showed 98% nucleotide and 100% amino acid identity to that of strain Yuri/52/99/JP (AB009876, GII/5-like) (Table 3) and showed a further 89% nucleotide identity to strain SaitamaT49/01/JP (AB112315, GII/5). In contrast, the nucleotide sequences of the 5'-ORF2 (5' terminus of ORF2) regions from three of these six isolates (Minato/3, Minato/6, and Minato/9) showed low homology to either Mc37/JP (81%) (AY237415, GII/10), SaitamaT49/01/JP (79%) (AB112315, GII/5), Toronto/TV24/93/CA (79%) (U02030, GII/3), or Hawaii/71/US (79%) (U07611, GII/1) (Fig. 2B; Table 3). Hence, the genotypes of these three strains were likely to be GII/5. We could not obtain sequence information for the 5'-ORF2 regions of the three remaining strains (Minato/4, Minato/13, and Minato/31) due to the low yields of the PCR products.

Another single-point recombination event may have occurred at the NV ORF1-ORF2 junction in Minato/14, which was isolated from the feces of crew member 14. The nucleotide sequence of the 5'-ORF2 region of Minato/14 revealed 95% nucleotide identity with the Neustrelitz/260/00/GE (AY772730, GII/1-like) strain and 75% identity with the SaitamaU4/00/JP (AB039777, GII/6) strain (Table 3). In contrast, the 3'-ORF1 sequence of Minato/14 showed only 70% homology with Neustrelitz/260/00/GE but showed 99% identity with the SaitamaU4/00/JP strain. It is therefore highly likely that the Minato/14 isolate of NV from case 14 is a recombinant of Neustrelitz/260/00/GE (GII/1-like) and SaitamaU4/00/JP (GII/6).

The nucleotide sequence of 3'-ORF1 of Minato/10 showed 96% nucleotide identity with the Neustrelitz/260/00/GE (GII/1-like) strain, but we could not obtain the corresponding sequence information for the 5'-ORF2 region of this isolate. The nucleotide sequences of the 5'-ORF2 regions of Minato/8 and Minato/17, from which the 3'-ORF1 sequences could not be obtained, were identical to that of Minato/6. Additionally, it is probable, therefore, that Minato/33 can be classified as GII/4, because its 5'-ORF2 sequence showed 86% nucleotide identity with the Bristol/93/UK (X76716, GII/4) strain.

In summary, therefore, one recombinant NV isolate, Minato/14, was identified from this outbreak by nucleotide sequence analysis as a recombinant NV strain of GII/6 and GII/1. In addition,

three genotypes (GII/1 [Minato/10], GII/4 [Minato/33], and GII/5 [Minato/6]) were subsequently classified in this epidemic.

(iv) SSCP analysis. For SSCP analysis, preliminary testing was performed to optimize the experimental conditions (including incubation temperatures and concentrations of samples and buffers). The SSCP results for the 3'-ORF1 and 5'-ORF2 regions of NV isolates from 22 samples are shown in Fig. 3. We identified nine different patterns and designated these as types A to type F for the 5'-ORF2 sequences and as types P to R for the 3'-ORF1 regions. The type A pattern was evident in seven cases (Fig. 3, lanes 1, 2, 4, 5, 8, 11, and 12). Among these seven isolates, five strains showed low nucleotide identities with the GII/1, GII/3, GII/5, and GII/10 reference strains (79 to 81%). The SSCP patterns of the 3'-ORF1 PCR products from these five samples showed a type P pattern (lanes 16, 17, 18, 21, and 22). Moreover, three of these five samples (Minato/3, Minato/9, and Minato/13) were identical to Yuri/52/99/JP (GII/5-like) following sequencing of the PCR products.

Minato/33, the 5'-ORF2 region of which was classified with the Bristol/93/UK (GII/4) strain, showed a type E pattern (Fig. 3, lane 14). The 3'-ORF1 region of Minato/33 showed a type P SSCP pattern (lane 22), although we could not obtain sequence information for the ORF1 region of this NV isolate. The 100 N-terminal bases of this 838-bp 3'-ORF1 PCR product could be shown as having a sequence identical to that of the Yuri/52/99/JP strain, but the remaining 700-bp sequence, including the C-terminal region, was ambiguous and demonstrated overlapping peaks.

The NV isolates from cases 10 and 14 (Minato/10 and Minato/14) revealed a type B SSCP pattern for the 5'-ORF2 region (Fig. 3, lanes 6 and 9), but, interestingly, the 3'-ORF1 SSCP patterns were different for these two samples. Minato/10 showed a type Q pattern (lane 19), but Minato/14 clearly had a type R pattern (lane 20), which contains an extra band compared to type Q. It is therefore highly possible that the isolate from case 14 was a recombinant NV of Neustrelitz/260/00/GE (GII/1-like) and SaitamaU4/00/JP (GII/6) as stated earlier. The type C (lane 10) and D (lanes 10 and 15) patterns that we identified also indicated additional NV genogroups, but the low yields of the PCR products in these cases pre-

FIG. 3. SSCP patterns of amplified products from 5'-ORF2 (a) and 3'-ORF1 (b) of NV. Each lane corresponds to an individual crew member. Electrophoretically distinguishable patterns for 5'-ORF2 were designated as types A through E (a), and those for 3'-ORF1 were designated as types P, Q, and R (b).

vented further SSCP analysis. Type F (lanes 3 and 7) showed multiple banding patterns, which we speculate to be the result of the coexistence of multiple strains of NV.

DISCUSSION

In our current study we describe our analysis of an outbreak of acute gastroenteritis under confined conditions, and we further identify coinfections with NV and ASV as a possible underlying cause of this localized epidemic. In addition, genotypic variations among the infecting NV strains were identified. The profile of the outbreak of acute gastroenteritis under study can be characterized by a typical clinical course, which was caused by NV infection and resulted in strong symptoms and illness for only a few days (1, 32). The epidemic curve that we generated from our data had a sharp single peak (Fig. 1), suggesting a point source. Moreover, as shown in Table 2, multiple infections with both NV and ASV could be detected in the specimens of crew members who became ill within 24 h of onset (0900 on 22 October) of the outbreak (cluster 1). However, either NV or ASV, but not both, was detectable in symptomatic individuals who developed illness after this 24-h period (cluster 2). It is therefore possible that the infection source contained both of these viruses and that the cluster 1 cases were the result of exposure to higher levels of these viruses than the cluster 2 cases. The source of the infection cannot be specified, although two possible causes are hypothesized. One of these is the consumption of contaminated seafood on 12, 14, or 15 October, and the other possible source is the two preceding cases of crew members who developed symptoms on 13 and 15 October, respectively. However, the possible involvement of either of these two factors as a source of the infection has not been confirmed following our investigations.

ASV strains have been found to be associated mainly with sporadic outbreaks of diarrhea in children, occurring in such settings as hospital wards, day care centers, kindergartens, and schools (7, 16, 27, 29). Previous reports showing that many individuals are positive for ASV antibodies indicate that this virus may be ubiquitous (25). Hence, the pathogenicity of ASV is still controversial, and much uncertainty about its role in disease remains (18, 27). We contend, however, that it is de-

sirable for the rate of ASV detection to be incorporated as the background rate in any future investigations of outbreaks caused by NV infection.

Our finding of asymptomatic cases, demonstrating multiple viral infections, has potentially very interesting implications for the transmission of both NV and ASV. Previous studies with volunteers have shown that as many as 30% of NV infections may be asymptomatic (15). The role of asymptomatic infection in the transmission of NV is not well understood at present (2, 10, 32), but both symptomatic and asymptomatic food handlers have been implicated in many food-borne outbreaks of gastroenteritis (6, 12, 37). It is highly possible, therefore, that individuals who display no signs of illness may be effective transmitters of these pathogenic viruses. Gallimore et al. previously reported that asymptomatic individuals excreted different NV variants, containing small mutations, which they identified from a hospital outbreak of gastroenteritis (13). However, conclusive evidence of the spread of pathogenic NV and ASV by asymptomatic carriers still remains elusive.

We also demonstrate from our current findings that in one NV isolate, a single-point recombination event may have occurred (GII/6-GII/1). Recombinant NV may be representative of the causative pathogen at the beginning of the outbreak, indicating that the recombination event did not occur during the viral propagation accompanying the outbreak, but at a much earlier stage. Using sequencing and SSCP analyses, we detected multiple NV variants from the infected individuals in our present study. We speculate that the multiple banding patterns are the result of the simultaneous amplification of multiple NV genes in a single specimen. This is therefore an example of NV of multiple genotypes being detected from an individual patient. When the infection source contains multiple viruses, such a mixed infection might be initiated. Additionally, it is essential when using SSCP analysis that variants can be detected, and this can best be achieved by optimizing the experimental conditions (3).

This incidence of the spread of acute gastroenteritis caused by NV has, therefore, different characteristics from common examples of such epidemics, which, with the exception of shellfish-related outbreaks, generally originate from infection by a single NV genotype (23). We also postulate that these may be

the result of both the viral load and the unusual, and as yet uncertain, circumstances surrounding this particular closed epidemic. Nilsson et al. previously showed that the accumulation of mutations in the protruding P2 domain of the NV capsid leads to structural changes and possibly a new antigenic phenotype (31). Persistent viral infection is considered to be an important determinant of genetic diversity for several viruses (33), and recombination and mutation may lead to the generation of new NV strains in this way.

In summary, DNA sequencing provides the most exhaustive body of data when one is undertaking analyses such as those described here but is both expensive and labor-intensive. SSCP is also suitable, however, for most molecular epidemiologic studies and is less costly and time-consuming. Moreover, previous SSCP analyses of several viruses, such as hepatitis C virus, feline coronavirus, bovine viral diarrhea virus, and plant viruses, have been reported (22, 24, 26, 39). Using a highly sensitive method for the detection of NV, minor variants would have been expected to make up quasispecies. Because of high infectivity rates detected in our current study, however, small amounts of such NV minor variants may be sufficient to infect an individual. Furthermore, the occurrence and persistence of an outbreak of this nature in a strictly closed setting may be another circumstance that will promote viral genetic diversity.

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