Virological, Serological, and Clinical Features of an Outbreak of Acute Gastroenteritis Due to Recombinant Genogroup II Norovirus in an Infant Home

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Norovirus (NV) is an important cause of acute nonbacterial gastroenteritis worldwide. Recently, several naturally occurring recombinant NVs have been reported. In January 2000, there was an outbreak of gastroenteritis in an infant home in Sapporo, Japan. Of 34 residents of the home that were less than 2 years old, 23 developed gastrointestinal symptoms and NV infection was confirmed by conventional reverse transcription-PCR to detect the RNA polymerase region of genogroup II NV. In this virus, the RNA polymerase region shared 86% nucleotide identity with Hawaii virus but only 77% with Mexico virus; however, its capsid region shared only 70% identity with Hawaii virus but 90% with Mexico virus. On the other hand, both regions shared a higher 96% nucleotide identity with Arg320 virus, which was found in Mendoza, Argentina, in 1995 and considered to be a recombinant of Hawaii and Mexico viruses. The findings indicate that the virus involved in the outbreak was similar and may have evolved from the Arg320 virus. Clinically the cases were more severe than those of previously reported sporadic or outbreak cases of NV infection.

Norovirus (NV) is an important cause of acute nonbacterial gastroenteritis in children and adults worldwide (6, 11). NV is classified in the calicivirus family together with vesivirus, lagovirus, and sapovirus. Caliciviruses have single-stranded, positive-sense RNA genomes of approximately 7.5 kb (17, 19, 25), which are organized into three open reading frames (ORFs) that encode the nonstructural proteins (ORF1), a capsid protein (ORF2), and a small basic protein (ORF3).

NVs have been divided into two distinct groups, genogroup I (GI) and genogroup II (GII) by nucleotide sequence analyses of the polymerase and capsid regions (14, 41). Each genogroup contains several genetic clusters. Only genotyping of the capsid region corresponds with the antigenic type (3).

Recently, several naturally occurring recombinant NVs have been reported (20, 21, 27, 40). By phylogenetic analysis of the complete genome, including the RNA polymerase and capsid regions, it has been shown that recombination usually occurs at the junction of the two regions. However, detailed analysis of the clinical and serological features to establish what effects recombination has on clinical severity or viral antigenicity was not possible, especially in infant cases, because the number of virologically confirmed outbreak cases due to recombinant NVs was small.

An outbreak of acute gastroenteritis due to recombinant NV of GII (NV/GII) occurred in an infant home in Sapporo, Japan, in 2000. In this study, the recombination of NV/GII was analyzed using a phylogenetic tree and capsid identity. Type-specific or cross-reactive serum immunoglobulin G (IgG) or IgM antibody responses were examined by an enzyme-linked immunosorbent assay (ELISA) using three kinds of recombinant virus-like particles (VLPs) which carry antigenically distinct NV capsid proteins. The clinical severity of this single outbreak was also estimated using a 0- to 20-point scoring system (35).

MATERIALS AND METHODS

Outbreak. In January 2000, there was an outbreak of acute gastroenteritis in an infant home in Sapporo, Japan. Of 34 residents (mean age, 13.2 months; range, 0 to 26 months) in the home, 23 (68%) developed gastrointestinal symptoms within a week, probably due to person-to-person transmission. The mean age of patients was 15.9 months (range, 6 to 25 months). No staff members reported gastrointestinal illness during the outbreak. A pediatrician at the home recorded the clinical features of the affected children. No indication of how the virus was introduced into this population was obtained.

Stool samples were obtained from all 23 patients and stored at −20°C for further analysis. We screened the stool samples for rotavirus and adenovirus by ELISA and for norovirus, sapovirus, and astrovirus by reverse transcription-PCR (RT-PCR).

Paired sera were obtained from 15 patients. Preinfection serum samples were collected from 1 to 3 months before the outbreak, and postinfection sera were collected 5 months after the outbreak. From 12 of the 15 patients, acute-phase sera, that is, 2 to 8 days after the onset of illness, were obtained. Serum samples were stored at −20°C until tested. We obtained stool and serum samples with the permission of the head of the institute, who acts as the patients’ guardian. There had been no other outbreak of gastroenteritis during this 8-month period.

Stool suspension and RNA extraction. Stool samples were prepared as an approximately 10% (wt/vol) suspension in 10 mM phosphate-buffered saline (pH 7.4) and clarified by centrifugation at 3,000 × g for 20 min. The supernatant was again clarified by centrifugation at 7,000 × g for 20 min. The second supernatant was stored at −20°C until tested. Viral RNA was extracted from the stool suspension by the cetrimide-thymol-bromide method (18). RNAs were stored at −20°C until tested.

RT-PCR. NV/GII was sought in these stool samples by RT-PCR using primers SR46 and SR33 (Table 1) derived from the RNA polymerase region as previously described (16). The nucleotide sequence of the 3.3-kb NV/GII genome from the RNA polymerase region to the 3′ end of the genome was then deter-
<table>
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<th>Primer</th>
<th>Sequence (5′→3′)</th>
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a −, negative; +, positive.
b Equivalent location within the Lordsdale virus genomic sequence (X86557, GenBank).

RESULTS

Nucleotide and amino acid sequences. On the basis of RT-PCR testing, 22 of the 23 patients with gastrointestinal symptoms were positive for NV/GII. All patients were negative for rotavirus, adenovirus, sapovirus, and astrovirus (data not shown). Five stool specimens from five patients were selected randomly, and 3.3-kb NV/GII gene sequences were determined. The resulting nucleotide sequences from these five NV strains were completely identical and contained 3,315 bp with a polyadenylated 3′ end. This NV/GII was named SN2000JA. The 5′ end of the cDNA encoded the C terminus of the ORF1 product (ORF1 included the RNA polymerase gene). This RNA polymerase sequence started at nt 1 and ended at nt 872, encoding a protein of 289 amino acids (aa). The second ORF started at nt 853 and ended at nt 2,499, encoding a protein of 548 aa, which is predicted to be the viral capsid protein. The third ORF started at nt 2,499 and ended at nt 3,263, encoding a small basic protein of 254 aa.

Sequence alignment of SN2000JA with Arg320, Hawaii, and Mexico viruses. Sequence alignment of the 3.3-kb SN2000JA shared 96% nucleotide identity with Arg320, which was reported to be a recombinant NV (20). The RNA polymerase region of SN2000JA shared 96% and 86% identity with Arg320 and Hawaii viruses, respectively, but 77% identity with Mexico virus. The capsid region shared 96% and 90% identity with Arg320 and Mexico viruses, respectively, but only 70% identity with Hawaii virus. The ORF3 region shared 96% and 87% identity with Arg320 and Mexico viruses, respectively, but only 69% identity with Hawaii virus.

For amino acids, the RNA polymerase region of SN2000JA shared 98% and 96% identity with Arg320 and Hawaii viruses, respectively, but 87% identity with Mexico virus. The capsid region shared 98% and 95% identity with Arg320 and Mexico viruses, respectively, but 73% identity with Hawaii virus. The ORF3 region shared 92% and 83% identity with Arg320 and Mexico viruses, respectively, but 71% identity with Hawaii virus.

Phylogenetic trees of RNA polymerase region, capsid region, and ORF3 region. The phylogenetic trees of the amino acid sequence were made using SN2000JA, Arg320, Hawaii, Mexico, Lordsdale, Melksham, Norwalk, Sh-5, Ueno7k, and Chiba viruses. Only the capsid regions of Sh-5 and Ueno7k viruses were included in phylogenetic trees. On the basis of the RNA polymerase region, SN2000JA grouped with Arg320 and Hawaii viruses (Fig. 1A), whereas on the basis of the capsid and ORF3 regions, SN2000JA was closely related to Arg320, Sh-5, and Mexico viruses (Fig. 1B and C).

Recombination analysis. To analyze recombination, the nucleotide identity window search was performed by using the SimPlot program version 2.5 (28). The detection of serum IgG and IgM antibody against NV. VLPs expressing capsid protein of Sh-5 (NV/GII), Ueno7k (NV/GII), or Chiba (NV/GI) virus were used as NV/GI or NV/GI antigens. The expression and purification of NV VLPs were performed as previously described (23, 24, 38). Serum samples were tested by ELISA using VLPs as previously described (26). The ELISA antibody titers were expressed as the highest dilution that gave specific absorbance of more than 0.15 (30).

Evaluation of severity of gastroenteritis. The clinical severity of acute gastroenteritis was estimated using a 0- to 20-point scoring system, which was previously used to assess the clinical severity of rotavirus gastroenteritis and to evaluate the efficacy of rotavirus vaccine (35). A score of ≤7 is considered to be mild, 8 to 10 is moderately severe, 11 to 13 is severe, and ≥14 is considered extremely severe (34).

Nucleotide sequence accession number. The sequence of SN2000JA was submitted to the DDBJ/EMBL/GenBank databases under accession number AB190457.

TABLE 1. Primers used for RT-PCR

For this purpose, five separate, mostly overlapping, RT-PCRs were performed one after another. The first RT-PCR product was obtained using primer NV36 (nucleotides [nt] 4212 to 4232) and SR33 (nt 4595 to 4615); the second product was obtained using forward primer SR46 (nt 4493 to 4513) and random primers (Invitrogen Corp., Carlsbad, CA). The third, fourth, and fifth RT-PCRs were performed using forward primer NV04 (nt 5124 to 5143), NV06 (nt 6663 to 6684), and NV07 (nt 7247 to 7267), respectively, which were newly designed sequentially on former sequence data. For reverse primer, random primers were used in the third and fourth RT-PCRs and oligo(dT)16 (Promega Corp., Madison, WI) was used in the final RT-PCR (Table 1). The amplification with these primer sets produced fragments that overlapped each other by 80 to 650 nt.

Cloning and sequencing RT-PCR products. Viral cDNA fragments separated by agarose gel electrophoresis were cloned into pCR2.1-TOPO vector using TOPO TA cloning kits (Invitrogen). The third, fourth, and fifth RT-PCRs were performed with Clustal W (39). A phylogenetic tree with 1,000 bootstrap strands.

Sequencing analysis. The nucleic acid and amino acid sequence alignments included Arg320 (AF190817), Hawaii (U07611), Mexico viruses, respectively, but only 70% identity with Mexico virus. The capsid region showed 96% and 90% identity with Arg320 and Mexico viruses, respectively, but only 70% identity with Hawaii virus. The ORF3 region showed 96% and 87% identity with Arg320 and Mexico viruses, respectively, but only 69% identity with Hawaii virus.

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Serological immune response. VLPs expressing the capsid protein of Sh-5, Ueno7k, or Chiba virus were used as antigens. The amino acid sequences of the capsid regions of Sh-5, Ueno7k, and Chiba viruses shared 98%, 72%, and 44% identity with SN2000JA, respectively. In preinfection sera, 10 of 15 children showed a low positive IgG antibody titer (1:100 to 1:400) against recombinant Sh-5 (rSh-5) virus; however, all 15 patients, including 1 patient whose stool sample was negative for NV/GII by RT-PCR, showed an increase of more than 16-fold 5 months after infection (Table 2). On the other hand, against recombinant Ueno7k (rUeno7k) and recombinant Chiba (rChiba) viruses, only one child (patient 9) and three children (patients 6, 7, and 9) showed weak fourfold increases 5 months after infection, respectively (Table 2).

From 12 of 15 children, acute-phase sera, that is, 2 to 8 days after the onset of illness, were obtained. Therefore, specific IgM ELISA antibody responses could be checked at three time points. In pre- and postinfection serum samples, no or only low positive (under 1:200) IgM responses against either VLP were found (Table 3). On the other hand, nine acute-phase sera, collected just over 6 days after the onset of disease, showed significant IgM responses (1:400 to 1:12,800) against rSh-5 only (Table 3).

Clinical features of the outbreak. The clinical features of the 23 patients are summarized in Table 4. The most common symptom was diarrhea (22/23 patients [96%]), followed by vomiting (15/23 patients [65%]). The mean durations of diarrhea and vomiting were 6.0 days (range, 1 to 24) and 1.2 days (range, 1 to 5), respectively. The maximum numbers of diarrhea and vomiting/day were 4.2 stools (range, 0 to 10) and 1.4 episodes (range, 0 to 5), respectively. Four patients had mild illness, 11 moderate, 3 severe, and 5 extremely severe. The
mean clinical severity of NV gastroenteritis was 9.9 points (range, 3 to 15), considered moderately severe. Fevers over 37.5°C were observed in 13 patients (57.5%) (data not shown).

These clinical parameters were compared with those of a former outbreak of NV gastroenteritis in this infant home (36) and of sporadic cases in Finland (34) (Table 4). The patients in those reports were also under 2 years of age. Although statistical comparison among these three studies could not be conducted because individual data were unavailable, the durations of diarrhea and vomiting in the present study were equal to or longer to than those of the previous studies, and the clinical severity scores were also equal to or higher than those of the previous studies.

**DISCUSSION**

We describe an outbreak of gastroenteritis due to a recombinant NV/GII strain, which probably resulted from a naturally occurring recombination. According to analysis of the sequences of the 3.3-kb genome, which encodes part of RNA polymerase (ORF1) and the entire ORF2 and ORF3, the RNA polymerase gene shared a high level of identity with Hawaii virus, but its capsid and ORF3 gene shared a high level of identity with Mexico virus. On the other hand, all three regions of the virus found in this outbreak shared their highest sequence identity with Arg320, which was isolated in 1995 and identified as a recombinant NV (20). Therefore, this virus, named SN2000JA, was strongly suggested to be a recombinant strain of Hawaii virus and Mexico virus similar to Arg320 virus.

Similar recombination cases for other single-stranded RNA viruses, such as astrovirus (4), poliovirus (22), echovirus (32), and enteroviruses (37), have been reported. There have been several reports of recombination occurring at the junction of ORF1 and ORF2 in NV (20, 21, 27, 40).

Three possibilities should be considered for the recombination event observed in this outbreak. First, the recombination observed may represent simultaneous infection with two or more
strains of NV. Second, the recombination event may have occurred before the onset of this outbreak, independent of the recombination event of Arg320 virus. Third, it is possible that the recombination occurred in the remote past, that these recombinant strains have been circulating in the world, and that Arg320 also originated from these strains. The first possibility is unlikely, because completely identical 3.3-kb sequences were confirmed in five stool samples from different patients which were collected within a week, and each sequence was repeated more than two times. However, the second and third possibilities cannot be resolved easily because of the limited evidence of recombination. The fact that 96% nucleotide sequence identity existed between the present NV and Arg320 virus, which was isolated in 1995, suggests that the present NV has evolved from Arg320. In other words, this may represent spread of an Arg320-like strain to Japan. There is also a possibility that the present NV and Arg320 virus, which was isolated in 1995, could have resulted from genetic evolution from a common single original recombinant strain.

All recombinant NV/GII strains, Arg320, Wortley, Saitama U1, Snow Mountain, and SN20000A viruses, have polymerase genes that are similar to Lordsdale or Hawaii virus; however, their capsid genes resemble Mexico, Hawaii, or Melksham virus (20, 21, 27, 40). Lordsdale-like viruses are the predominant global strain of NV (5, 7, 8, 9, 13, 15, 29, 31); therefore, recombinant NVs sharing high identity with Lordsdale virus are likely to be reported most frequently.

Because many recombinant NV strains are expected to exist worldwide, conventional RT-PCR methods to detect only the RNA polymerase region of NV are insufficient to classify the NV field strain correctly. With recombinant strains, a series of nucleotide sequence analyses from polymerase to capsid regions is necessary to classify a cluster of norovirus.

In this outbreak, a number of serial blood samples were obtained at the preinfection, acute infection, and postinfection phases of this outbreak of gastroenteritis due to recombinant NV/GII. This enabled a detailed analysis of the serological responses against homologous or heterologous NV, as ELISA antigens carrying homologous (NV/GII) or heterologous capsid (NV/GI and NV/GII) were prepared. An ELISA using VLPs, which express capsid protein of NV, has been shown to be more sensitive and specific for detecting antibody responses than an ELISA using native NV antigen from stool (12). This implies that the antigenicity of NV depends almost entirely on its capsid protein.

In the present study, ELISA IgG responses against homologous capsid protein increased more than 16-fold 5 months after infection, but the responses against heterologous proteins, regardless of genotype (GI or GII), were less than 4-fold. Therefore, the >16-fold increase in paired samples should help to determine the causative antigens. On the other hand, ELISA IgM responses were more genotype specific and were observed only in the early convalescent phase of infection (6 to 8 days). As a result, one-point serum samples collected in the early convalescent phase may be useful for the early diagnosis of NV gastroenteritis and determination of the NV genotype.

Clinical features of this outbreak, such as duration of diarrhea and vomiting and clinical severity score, were equal to or a little more severe than those in former outbreaks in the same infant home (36) and in sporadic infant cases in Finland (34) (Table 4). Although there had been no analysis on recombination of these former NV strains, it could be said that recombination among NVs may increase their pathogenicity. However, it should be mentioned that epidemics of gastroenteritis due to some recombinant NVs with possible reduced pathogenicity might not be estimated properly, because they could not be identified.
be less easily recognized. Age factors may also influence pathogenicity; some asymptomatic infections in adults associated with recombinant NV were recently reported (10). The clinical and biological significance of recombinant NV strains should be determined by further studies on several NV outbreaks, in which consecutive analysis of the polymerase and capsid regions is available.

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REFERENCES
