

co-circulating in Japanese children with acute gastroenteritis (Fig. 2). It was found that NoV strains in the study with the same genotype had high homology with each other, ranging from 95% to 100% even when they were detected in different years and different areas in Japan.

DISCUSSION

Norovirus is one of the important causes of sporadic cases and outbreaks of gastroenteritis worldwide [Koo et al., 1996; Holtby et al., 2001; Lopman et al., 2004]. Out of 3,864 fecal specimens tested in the study, 11% were

positive for NoV by RT-PCR. This finding is in agreement with previous reports on molecular epidemiology of NoV infection worldwide in which its prevalence was shown ranging from 10% to 60% [Love et al., 2002; Marks et al., 2003]. The finding also suggested that from acute gastroenteritis cases in children in five regions of Japan about 11% might be due to NoV and 89% might be caused by other etiologic agents. The result also confirmed NoV as one of the important enteropathogens responsible for viral gastroenteritis among children in Japan. In some reports, NoV was prevalent during the cold season, and several studies did not find a seasonal correlation [Noel et al., 1997; Mounts et al., 2000; Otsu

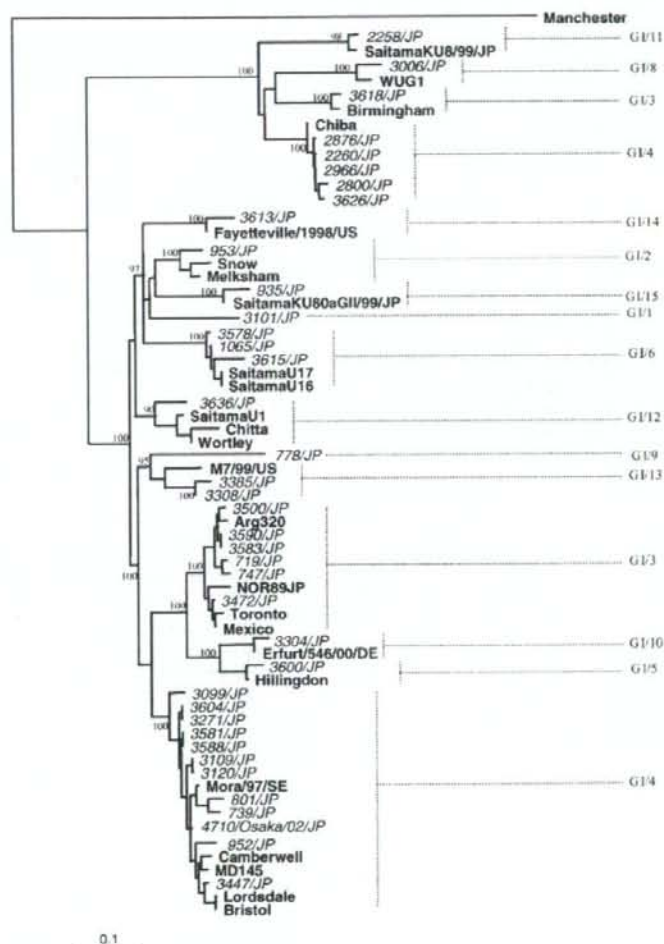


Fig. 2. Phylogenetic tree of nucleotide sequences of NoV. The tree was constructed from partial nucleotide sequences of capsid region of NoV isolates detected in Japan. Reference strains of NoV were selected from the DDBJ/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. Manchester strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values.

et al., 2003]. The main peak of NoV infection in Japan was between November, December, and January. Obviously, there are two peaks of viral infection causing acute gastroenteritis in Japan; one peak associated with NoV infection lasts from November to January known as early winter in this study and another peak associated with rotavirus infection lasts from February to April known as late winter [Yoshinaga et al., 2006]. Furthermore, it was interesting that the highest incidence of NoV infection fell into the 1-year old group, and the rate of incidence decreased with increasing age over 2 years. Quite possibly, children aged 1 year might lack antibody protection to NoV; whereas by the time children have reached 2 years old they begun to acquire viral immunity.

The results of this study showed that all Japanese NoV isolates belonged to two distinct genogroups I and II (GI, GII) and these represented 3% and 97%, respectively. This indicated that NoV GII was the dominant group causing acute gastroenteritis among Japanese pediatric population. The distribution of NoV genotypes was also investigated within a time-line period from 1995 to 2001. Interestingly, phylogenetic analysis of the partial capsid gene of NoVs identified a wide range of genotypes (up to 16) had been co-circulating and caused diarrheal illness among children in Japan during that time. In this study, a rare NoV genotype (GII/9), which has not been detected in Japan, was identified. Moreover, there was only one strain, which matched closely this GII/9 strain in the DDBJ/GenBank database. The other NoV strains, such as GII/13, which have a few homologous strains in the DDBJ/GenBank database, were also detected. Taken together, it is noteworthy that NoV strains detected among Japanese pediatric population with acute gastroenteritis demonstrated a great genetic diversity.

According to other reports published by different groups of investigators, NoV belonging to the Lordsdale cluster (GII/4) represented the highest detection in sporadic gastroenteritis among infants and children not only in Japan but also many other countries who conduct NoV surveillance [Lopman et al., 2002; Nicollier-Jamot et al., 2003; Ueki et al., 2004]. Generally, GII/4 detected in the present study was found to be the dominant genotype in causing acute gastroenteritis in Japan. However, it was interesting that GII/3 sometimes dominated GII/4 in some regions of Japan during different periods of time. In 1996–1997, the detection rate of GII/3 in Tokyo was very high as 76.9% whereas that of GII/4 was only 23.1%. A similar pattern of NoV infection was also identified in Sapporo and Maizuru in 1997–1998. Further epidemiologic studies should be conducted to determine whether strains from GII/3 continue to be dominant in Japan in future.

Another interesting feature of the present study was the temporary increase of NoV GII/6 strains, which became the second predominant NoV genotype causing the illness among children in Japan in Maizuru during 1995–1996 and 1998–1999. These results suggested that the NoV GII/6 were also an important cause of

sporadic cases of acute gastroenteritis. This sudden predominance of NoV GII/6 strains indicated that the pediatric population might lack antibody protection to these strains, whereas by the time they have begun to acquire viral immunity or NoV GII/6 strains could be more virulent at that time.

In conclusion, diarrhea due to NoV infection is still a health burden in Japan. This report also stresses the great genetic diversity and the importance of NoV causing the diarrhea in Japan. Moreover, such study of the molecular epidemiology of NoV provides knowledge on the diversity of genotypes found in humans. Continuous monitoring of the NoV genotypes should be continued for the control of diarrheal disease due to NoV infection to be successful.

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ORIGINAL ARTICLE

Viral Gastroenteritis and Genetic Characterization of Recombinant Norovirus Circulating in Eastern Russia

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SUMMARY

From November 2003 to March 2004 a total of 100 fecal specimens from infants and children with acute gastroenteritis in the city of Birobidzhan, Eastern Russia were tested for the presence of diarrheal viruses by RT-multiplex PCR. Of these, 74 fecal specimens were positive for diarrheal viruses and this represented 74%. Among the diarrheal viruses detected, group A rotavirus was the most prevalent (67%; 67 of 100), followed by norovirus (4%; 4 of 100), group C rotavirus (1%, 1 of 100), sapovirus (1%; 1 of 100), and hepatitis A virus (1%; 1 of 100). It was found that 86.6% (58 of 67) of group A rotavirus were serotyped as G3. Sapovirus and hepatitis A virus were genetically determined to belong to GI/1 and subgenotype 1A, respectively. Interestingly, all norovirus isolates in the study turned out to make a novel cluster when polymerase-based grouping was performed. It is noteworthy to point out that these norovirus isolates were further genetically characterized as naturally occurring recombinants, which were firstly found circulating in the Russian population studied. Breakpoint analysis of recombinant norovirus showed that the recombination site was at the open reading frame (ORF)1/ORF2 overlap. This is the first report of the existence of acute gastroenteritis caused by recombinant norovirus in Eastern Russia. (Clin. Lab. 2006;52:247-253)

KEY WORD

norovirus, recombination, Eastern Russia

INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality of children due to gastroenteritis is greater in developing than in the developed countries [1]. It has been well established that virtually every child becomes infected with a rotavirus at least once by 3 years of age [2]. Rotaviruses are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with group A, B and C rotaviruses. Of

these, group A rotavirus is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [3]. Apart from group A rotavirus as the most common cause of gastroenteritis, norovirus is also considered to be a global enteropathogen. This virus is associated with sporadic cases and outbreaks of acute gastroenteritis in such settings as kindergartens, schools, nursing homes for the elderly, and among military recruits [4, 5]. The transmission routes of this virus are classified into foodborne, airborne, person-to-person spread and perhaps by some other unknown modes [4, 6]. However, norovirus is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make norovirus a major public health concern [7]. Norovirus (NoV, formerly known as "Norwalk-like virus") is the distinct genus within the family *Caliciviridae*. Norovirus contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The norovirus genome contains three ORFs (ORF1, 2 and

3). The ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase (RdRp), ORF2 encodes the capsid protein (VP1), and ORF3 encodes a small capsid protein (VP2). Human norovirus is still unculturable by standard culture with different cell lines. However, expression of either VP1 alone or both VP1 and VP2 using recombinant baculoviruses form the virus-like particles (VLPs) that are morphologically and antigenically similar to the native virion [8]. The prototype strain of norovirus is the Norwalk virus (Hu/NoV/Norwalk virus/1968/US), which was originally discovered during an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA in 1968 [9]. Immunological and seroepidemiologic studies indicate a worldwide distribution of norovirus [4, 10, 11]. Moreover, it was found that the serum antibody level to norovirus was lowest in the first year of life, rising after two years of age [12].

The objectives of this study were to determine the prevalence of diarrheal viruses in fecal specimens from infants and children with acute gastroenteritis in the city of Birobidzhan, Eastern Russia and to characterize the detected viruses according to type. Additionally, the genetic analysis of norovirus is also described.

MATERIALS AND METHODS

Fecal specimens

A total of 100 fecal specimens were collected from infants and children with acute gastroenteritis in the city of Birobidzhan, Eastern Russia, during the period of November 2003 to March 2004. The fecal specimens were diluted with distilled water to 10% suspensions and clarified by centrifugation at 10,000 \times g for 10 min. The supernatants were collected and stored at -30°C until use for the detection of diarrheal viruses.

Extraction of viral genome

The viral genomes were extracted from 140 μl of 10% fecal suspensions using a QIAamp spin-column technique according to the manufacturer's instructions (QIAGEN[®], Hilden, Germany).

Reverse transcription (RT)

For reverse transcription (RT), 4 μl of extracted viral genome was added to a reagent mixture consisting of 1 μl of 5x First strand buffer (Invitrogen, Carlsbad, CA, USA), 0.4 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.4 μl of 10 mM DTT (Invitrogen), 0.4 μl (200 U/ μl) of superscript reverse transcriptase III (Invitrogen, Carlsbad, CA, USA), 0.2 μl (1 $\mu\text{g}/\mu\text{l}$) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.3 μl (33 U/ μl) of RNase Inhibitor (Toyobo, Osaka, Japan), and 1.3 μl MilliQ water. The total of the reaction mixture was 8 μl . The RT step was carried out at 50°C for 1 h, followed by 99°C for 5 min and then held at 4°C [13].

Polymerase chain reaction (PCR)

Multiplex PCR with specific primers and protocols as previously published was performed for the detection of three groups of diarrheal viruses [13]. Identification of the first group of viruses was performed with the specific primers Beg9 and VP7-1', B5-2 and B3-3, G8NS1 and G8NA2, Ad1 and Ad2 for group A, B, and C rotaviruses and adenovirus with four different amplicon sizes of 395 bp, 814 bp, 352 bp, and 482 bp, respectively in one PCR tube. For the detection of the second group of viruses, the primers PreCAP1 and 82b; G1SKF and G1SKR; COG2F and G2SKR, SLV5317 and SLV5749 specifically generated four different sizes of amplicons of 719 bp, 330 bp, 387 bp and 434 bp for astrovirus, norovirus (GI, GII), and sapovirus, respectively. For the third group, the primers F1 and R1, P3 and P4, 2s and 2as, and MMU42 and MMU43 specifically generated four different sizes of amplicons of 440 bp, 267 bp, 146 bp, and 219 bp for enteroviruses, hepatitis A and E viruses and influenza A virus, respectively. The norovirus polymerase region was also amplified to identify recombinant norovirus using previously described primers [14]. The full length of the capsid and polymerase regions was amplified with the method previously presented by Katayama *et al* [15]. Exactly 1 μl of cDNA was mixed with a reagent mixture containing 1.3 μl of 10x Taq DNA polymerase buffer (Promega, Madison, WI, USA), 1 μl of dNTPs (2.5 mM/ μl), 0.2 μl of each specific primer (33 μM), 0.06 μl (5 U/ μl) of Taq DNA polymerase (Promega, Madison, WI, USA). MilliQ water was added to make up a total volume of 11 μl . PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 7 min, and then held at 4°C .

Group A rotavirus serotyping

Serotyping of group A rotavirus was performed using the protocol of the method previously presented by Das *et al* [16]. The nearly full length of the VP7 gene was reversely transcribed and then further amplified with primers 9con1 and End9. Exactly 3 μl of RNA plus 0.3 μl of 50% DMSO was mixed with a reagent mixture containing 2.5 μl of 10x Taq DNA polymerase buffer (Promega, Madison, WI, USA), 2 μl of dNTPs (2.5 mM/ μl), 0.4 μl of each specific primer (33 μM), 0.5 μl (200 U/ μl) of superscript reverse transcriptase III (Invitrogen, Carlsbad, CA, USA), 0.2 μl (33 U/ μl) of RNase Inhibitor (Toyobo, Osaka, Japan), 0.1 μl (5 U/ μl) of Taq DNA polymerase (Promega, Madison, WI, USA), MilliQ water was added to make up a total volume of 25 μl . The expected size of the PCR product generated from the nearly full-length VP7 gene was 1,025 bp in length. The second amplification was performed using the first PCR product as the template with G-genotype specific mixed primers (9T1-1, 9T1-2, 9T-3P, 9T-4 and 9T-B) for downstream priming and 9con1 for upstream priming in an amplification of VP7 genes of

Table 1: Distribution of Diarrheal viruses circulating among infants and children with acute gastroenteritis in the city of Birobidzhan, Eastern Russia

Date of fecal specimen collection	Number of specimens tested	Fecal specimens positive for diarrheal virus (%)	Target virus (%)				
			Group A rotavirus	Norovirus GII	Sapovirus	Group C rotavirus	Hepatitis A virus
11.2003 – 3.2004	100	74 (74)	67 (67)	4 (4)	1 (1)	1 (1)	1 (1)

Table 2: Characteristics of norovirus infected children with acute gastroenteritis in the city of Birobidzhan, Eastern Russia

Number	Patient	Gender	Age (month)	Date of fecal collection	Norovirus strain	Diarrhea	Fever	Vomiting	Headache	Abdominal pain
1	78	Female	18	07.02.2004	HU/78/04/RU	+	+	+	-	-
2	83	Male	20	09.02.2004	HU/83/04/RU	+	-	+	-	-
3	88	Male	15	10.02.2004	HU/88/04/RU	+	-	+	-	-
4	92	Male	28	13.02.2004	HU/92/04/RU	+	-	+	+	+

G1-G4 and G9, respectively. These primers specifically generated five different sizes of amplicons of 158 bp, 224 bp, 466 bp, 403 bp, and 110 bp for G1, G2, G3, G4, and G9, respectively. Exactly 1 µl of the first PCR product was mixed with a reagent mixture containing 2.5 µl of 10x Taq DNA polymerase buffer (Promega, Madison, WI, USA), 1 µl of dNTPs (2.5 mM/µl), 0.4 µl of each specific primer (33 µM), 0.1 µl (5 U/µl) of Taq DNA polymerase (Promega, Madison, WI, USA), and 18 µl MilliQ water. The total of the reaction mixture was 25 µl. PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 1 min, 45 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 7 min, and then held at 4 °C.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min and then visualized under ultraviolet (UV) light; the results were recorded by photography.

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of the PCR products (DNA) positive for diarrheal virus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). A phylogenetic tree with 1,000 bootstrap replicates of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3)

was used to compare recombinant norovirus sequences. The nucleotide sequence data of norovirus strain HU/78/04/RU had been submitted to the DDBJ DNA/GenBank database and had been assigned accession number AB242258. Reference norovirus strains and accession numbers used in this study were as follows: Manchester (X86560), Melksham (X81879), Toronto24/91/CA (U02030), Lordsdale (X86557), Camberwell (AF145896), MD145 (AY032605), Hawaii (U07611), Mora/97/SE (AY081134), and Bristol (X86557).

RESULTS

Epidemiology of viral infections

A total of 100 fecal specimens collected from infants and children with acute gastroenteritis in the city of Birobidzhan, Eastern Russia during the period of November 2003 to March 2004 were examined for the presence of diarrheal viruses. Among the patients from whom the fecal specimens had been collected, the youngest was 1 month old, the oldest was 47 months, and the average age was 15 months. Of all infants and children with acute gastroenteritis, 58% (58 of 100) were male. Diarrheal viruses were detected in 74 out of 100 (74%) specimens tested. Of these, group A rotavirus was the most prevalent (67%) followed by norovirus (4%). Both group C rotavirus and sapovirus were the next with 1%. Interestingly, one fecal specimen was found positive for hepatitis A virus in this study (Table 1).

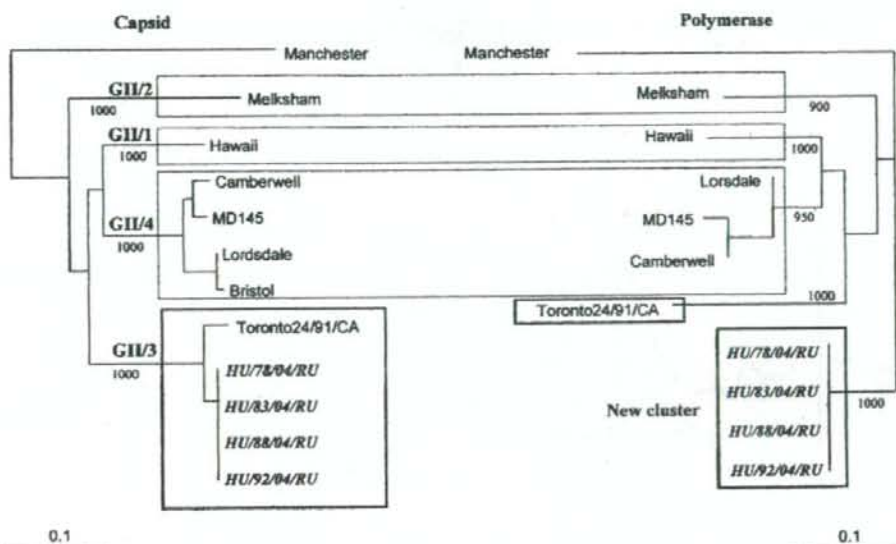


Figure 1: Observation of changes of norovirus genotypes on the basis of phylogenetic trees of nucleotide sequences. The trees were constructed from partial nucleotide sequences of capsid and polymerase regions of Russian isolates of norovirus genogroup II. Reference strains of norovirus were selected from DDBJ/GenBank under the accession number indicated in the text. The Russian norovirus is highlighted in italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. The Manchester strain was used as an out-group strain for phylogenetic analysis.

Group A rotavirus was further characterized by serotyping. It was found that 86.6% (58 of 67) of group A rotavirus were serotyped as G3 and 13.4% (9 of 67) were mixed infections with different G-types including one triple infection with G1, G2 and G3. Other diarrheal viruses were subjected to sequencing analysis. Sapovirus and hepatitis A virus were genetically determined to belong to genogroup I genotype 1 (GI/1) and subgenotype 1A, respectively. Group C rotavirus had the closest match (99%) at the nucleotide level with the strain Javeriana isolated from Colombia.

Clinical manifestations

All clinical symptoms from the Russian infants and children with viral gastroenteritis during the research period were reported. All of them had diarrhea. Of these, 23% passed watery stools 6-10 times per day. The symptoms were accompanied by vomiting (74.3%), fever (67.6%), cough (9.5%), abdominal pain (5.4%), and headache (1.4%). Infants and children with vomiting vomited 1-10 times per day. The fever rose to 39.6 °C. No mucus or blood was found in the feces.

Detection of norovirus infection

It was found that four norovirus genogroup II (NoV GII) isolates were identified in the fecal specimens collected from the Russian patients No. 78, 83, 88 and 92 by RT-multiplex PCR in the present study, and this accounted for 4% (Table 2). The age ranged from 18 to 28 months. All norovirus infected patients were detected during February 2004 and had diarrhea as well as vomiting. Only one patient had a fever of 38.4 °C. No norovirus genogroup I (NoV GI) was identified in the study.

Nucleotide sequencing and phylogenetic analysis of norovirus genotype

The PCR products of norovirus were sequenced in order to further characterize the genetic relationship among the norovirus isolates detected in infants and children with acute gastroenteritis in Eastern Russia. Their partial nucleotide sequences were compared to each other as well as to those of reference norovirus strains available in the DDBJ DNA/GenBank database by BLAST. The nucleotide sequence of the 5' end of the norovirus capsid gene was determined by direct sequencing with the amplified fragments. A total of 4 norovirus nucleo-

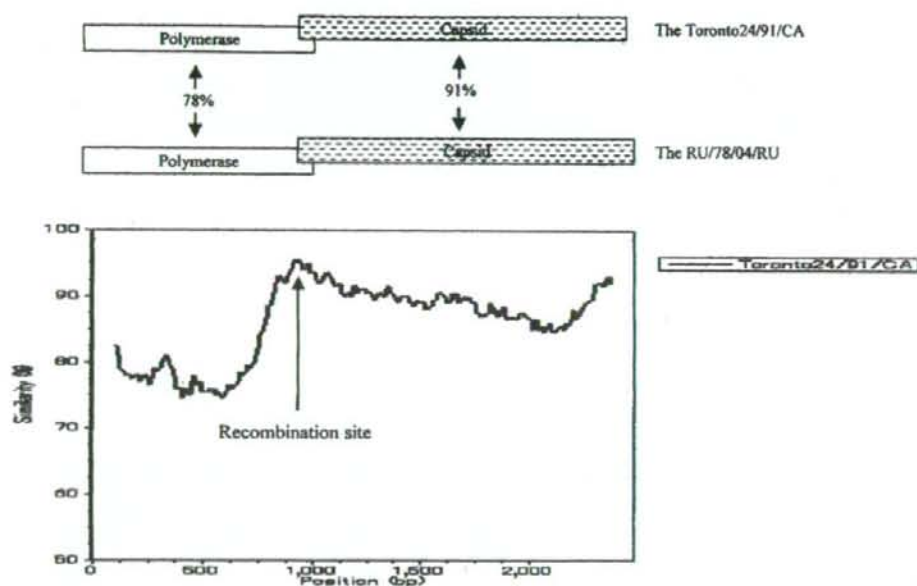


Figure 2: Genetic characterization of recombinant norovirus: the SimPlot analysis of strains HU/78/04/RU and the Toronto24/91/CA. The HU/78/04/RU was used as query strain and the Toronto24/91/CA was used as reference strain. Low and high homologies with polymerase and capsid regions among them were found.

tide sequences were analyzed by phylogenetics and grouped using the recent norovirus capsid region classification schemes of Kageyama *et al.*, 2004 [7]. Figure 1 shows that all norovirus GII sequences cluster into only one distinct genotype 3 (known as the Toronto24/91/CA virus cluster). The homology on the nucleotide level among the NoV GII isolates detected in this study was 100%. The identity of 91%–97% between these norovirus isolates and other norovirus reference strains in the same genotype was also noted.

Nucleotide sequencing and genetic analysis of norovirus polymerase

To further analyze the genetic characteristics of noroviruses from Eastern Russia, the partial polymerase regions of all NoV with GII/3 capsid were additionally amplified and sequenced. Interestingly, all norovirus isolates were not classified into any previously published norovirus cluster, even with the Toronto24/91/CA when polymerase-based grouping was performed (Figure 1). The nucleotide levels of the polymerase region of these norovirus isolates had low homologies, ranging from 57% to 78%, with other norovirus reference strains. Taken together, the norovirus isolates in Eastern Russia represented a novel cluster based on polymerase

sequences. It was found that the HU/78/04/RU shared a low level of sequence identity (78%) in the RNA polymerase region and a high level of nucleotide identity (91%) in the capsid region with the Toronto24/91/CA. Therefore, these 4 norovirus isolates were expected to be recombinants with GII/3 capsid and novel polymerase.

Recombination in norovirus ORF1/ORF2 overlap

As mentioned above, 4 isolates from GII/3 had high homology (100%) at the nucleotide level of capsid and polymerase. The findings clearly demonstrated that they very likely represented the same strain. Furthermore, they were also suspected to be recombinant noroviruses based on their partial capsid and polymerase sequences. To localize the potential recombination site and to understand a possible recombination mechanism of these recombinant noroviruses, the complete capsid and polymerase regions of one representative isolate, HU/78/04/RU, were determined. When the nucleotide sequence of the HU/78/04/RU was compared with that of the Toronto24/91/CA using the SimPlot software, an apparent site of genetic recombination was found in the ORF1/ORF2 overlap. After this junction, the capsid genes of these two strains were highly identical. How-

ever, before this junction the homology was notably different, and the SimPlot analysis showed a sudden drop in the nucleotide identity for the HU/78/04/RU (Figure 2).

DISCUSSION

Viral gastroenteritis is still a health burden in developed and developing countries [1]. In this study, diarrheal viruses were detected in 74% fecal specimens tested. The finding suggested that acute gastroenteritis in infants and children in the city of Birobidzhan, Eastern Russia, might to about 74% be due to diarrheal viruses and 26% caused by other etiologic agents. Among the detected diarrheal viruses, group A rotavirus was the most prevalent and became a leading cause of viral gastroenteritis in infants and children in Eastern Russia, followed by norovirus, group C rotavirus and sapovirus. Interestingly, one fecal specimen turned out to be positive for hepatitis A virus by RT-PCR. This virus was isolated from a 1-year old male child who was admitted to hospital with the clinical manifestation of acute gastroenteritis. This provides evidence that the hepatitis A virus is one of the enteropathogens responsible for viral gastroenteritis among infants and children in Eastern Russia. The results of the study also confirmed the existence of many different co-circulating diarrheal viruses and their important role in causing diarrheal illness in Eastern Russia.

At present, amplification of the genome by multiplex PCR assays used in the present study has been recognized as a convenient and powerful alternative for molecular diagnosis [17-19]. These multiplex PCR assays have demonstrated high sensitivity and specificity, which are comparable to those of monoplex PCR. In case of mixed infection, multiplex PCR can detect up to 4 target viruses with different viral loads in only a single PCR tube [17-19]. Furthermore, primer selection for the target viruses in these multiplex PCR assays was based on the sizes of the amplicons generated. These primers produced different PCR products for different target viruses. Thus, these amplicons could be visualized and easily differentiated by agarose gel electrophoresis [17-19]. However, we also used different methods such as serotyping for group A rotavirus and sequencing for norovirus, sapovirus, hepatitis A virus, and group C rotavirus to confirm the PCR results.

RNA recombination is a mechanism for virus evolution [20]. There is now a fairly rich literature documenting recombination in norovirus. The first naturally occurring recombinant norovirus was the prototype Snow Mountain virus in the US [21]. Later, several recombinant noroviruses causing sporadic cases and outbreaks of acute gastroenteritis were reported worldwide, such as the Thai isolate Mc37 [22], the Japanese isolate Saitama U1 [15], the Australian isolate Sydney C14/02/AU [23], the German isolate BRA/2.2/98/DEU [24], and the Arg302 from Argentina [25] but no recombinant norovirus from Russia. In this study, the capsid sequences of

the HU/78/04/RU and the Toronto24/91/CA were highly identical, but the polymerase sequence of the HU/78/04/RU was distinctly different from that of the Toronto24/91/CA. Furthermore, an apparent site of genetic recombination of this norovirus was found in the ORF1/ORF2 overlap. This pattern of genetic characterization of the HU/78/04/RU implied a naturally occurring recombinant norovirus with GII/7 capsid and novel polymerase and would be the first recombinant norovirus identified in Russia. The phylogenetic analysis of the polymerase and capsid sequences of the four norovirus isolates in the present study demonstrated only one recombinant NoV GII sequence. Moreover, they had high homology (100%) at the nucleotide level and the amino acid level. It was indicative that they came from the same source of norovirus infection. However, in order to monitor the quality of extraction and RT-PCR, Hu/NoVGII/Maizuru/5188 and MilliQ water were used as positive and negative controls, respectively. To avoid false positive results of the experiments, instructions to prevent cross-contamination were strictly followed [26]. Therefore, contamination leading to the detection of these NoV isolates was unlikely.

Recombination is one of the major driving forces of viral evolution [27]. Recombination is known to depend on various immunological and intracellular constraints that allow the recombinant virus to adapt to different environments and to rapidly emerge as the predominant population [20]. Recombinant viruses are all alike in that they successfully pass through five stages: i) successful co-infection of a single host, ii) successful co-infection of a single cell, iii) efficient replication of both parental strains, iv) template switching, v) purifying selection for viable recombinants to be transmitted [20]. In this study, four recombinant noroviruses were recovered from children with clinical manifestations of acute gastroenteritis in Eastern Russia. This observation clearly indicated that these Russian norovirus isolates theoretically fulfilled all prerequisites for recombination.

Norovirus capsid is reported to contain the determinants which are important for immune recognition [28]. The capsid gene has been predicted to be well suited for the genotype classification of circulating norovirus strains [15]. In this study, the norovirus isolates in Eastern Russia were recognized to belong to two distinct norovirus clusters (GII/3 and novel cluster) by capsid- and polymerase-based groupings. Moreover, the recent demonstration of recombination in an increasing number of norovirus suggests that it is a more general event than was previously realized. Thus, the phylogenetic classification of norovirus based only on the nucleotide sequence of the capsid gene is rather questionable. We suggest that classification of norovirus strains should rely not only on the capsid sequence but also on the polymerase sequence.

In conclusion, even though the fecal specimens were collected during a short period of time (5 months), this report is still the first indication to demonstrate the

diversity of diarrheal viruses, especially recombinant norovirus co-circulating among infants and children with acute gastroenteritis in Eastern Russia. Our results have described the genetic characterization of naturally occurring recombinant noroviruses as well as increased the evidence for the worldwide distribution of recombinant noroviruses.

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ORIGINAL ARTICLE

**Detection and Genetic Characterization of Norovirus Strains
Circulating among Infants and Children with Acute Gastroenteritis
in Japan during 2004-2005**

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SUMMARY

A total of 752 fecal specimens collected during the period of July 2004 to June 2005 from infants and children with acute gastroenteritis from four different regions (Maizuru, Tokyo, Sapporo, and Osaka) of Japan were tested for the presence of norovirus by RT-PCR. It was found that 139 (18.5%) fecal specimens were positive for norovirus. Norovirus infection was detected almost all year round with the highest prevalence in January. Norovirus GII was the most predominant genogroup (98.6%; 137 of 139). The genotypes detected in this study were GI/1, GII/1, GII/3, GII/4, and GII/6. Of these, NoV GII/4 (known as the Lordsdale virus cluster) was re-emerging and became the leading genotype (77.7%). Meanwhile, the incidence of NoV GII/3 (known as the Arg320 virus cluster) has dropped rapidly, accounting for only 15.8%. Another interesting feature of the study was the identification of Picton03/AU-like recombinant NoV for the first time in Japan. Based on the genetic analysis, it was interesting to note that NoV GII/4 in 2004-2005 made a distinct cluster in comparison to other NoV GII/4 circulating in 2002-2003 and 2003-2004. Of note, "new recombinant variant designated GIIB" within NoV GII/3, which was first detected in Saga City, Japan in 2003-2004 in only one case, had increased, spreading widely in Japan and representing 45.5% (10 of 22). Further epidemiological studies should be conducted to determine whether this new recombinant variant strain will be dominant in Japan in the coming year. (Clin. Lab. 2006;52:519-525)

KEY WORD

RT-PCR, norovirus, gastroenteritis, Japan

INTRODUCTION

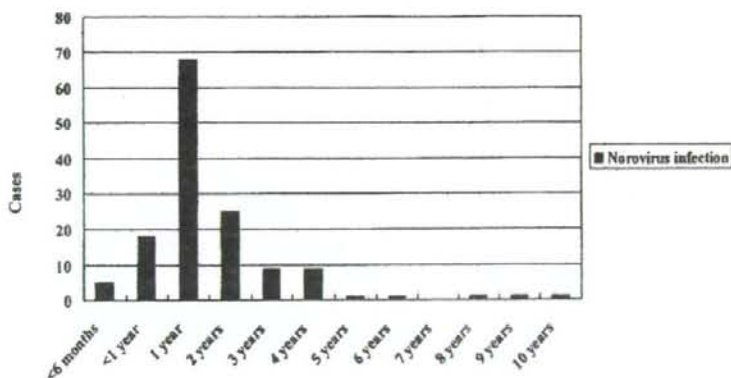
Viral gastroenteritis is one of the most common illnesses in humans worldwide and has a great impact on people [1]. The mortality among children due to acute gastroenteritis is greater in developing than in developed countries. Annual mortality associated with acute gastroenteritis was estimated to be 2.1 million in 2000 [2,

3]. Among different kinds of diarrheal viruses, norovirus (NoV) is also considered to be a significant global enteropathogen, being a major cause of acute gastroenteritis in infants and young children worldwide [4, 5]. The transmission routes of this virus are classified into food-borne, water-borne, air-borne, person-to-person spread and might be by some other unknown modes [5, 6]. NoV is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make NoV a major public health concern [7].

NoV (formerly known as "Norwalk-like virus") is the distinct genus within the family *Caliciviridae*. NoV is one of the leading agents of acute gastroenteritis world-

Table 1: Distribution of norovirus genotypes circulating among infants and children with acute gastroenteritis in four different regions of Japan

Date of fecal specimen collection	Number of specimens tested	Fecal specimens positive for norovirus (%)	Genogroup I		Genogroup II		
			Genotype 1	Genotype 1	Genotype 3	Genotype 4	Genotype 6
July 2004-June 2005	752	139 (18.5)	2 (1.4)	1 (0.7)	22 (15.8)	108 (77.7)	6 (4.4)

**Figure 1: Age-related distribution of NoV infection among infants and children with acute gastroenteritis in four different regions (Maizuru, Tokyo, Sapporo, and Osaka) of Japan during the period of July 2004 to June 2005.**

wide and causes outbreaks in various epidemiological settings such as restaurants, schools, day care centers, hospitals, nursing homes, and cruise ships [2, 5, 6]. The prototype strain of NoV is the Norwalk virus, which was originally discovered in an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA in 1968 [8]. NoV contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The NoV genome contains three ORFs (ORF1, 2 and 3). The ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase (RdRp), ORF 2 encodes the capsid protein (VP1), and ORF3 encodes a small capsid protein (VP2). Based on the sequence analysis of the capsid gene, NoV is divided into genogroups I and II known to infect humans. A recent study indicated that NoV GI and NoV GII could be classified into 14 and 17 genotypes, respectively [9]. To date, NoV is still uncultivable by standard culture with different cell lines. However, expression of either VP1 alone or both VP1 and VP2 using recombinant baculoviruses formed the virus-like particles (VLPs) that are morphologically and antigenically similar to the native virion [10].

The objectives of this study were: to determine the incidence of NoV infections in infants and children with acute gastroenteritis in four different regions of Japan during 2004 and 2005, to characterize NoV detected according to genogroup and genotype, and to describe the genetic diversity among them. Additionally, the age-related distribution and seasonal pattern of NoV infection were also determined.

MATERIALS AND METHODS

Fecal specimens

A total of 752 fecal specimens were collected from sporadic cases of acute gastroenteritis in four pediatric clinics, encompassing four different regions (Maizuru, Tokyo, Sapporo, and Osaka) of Japan during the period of July 2004 to June 2005. A 10% fecal suspension was prepared in distilled water and clarified by centrifugation at 10,000 x g for 10 min. The supernatant was collected and stored at -30 °C until use.

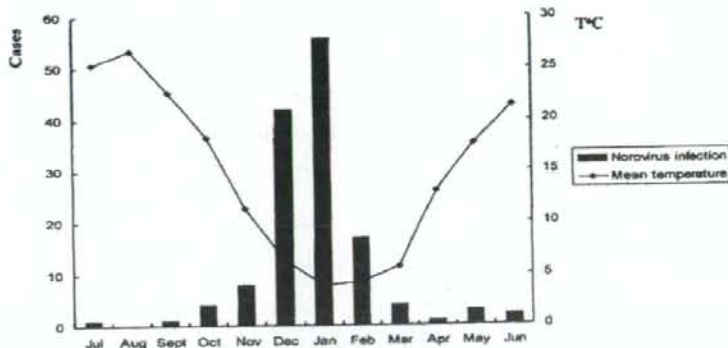


Figure 2: Seasonal variation of NoV infection among infants and children with acute gastroenteritis in four different regions (Maizuru, Tokyo, Sapporo, and Osaka) of Japan during the period of July 2004 to June 2005. The mean temperature in Japan is also shown.

Extraction of viral genome

The viral genomes were extracted from 140 µl of 10% fecal suspensions using the QIAamp viral RNA Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions in which multiple washing steps in the process of RNA extraction eliminate inhibitors in fecal specimens.

Reverse transcription (RT)

For reverse transcription (RT), 4 µl of extracted viral genome were added to the reaction mixture containing 1 µl of 5x first strand buffer (Invitrogen, Carlsbad, CA, USA), 0.4 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.4 µl of 10 mM DTT (Invitrogen), 0.4 µl (200 U/µl) of superscript reverse transcriptase III (Invitrogen, Carlsbad, CA, USA), 0.2 µl (1 µg/µl) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.3 µl (33 U/µl) of RNase inhibitor (Toyobo, Osaka, Japan), and 1.3 µl MilliQ water. The total volume of the reaction mixture was 8 µl. The RT step was carried out at 50 °C for 1 h, followed by 99 °C for 5 min and then held at 4 °C [11].

Polymerase chain reaction (PCR)

The NoV genogroups were identified by PCR method using specific primers as previously described [11]. Two pairs of specific primers GISKF (CTGCCCGAATTGTAATGA) and G1SKR (5'-CCAACCCARCCATT-RTACA), and COG2F (CARGARBCNATGTTYAGRTGGATGAG) and G2SKR (CCRCNCGCATRHCCRTT-RTACAT) [where B was C, G or T; H was A, C or T; N

was any base; R was A or G, and Y was C or T] that amplify both the polymerase gene and the capsid gene of NoV were used to detect NoV GI and NoV GII, respectively. These primers specifically generated two different sizes of amplicons of 330 bp and 387 bp for NoV GI and NoV GII, respectively. The PCR was carried out with 1 µl of cDNA in 10 µl of the reaction mixture containing 10x Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/µl), primers (33 µM), Taq DNA polymerase (5 U/µl) (Promega, Madison, WI, USA) and MilliQ water. The PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min and then visualized under ultraviolet (UV) light. The results were recorded by photography.

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of PCR products (DNA) positive for NoV were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.). Sequence analysis was performed using CLUSTAL X software (Version 1.6). A phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using

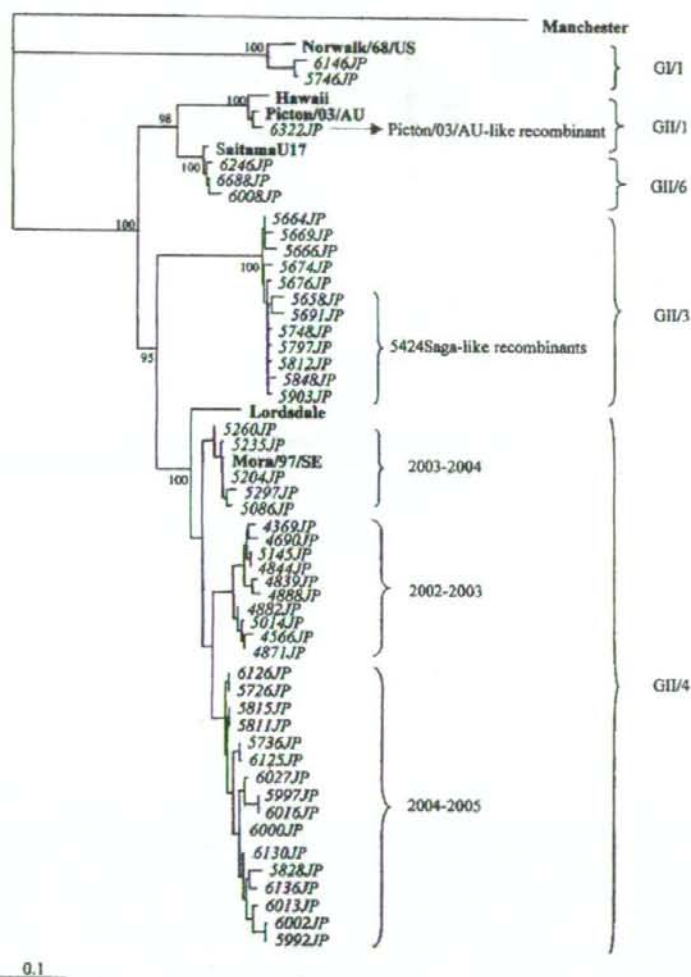


Figure 3: Phylogenetic tree of the nucleotide sequences of NoV. The tree was constructed from partial nucleotide sequences of the capsid region of NoV GII strains detected in Japan. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV is highlighted in italics. The Manchester strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values.

Kimura's two-parameter method (PHYLIP). Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Norwalk/68/US (M87661), Hawaii (U07611), Picton/03/AU (AY919139), SaitamaU17 (AB039779), Lordsdale (X86557), and Mora/97/SE (AY081134).

RESULTS

Molecular epidemiology of norovirus infections

A total of 752 fecal specimens collected from infants and children with acute gastroenteritis from four different regions of Japan during the period from July 2004 to

June 2005 were examined for the presence of NoV. NoV was detected in 139 out of 752 (18.5%) specimens tested. The detection rates of NoV were different between the four regions, including 15.2% in Maizuru, 29.4% in Sapporo, 16.7% in Tokyo, and 22.5% in Osaka. The highest prevalence of NoV was found in infants and children aged 1 year (68 of 139, 48.9%). Only 5 cases (3.6%) of NoV infection were identified among infants aged less than 6 months (Figure 1). It was also found that infants and children younger than 3 years had a high rate of NoV infection (116 of 139, 83.5%). NoV was detected almost all year round (Figure 2). However, none of NoV was detected in August 2004. The NoV incidence was found to be highest in January (56 of 139, 40.3%), followed by December (42 of 139, 30.2%), and February (27 of 139, 19.4%). The lowest NoV detection rate was in July, September, and April (1 of 139, 0.7%).

Nucleotide sequence and phylogenetic analyses of NoV genotypes

The partial nucleotide sequences of the capsid gene of NoV detected in this study were compared to each other as well as to those of NoV reference strains available in the DDBJ DNA/GenBank database by BLAST. A total of 139 NoV nucleotide sequences, including 2 of NoV GI and 137 of NoV GII were analyzed by phylogenetic grouping based on the recent NoV capsid region classification schemes described by Kageyama et al., 2004 [9]. It was found that the NoV GI sequences clustered into one distinct group with GI/1, which was represented by the Norwalk/86/US virus cluster (Figure 3). The NoV GI strains in Japan showed 98%-100% nucleotide sequence identities with those of other human NoV reference strains.

Of 137 NoV GII sequences, four distinct genotypes, GII/1, GII/3, GII/4, and GII/6, were identified (Table 1). Of these, the GII/4 (known as the Lordsdale virus cluster) was the most predominant genotype with a prevalence rate of 77.7%, followed by 15.8% for GII/3 (the Arg320 virus cluster), 4.4% for GII/6 (the Seacroft virus cluster), and 0.7% for GII/1 (the Picton/03/AU virus cluster). It was found that NoV strains in the study of the same genotype shared a high homology with each other, ranging from 98% to 100% even when they were detected in different regions of Japan. The nucleotide sequence identities ranged from 57% to 99% when NoV GII strains detected in this study were compared with those of the reference strains previously registered in the DDBJ DNA/GenBank database.

Nucleotide sequence and genetic analyses of the NoV RNA polymerase gene

To further investigate the evolution of NoV in Japan, the RNA polymerase sequences of all NoV strains were additionally analyzed. Of 22 NoV strains with GII/3 capsid, 10 shared a high homology (97%-99%) with the NoV strain 5424Saga in both polymerase and capsid genes. The NoV strain 5424Saga was previously reported as a recombinant virus with GII/3 capsid and GIIb

polymerase. The findings suggested that these 10 NoV strains were also all recombinant viruses. Interestingly, another NoV strain, the 6322JP, was grouped with NoV reference Picton/03/AU, which was designated as a rare recombinant virus in Australia. Polymerase and capsid genes of the 6322JP were homologous with the Picton/03/AU, ranging from 99% to 100% at the nucleotide and amino acid levels. Taken together, the results indicated that the 6322JP was also the recombinant strain with GII/1 capsid and GIIb polymerase. In contrast, the genotypes of all NoV strains belonging to GI/1 (the Norwalk/68/US virus cluster), GII/4 (the Lordsdale virus cluster), GII/6 (the SaitamaU17 virus cluster), and of other GII/3 strains (the Arg320 virus cluster) remained the same, no matter whether the polymerase or capsid regions were analyzed.

DISCUSSION

Viral gastroenteritis is still a health burden in developed and developing countries [12, 13]. It has been reported that NoV is a major agent causing non-bacterial gastroenteritis and is globally associated with sporadic cases and outbreaks of this illness. NoV infection causes acute gastroenteritis in all age groups, though it occurs predominantly in young children [2, 14]. In the present study two pairs of specific primers (G1SKF and G1SKR, COG2F and G2SKR) were used to detect NoV. In the molecular epidemiology of NoV from 66 outbreaks of acute gastroenteritis in a variety of settings, including restaurants, schools, hotels, dormitories, and nursing homes in Saitama, Japan during the period of 1997 to 2002, 19 different NoV genotypes were identified when PCR with these primers was conducted [9]. Moreover, these primers could detect NoV not only in feces but also in sewage, treated wastewater, river water, and oysters [15, 16]. Taken together, these primer sets could amplify NoV strains of wide diversity and different origins. In this study it was found that infants and children aged less than 3 years had a high rate of NoV infection, which accounted for 83.5%. Out of 752 fecal specimens tested, 18.5% were determined to be positive for NoV by RT-PCR. These results were consistent with previously published reports on NoV epidemiology worldwide in which its prevalence was shown to range from 10% to 60% or more [2, 5, 17-19]. The finding suggested that from acute gastroenteritis in infants and children in four regions of Japan about 18.5% might be due to NoV and 81.5% caused by other etiologic agents. The result also confirmed NoV as one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. All fecal specimens were also screened for group A rotavirus and adenovirus. It was found that 82 specimens (10.9%) were positive for group A rotavirus and 34 specimens (4.5%) were positive for adenovirus. Of these, two coinfections between group A rotavirus and NoV GII, and another co-infection between adenovirus and NoV GII

were identified (data not shown). In some reports, NoV was prevalent in the cold season, whereas several studies did not find a seasonal correlation [20-24]. In strong agreement with the surveillance on pediatric cases of viral gastroenteritis in Japan, the main peak of NoV infection was between November, December, and January [25, 26]. In this study the highest incidence of cases was in the 1-year age group, and the incidence decreased with increasing age over 1 year. Quite possibly, 1-year old children might lack antibody protection to NoV, whereas by the time they have reached the age of 2 years they have begun to acquire viral immunity.

The results of the study showed that all Japanese NoV isolates belonged to two distinct genogroups, GI and GII, and these represented 1.4% and 98.6%, respectively. The results indicated that NoV GII was the dominant group causing acute gastroenteritis among Japanese pediatric populations. Extensive epidemiological studies of NoV infection worldwide, including Japan, which characterized NoV strains and identified the prevalent genotypes circulating in infants and children with acute gastroenteritis, have indicated that NoV GII/4 was the most prevalent genotype [5, 9, 23, 25]. However, the emergence of new NoV GII/3 was identified, and this strain became the leading genotype (43.9%) in Japan during 2003 to 2004 [27]. At the same time, the prevalence of NoV GII/4 rapidly dropped from 75.6% in 2002-2003 [28] to 35.1% in 2003-2004 [27]. In this study, the changing pattern of genotype distribution of NoV infection in children with acute gastroenteritis has been demonstrated. Of note, the NoV GII/4 re-emerged to be the most prevalent with a high frequency (77.7%) compared to the lower frequency of NoV GII/3 (15.8%) and NoV GII/6 (4.4%), which were the second and third prevailing genotypes, respectively. We hypothesized that the insufficient antibody protection from acquired viral immunity against NoV GII/4 in Japanese pediatric populations was due to the lack of immunization by the previous NoV GII/4 infection during 2003-2004. This hypothesis was in strong agreement with recent findings that the detection rate of NoV GII/4 infection was low during 2003-2004 [27]. Interestingly, NoV GII/4 strains detected in this study (2004-2005) made a distinct cluster, which was separate from NoV strains in 2002-2003, and 2003-2004 even all of them belonged to the same genotype. However, this might be due to the co-existence of multiple factors such as changes of climate, water, and others.

Another interesting finding of this study was the identification of the Picton/03/AU-like strain. The Picton/03/AU was isolated from an outbreak of vomiting and diarrhea at a care facility for the elderly in New South Wales, Australia in July 2003 and had been reported to be a rare recombinant with GII/1 capsid and GIIb polymerase [29]. Surprisingly, our strain, the 6322JP, was not recovered from an elderly patient but from a 1-year old male child with acute gastroenteritis in Maizuru City in 2005. The 6322JP shared a high identity with the Picton/03/AU and therefore it also was a NoV recom-

binant. This is the first report of the detection of the rare Picton/03/AU-like recombinant in Japan. More interestingly, the high detection rate of "new NoV variant with GIIb polymerase" 5424Saga-like strains was identified. "New NoV variant with GIIb polymerase" was recently found to cause outbreaks as well as sporadic cases of acute gastroenteritis throughout European countries [30-32]. In Japan, the strain 5424Saga, recognized as a "new NoV variant with GIIb polymerase", was first recovered from a male patient aged 2 years who developed symptoms of acute gastroenteritis in Saga City in 2003 and had been reported to be a recombinant with GII/3 capsid and GIIb polymerase based on the genetic analysis [27]. The sudden increase in the number of the variant strain from 4% in 2003-2004 to 45% in 2004-2005 indicated that this variant was still virulent in causing the illness in Japan. Further surveillance of diarrheal viruses should be conducted to determine whether this recombinant NoV variant will be dominant in Japan in the coming year.

Acknowledgements

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Novel Recombinant Norovirus in China

To the Editor: Norovirus (NoV), the distinct genus within the family *Caliciviridae*, is a major cause of sporadic cases and outbreaks of acute gastroenteritis in humans (1). NoV possesses a positive-sense, single-stranded RNA genome surrounded by an icosahedral capsid. The NoV genome contains 3 open reading frames (ORFs). ORF1 encodes non-structural proteins, ORF 2 encodes capsid protein (VP1), and ORF3 encodes a small capsid protein (VP2). NoV is still uncultivable by standard culture with different cell lines. However, expression of either VP1 or both VP1 and VP2 with recombinant baculoviruses formed viruslike particles that are morphologically and antigenically similar to the native virion (2).

A fecal specimen was collected from an infant hospitalized with acute gastroenteritis in Kunming, China, in November 2004 and was tested for diarrheal viruses in a cooperative laboratory in Japan. The viral genome was extracted by using a Qiagen kit (Qiagen, Hilden, Germany). Polymerase chain reaction with specific primers resulted in the identification of astrovirus, rotavirus, sapovirus, adenovirus, and NoV genogroup I (GI) and GII (3). NoV polymerase was also amplified to identify recombinant NoV with primers Yuri22F and Yuri22R (4). Products were sequenced directly, and sequence analysis was performed by using ClustalX and SimPlot.

The fecal specimen was positive for NoV GII. The Figure shows that the 146/Kunming/04/China sequence clustered into the distinct GII genotype 7 (Leeds/90/UK cluster). 146/Kunming/04/China was classified into the Saitama U4 cluster (GI/6) when polymerase-based grouping was performed. Altogether, 146/Kunming/04/China was expected to be the

recombinant NoV with GII/7 capsid and GII/6 polymerase.

To eliminate the possibility of coinfection with 2 different NoV genotypes, to localize the potential recombination site, and to clarify a possible recombination mechanism, the ORF1/ORF2 overlap and flanking polymerase and capsid regions of 146/Kunming/04/China was amplified with primers Yuri22F and GIISKR to produce a 1,158-bp amplicon (3,4). When the sequence of 146/Kunming/04/China was compared with that of Saitama U4 by using SimPlot, a recombination site was found at the ORF1/ORF2 overlap. Before this junction, 146/Kunming/04/China and Saitama U4

were homologous. After the ORF1/ORF2 overlap, however, the homology was notably different. SimPlot showed a sudden drop in the nucleotide identity for 146/Kunming/04/China. ClustalX showed that 146/Kunming/04/China shared a high identity (93%) in the polymerase region and a low identity (78%) in the capsid region with Saitama U4. In contrast, high identity (95%) in the capsid region was found between 146/Kunming/04/China and Leeds/90/UK. Since Leeds/90/UK polymerase was not available in GenBank, the polymerase homology between 146/Kunming/04/China and Leeds/90/UK was unknown. Polymerase of 146/Kunming/04/China was almost

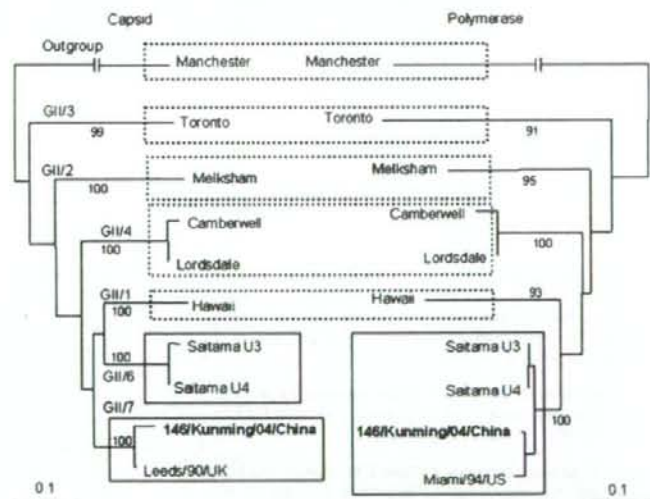


Figure. Changes in norovirus (NoV) genotypes on the basis of phylogenetic trees of nucleotide sequences of 146/Kunming/04/China. Trees were constructed from partial nucleotide sequences of capsid and polymerase regions of 146/Kunming/04/China. 146/Kunming/04/China is boldface. Dashed boxes indicate the maintenance of genotypes of reference NoV strains, and solid boxes indicate the involvement of NoV genotypes with recombinant NoV 146/Kunming/04/China. A phylogenetic tree with 100 bootstrap resamples of the nucleotide alignment datasets was generated by using the neighbor-joining method with ClustalX. The genetic distance was calculated by using the Kimura 2-parameter method (PHYLIP). The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. Manchester strain was used as an outgroup strain for phylogenetic analysis. The nucleotide sequence of NoV strain 146/Kunming/04/China had been submitted to GenBank and has been assigned accession no. DQ304651. Reference NoV strains and accession nos. used in this study are as follows: Manchester (X86560), Toronto (U02030), Melksham (X81879), Camberwell (AF145896), Leeds/90/UK (AJ277608), Lordsdale (X86557), Hawaii (U07611), Saitama U3 (AB039776), Saitama U4 (AB039777), and Miami/94/US (AF414410).

identical with that of Saitama U4, but the capsids of 146/Kunming/04/China and Leeds/90/UK were distinctly different from that of Saitama U4. This genetic pattern of 146/Kunming/04/China implied a novel, naturally occurring recombinant NoV with GII/7 capsid and GII/6 polymerase.

RNA recombination is a mechanism for virus evolution (5). Literature documenting recombination in NoV is fairly rich, but none is from China (6). Therefore, 146/Kunming/04/China was not only the first but also the first recombinant NoV from China. This isolate shared the closest sequences of polymerase and capsid with Saitama U4 and Leeds/90/UK, respectively. Strain Saitama U4 was detected in 1997 in Japan (7), whereas strain Leeds/90/UK was detected in 1990 in the United Kingdom (8). Quite possibly, Saitama U4 and Leeds/90/UK were parental strains of 146/Kunming/04/China. However, the distant geographic relationship of these strains obscured evidence of where and when the recombination event occurred. This phenomenon also suggested that these parent strains or this progeny strain might be more prevalent than is often assumed.

Recombination depends on various immunologic and intracellular constraints. Recombinant viruses are all alike in that they successfully pass through 5 stages: 1) successful co-infection of a single host, 2) successful co-infection of a single cell, 3) efficient replication of both parental strains, 4) template switching, and 5) purifying selection (9). In this study, 146/Kunming/04/China was recovered from a patient with diarrhea, fever, and vomiting. This observation indicated that this strain theoretically fulfilled all prerequisites for recombination.

The NoV capsid is predicted to be well suited for genotype classification (10). In this study, 146/Kunming/04/China belonged to 2 distinct genotypes, 7 and 6, by capsid- and poly-

merase-based groupings, respectively. Moreover, the recent demonstration of recombination in an increasing number of NoVs suggests that it is a more widespread event than was previously realized. Consequently, the phylogenetic classification of NoV on the basis of on capsid sequence is questionable. We suggest that classification of NoV strains should rely on not only capsid sequence but also polymerase sequence.

In conclusion, our results described the genetic characterization of novel, naturally occurring recombinant NoV and increased evidence for the worldwide distribution of recombinant NoV. This report is the first to describe acute gastroenteritis caused by recombinant NoV in China and warns of the threat it poses.

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Detection of Norovirus Antigens from Recombinant Virus-Like Particles and Stool Samples by a Commercial Norovirus Enzyme-Linked Immunosorbent Assay Kit

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The commercial norovirus enzyme-linked immunosorbent assay kit was evaluated for its reactivity to recombinant virus-like particles and the detection of natural viruses from stool samples of Japanese infants and children with sporadic acute gastroenteritis compared to reverse transcription-PCR. The kit had a sensitivity of 76.3% and a specificity of 94.9%. Our results clearly indicated that the kit allows the detection of the most prevalent genotype, GII/4. In order to increase the sensitivity of the kit, the reactivity with norovirus of GII/3 and GII/6 genotypes needs to be improved.

Norovirus (NoV) is one of the leading etiologic agents of nonbacterial sporadic acute gastroenteritis (AGE) in infants and children, and outbreaks of this infection may be due to contaminated water or food. At present, the reverse transcription-PCR (RT-PCR) assay is widely used to detect NoV in diarrheal stool samples. The development of immunological methods to detect NoV has been delayed due to the lack of viruses in cell culture and to diverse genotypes with distinct antigenicities. NoVs are currently divided into five genogroups, and most human NoV strains belong to two genogroups: genogroup I (GI) and genogroup II (GII). Furthermore, each genogroup contains at least 15 and 18 genotypes, respectively (13). RT-PCR is an expensive and complicated technique, and its use requires special equipment and skills. Thus, a faster and simpler method is needed. At present, three commercial enzyme-linked immunosorbent assay (ELISA) kits are available, the IDEIA NLV kit from Dako Cytomation, Ltd. (Ely, United Kingdom), the SRSV(II)-AD kit from Denka Seiken Co., Ltd. (Tokyo, Japan), and the RIDASCREEN norovirus (R-Biopharm AG, Darmstadt, Germany). According to previous evaluations of the ELISA kits, the first two kits cannot effectively replace RT-PCR for NoV detection due to their low sensitivities and/or specificities (1, 3, 19). To date, the RIDASCREEN norovirus assay has only been evaluated by one Australian group using outbreak specimens (2). No research has hitherto been conducted using recombinant virus-like particles (rVLPs) and sporadic stool samples.

Therefore, using the RIDASCREEN norovirus ELISA kit, we set out to measure the reactivity of 16 kinds of rVLPs, to detect the presence of NoV in fecal samples from infants and

children with sporadic AGE in Japan, and to compare the sensitivity and specificity data with those obtained with the RT-PCR.

We previously expressed one rVLP (strain 1207, GII/4) (14). The other 15 rVLPs were prepared from NoV isolated from stool samples among infants and children with diarrhea between 1995 and 2003 in Japan. The genotypic classification of these NoV was performed based on the method described by Kageyama et al. (6). These are genotypes 1 (strain 4656), 3 (strain 3634), 4 (strain 2876), 8 (strain 3006), and 11 (strain 2258) in genogroup I and genotypes 1 (strain 3101), 2 (strain 2840), 3 (strain 3229), 5 (strain 3611), 6 (strain 3612), 7 (strain 419), 12 (strain 2087), 13 (strain 3385), 14 (strain 2468), and 15 (strain 3625) in genogroup II. The production of recombinant bacmids was performed using the baculovirus expression system with Gateway Technology (Invitrogen Japan, Tokyo) and the transfection of bacmids into insect cells, as well as the purification of rVLPs, was performed according to the method of Hansman et al. (5). We used two sense primers: attBINVG1 (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAT GAT GGC GTC TAA GG) for GI strains and attBINVGII (GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC CAT GAA GAT GGC GTC GAA TGA) for GII strains. Purified rVLPs from the cultured supernatants of the insect cells were examined for particle formation by electron microscopy. Protein concentration of each rVLP was measured by BCA Coomassie protein assay (Pierce Biotechnology, Inc., Rockford, IL), and 150 µg/ml was prepared as stock solutions. The assays were started from 10 µg/ml as the highest concentration.

The rVLPs stock solutions were serially threefold diluted with the sample dilution buffer in the the RIDASCREEN norovirus ELISA kit and used to determine the minimal concentration of each rVLP for detection by ELISA according to the manufacturer's manual. All of the assays except that for

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