TABLE II. Distribution of NoV Genotypes in Five Regions of Japan From 1995 to 2001

				No. (%) of genotypes of GII (1995–1996)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	73	0	0	0	0	0	0	0	0	0
Tokyo	98	0	11	0	0	8 (72.7)	0	3 (27.3)	0	0
Maizuru	265	0	24	0	0	20 (83.3)	0	3 (12.5)	1 (4.2)	0
Osaka	—	—	—	—	—	—	—	—	—	—
Saga	—	—	—	—	—	—	—	—	—	—
Total	436	0	35	0	0	28 (80)	0	6 (17.1)	1 (2.9)	0

				No. (%) of genotype of GII (1996–1997)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	50	0	2	0	0	0	0	1 (50)	1 (50)	0
Tokyo	71	0	13	0	10 (76.9)	3 (23.1)	0	0	0	0
Maizuru	239	0	16	1 (6.3)	1 (6.3)	10 (62.5)	1 (6.3)	0	1 (6.3)	G9, G10
Osaka	—	—	—	—	—	—	—	—	—	—
Saga	—	—	—	—	—	—	—	—	—	—
Total	360	0	31	1 (3.2)	11 (35.5)	13 (41.9)	1 (3.2)	1 (3.2)	2 (6.5)	2 (6.5)

				No. (%) of genotypes of GII (1997–1998)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	62	0	16	0	6 (37.5)	4 (25)	0	5 (31.3)	1 (6.3)	0
Tokyo	93	0	16	0	0	16 (100)	0	0	0	0
Maizuru	249	0	16	0	11 (68.8)	5 (31.3)	0	0	0	0
Osaka	96	0	23	0	10 (43.5)	11 (47.8)	0	1 (4.4)	1 (4.4)	0
Saga	—	—	—	—	—	—	—	—	—	—
Total	500	0	71	0	27 (38.0)	36 (50.7)	0	6 (8.5)	2 (2.8)	0

				No. (%) of genotypes of GII (1998–1999)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	43	0	2	0	0	2 (100)	0	0	0	0
Tokyo	80	0	7	0	0	5 (71.4)	0	1 (14.3)	1 (14.3)	0
Maizuru	248	0	21	0	3 (14.3)	12 (57.1)	0	6 (28.6)	0	0
Osaka	134	0	23	0	7 (30.4)	12 (52.2)	0	4 (17.4)	0	0
Saga	87	0	7	0	7 (100)	0	0	0	0	0
Total	592	0	60	0	17 (28.3)	31 (51.7)	0	11 (18.3)	1 (1.7)	0

				No. (%) of genotypes of GII (1999–2000)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	56	0	3	0	3 (100)	0	0	0	0	0
Tokyo	49	GI/4, GI/11	7	0	2 (28.6)	4 (57.1)	1 (14.3)	0	0	0
Maizuru	387	GI/4, GI/3	57	5 (8.8)	5 (8.8)	44 (77.2)	1 (1.8)	1 (1.8)	0	G14
Osaka	121	GI/4, GI/8	14	0	6 (42.9)	7 (50)	0	1 (7.1)	0	0
Saga	153	GI/4	8	0	1 (12.5)	3 (37.5)	0	3 (37.5)	1 (12.5)	0
Total	766	7	89	5 (5.6)	17 (19.1)	58 (65.2)	2 (2.3)	5 (5.6)	1 (1.1)	1 (1.1)

				No. (%) of genotypes of GII (2000–2001)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	44	0	5	0	1 (20)	4 (80)	0	0	0	0
Tokyo	37	0	2	0	0	2 (100)	0	0	0	0
Maizuru	365	0	22	0	3 (13.6)	16 (72.7)	0	0	0	G10, G13
Osaka	108	GI/3, GI/4	23	0	7 (30.4)	7 (30.4)	2 (8.7)	4 (17.4)	1 (4.3)	G14, G15
Saga	106	0	15	0	3 (20)	11 (73.3)	0	0	0	G1
Total	660	4	67	0	14 (20.9)	40 (59.7)	2 (3)	4 (6)	1(1.5)	6(9)

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

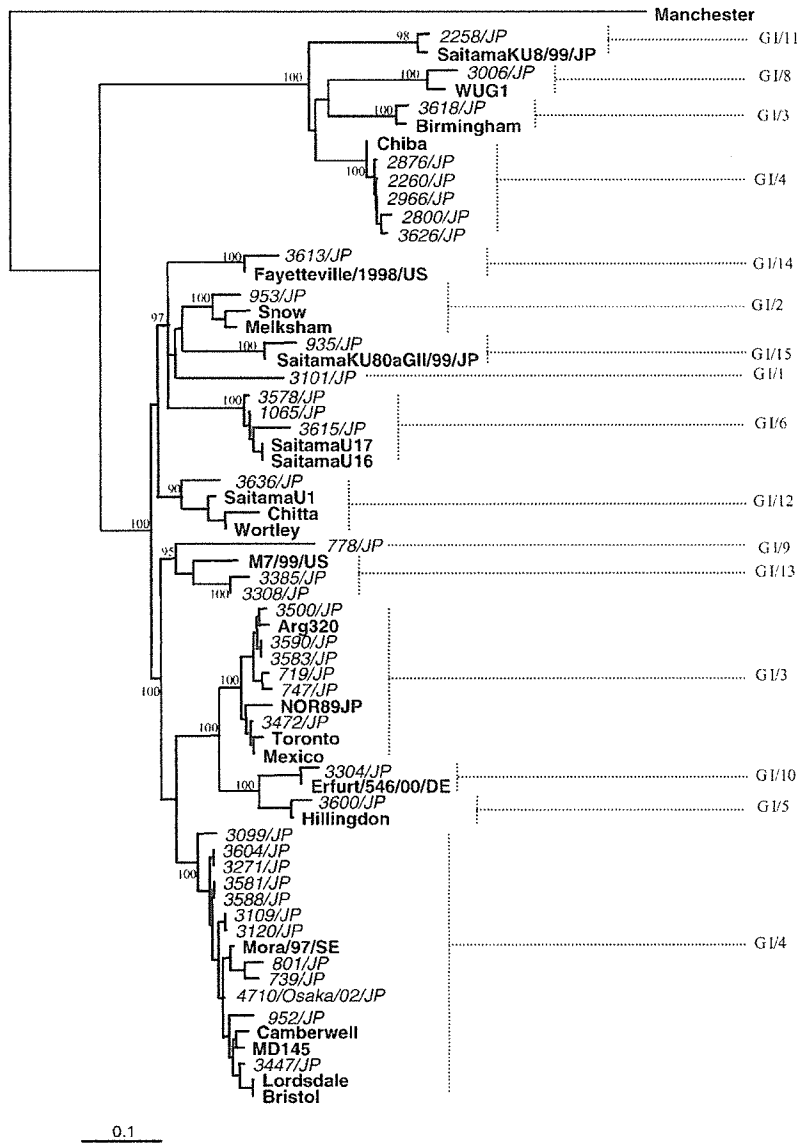

Fig. 2. Phylogenetic tree of nucleotide sequences of NoV. The tree was constructed from partial nucleotide sequences of capsid region of NoV isolates detected in Japan. Reference strains of NoV were selected from the DDBJ/GenBank database under the accession numbers indicated in the text. Japanese NoV 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.
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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

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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 x 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 (1µg/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 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 cheildren with acute gastroenteritis in the city of Birobidzhan, Eastern Russia

Number	Patient	Gender	Age (month)	Date of fecal collection	Norovirus stain	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).

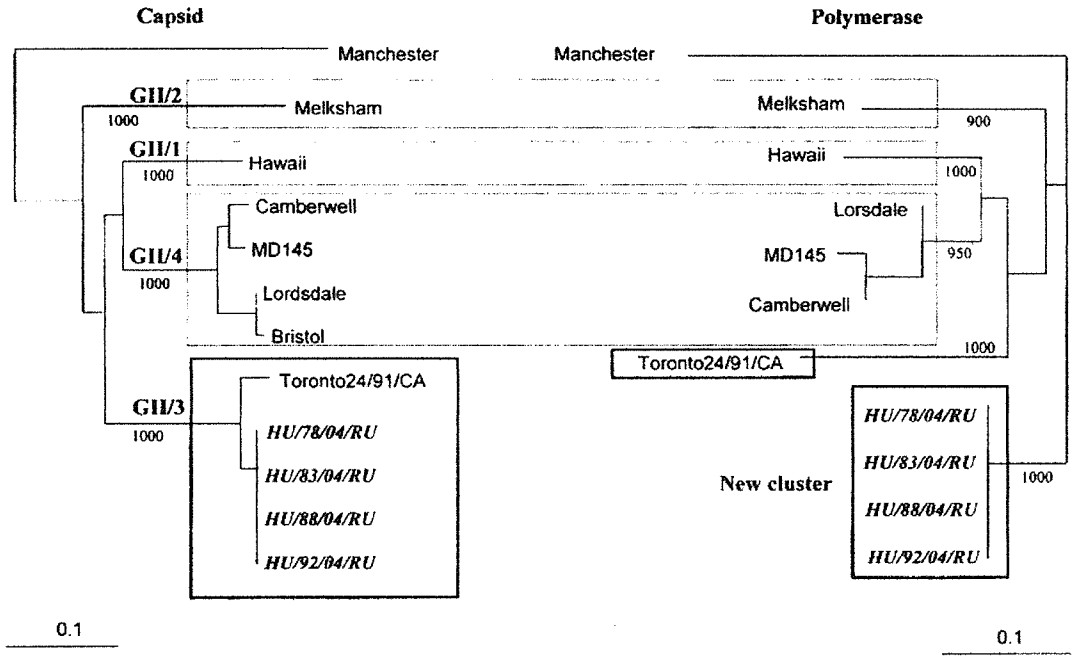

Figure 1: Observation of changes of norovirus genotypes on the basis of phylogenetic trees of nucleotide sequences. The trees were constructed from partial nucleotide sequences of capsid and polymerase regions of Russian isolates of norovirus genogroup II. Reference strains of norovirus were selected 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. Sapovi-rus and hepatitis A virus were genetically determined to belong to genogroup I genotype 1 (GI/1) and subgeno-type 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-

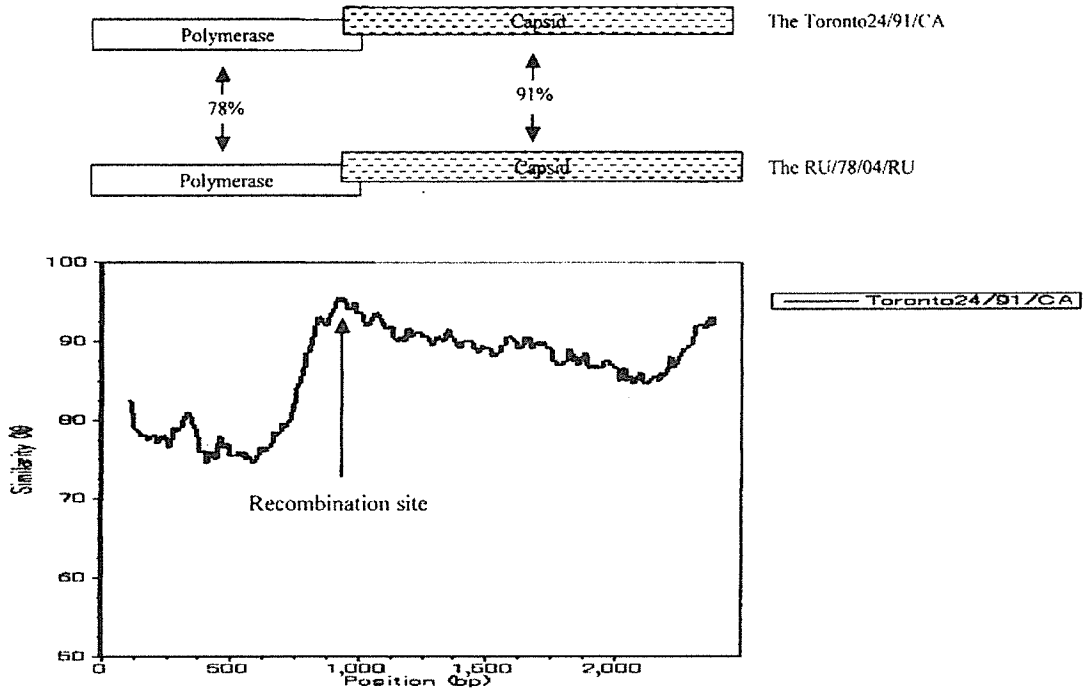

Figure 2: Genetic characterization of recombinant norovirus: the Simplot analysis of strains HU/78/04/RU and the Toronto24/91/CA. The HU/78/04/RU was used as query strain and the Toronto24/91/CA was used as reference strain. Low and high homologies with polymerase and capsid regions among them were found.

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 polymarse 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.

Acknowledgements

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SHORT COMMUNICATION

Novel Intragenotype Recombination in Sapovirus

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SUMMARY

Based on the genetic analysis, a novel, naturally occurring recombination between two distinct sapovirus subtypes (subtype a and subtype b) within genogroup I genotype 1 was identified. Breakpoint analysis of recombinant sapovirus showed that the recombination site was at the polymerase-capsid junction. This is the first report of the existence of acute gastroenteritis caused by intragenotype recombinant sapovirus. The results also provided evidence that the natural recombination occurs not only in sapovirus genogroup II but also in sapovirus genogroup I. (Clin. Lab. 2006;52:363-366)

KEY WORDS

Sapovirus, recombination, intragenotype, Japan

The study

Acute gastroenteritis is a common disease with a high morbidity reported worldwide. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths (1, 2). Sapovirus is recognized as a global enteropathogen, being a significant cause of acute gastroenteritis in such settings as kindergartens, schools, and nursing homes (3, 4). Immunological and seroepidemiologic studies have indicated a worldwide distribution of sapovirus (3, 5). Sapovirus (formerly known as "Sapporo-like virus") is the distinct genus within the family *Caliciviridae*. The sapovirus genome contains two ORFs. The ORF1 encodes non-structural and capsid proteins while ORF2 encodes a small protein. Sapovirus is divided into five genogroups, among which only genogroups I, II, IV, and V are known to infect humans (6).

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A fecal specimen was collected from a 10-month old male child with acute gastroenteritis in Maizuru City, Japan in November 2005. The fecal specimen was diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 x g for 10 min. The supernatant was collected and the viral genomes were extracted by using a QIAamp Viral RNA kit (QIAGEN®, Hilden, Germany). Using PCR with specific primers as previously reported resulted in the identification of the first group of diarrheal viruses including astrovirus, norovirus, and sapovirus and the second group including rotavirus and adenovirus (7). Polymerase was also amplified to detect recombinant sapovirus using primers SR80 and JV33 (8). Products were sequenced directly on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using CLUSTAL X (Version 1.6). A phylogenetic tree with 1000 bootstrap resamples 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 sapovirus sequences. The nucleotide sequence data of sapovirus strain 6728/05/Maizuru/JP had been submitted to GenBank and had been assigned accession number

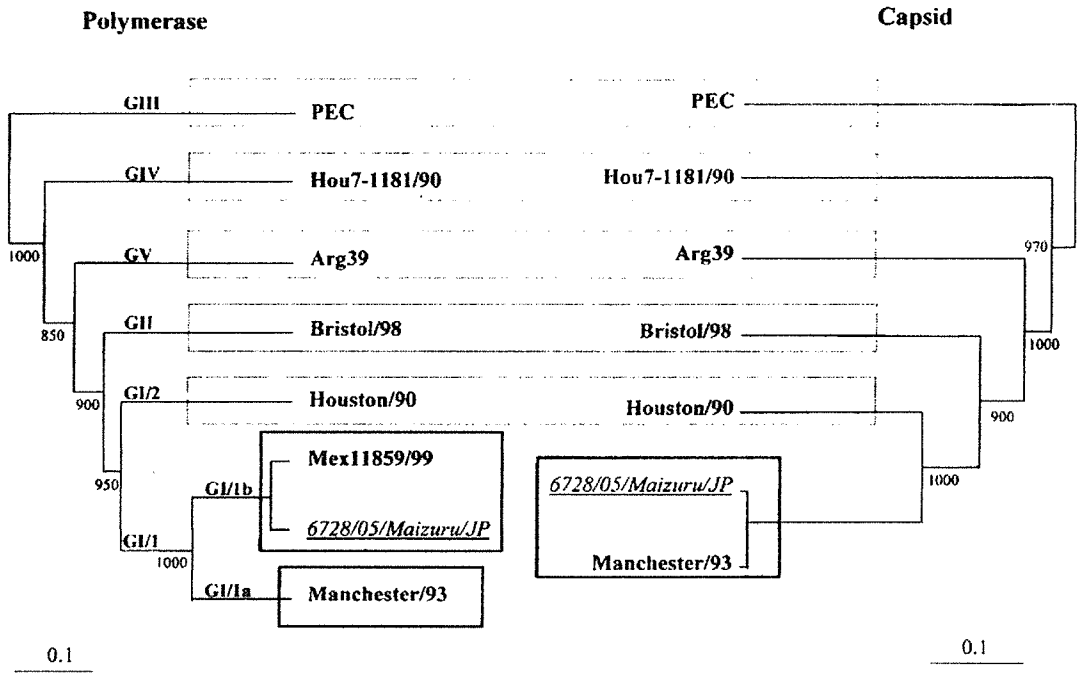

Figure 1: Observation of changes of sapovirus subtypes on the basis of phylogenetic trees of nucleotide sequences of the 6728/05/Maizuru/JP. The trees were constructed from partial nucleotide sequences of the capsid and polymerase regions of the 6728/05/Maizuru/JP. The 6728/05/Maizuru/JP is underlined and highlighted in italics. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. PEC strain was used as an out-group strain for phylogenetic analysis.

DQ395300. Reference sapovirus strains and accession numbers used in this study were as follows: PEC (AF182760), Arg39 (AY289803), Manchester/93 (X86560), Hou7-1181/90 (AF435814), Mex11859/99 (AY157857), Sapporo (U65427), Plymouth (X86559), Houston/86 (U95643), Dresden (AY694184), Houston/90 (U95644), and Bristol/98 (AJ249939).

The fecal specimen was positive for sapovirus. Figure 1 shows that the sequence of the 6728/05/Maizuru/JP was classified into genogroup I genotype 1a (GI/1a) known as the Manchester/93 cluster. Interestingly, the 6728/05/Maizuru/JP with GI/1a capsid belonged to the Mex11859/99 cluster (known as GI/1b) when polymerase-based grouping was performed. Taken together, the 6728/05/Maizuru/JP was expected to be a recombinant sapovirus with GI/1a capsid and GI/1b polymerase.

To eliminate the possibility of co-infection with two different sapovirus subtypes, to localize the potential recombination site as well as to understand a possible recombination mechanism of the novel recombinant sapovirus, flanking polymerase and capsid regions with their junction of the 6728/05/Maizuru/JP were amplified with primers SR80 and SLV5749 to produce 1,151-bp amplicon (7, 8). When the nucleotide sequence of the 6728/05/Maizuru/JP was compared with that of the Manchester/93 using SimPlot, an apparent region of genetic recombination was found at the polymerase-capsid junction. Before this junction, the homology of the 6728/05/Maizuru/JP and the Manchester/93 was different. After this junction, however, they were highly homologous. Using CLUSTAL X, the 6728/05/Maizuru/JP shared a low level of nucleotide identity (89%) in the polymerase region and a high level of the nucleotide identity (99%) in the capsid region with the Manchester/93. In contrast, there was a high level of identity (97%) in the polymerase region between the 6728/05/Maizuru/JP and the Mex11880/99. Since the capsid sequence of the Mex11859/99 was not available in GenBank, the homology in the capsid region between the 6728/05/Maizuru/JP and the Mex11859/99 was unknown. Altogether, the capsid sequences of the 6728/05/Maizuru/JP and the Manchester/93 were almost identical, but the polymerase sequences of the 6728/05/Maizuru/JP and the Mex11859/99 were different from that of the Manchester/93. This pattern of the genetic characterization of the 6728/05/Maizuru/JP implied a novel, naturally occurring recombinant sapovirus with GI/1a capsid and GI/1b polymerase.

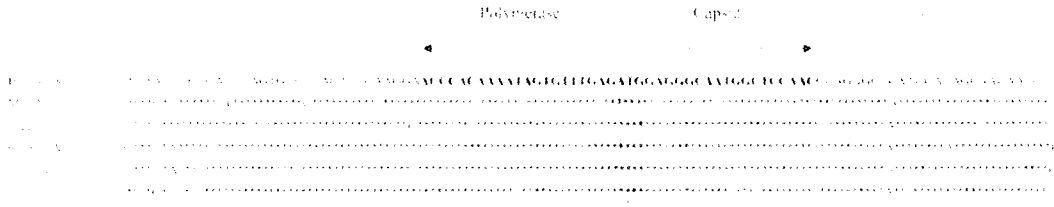

Figure 2: Nucleotide alignment of the 6728/05/Maizuru/JP, the Manchester/93, and reference sapovirus strains of GI/1 in GenBank, showing the highly conserved polymerase and capsid junction which is highlighted in bold type. The asterisks represent conserved nucleotides. The shaded nucleotides represent the putative capsid start codons.

RNA recombination is a mechanism for virus evolution (9). Even though there are now many molecular epidemiological studies on sapovirus infection worldwide, reports documenting recombination in sapovirus are still limited. To date, only 2 kinds of recombinant sapoviruses have been reported as intergenotype recombination and intergenogroup recombination (10, 11). Based on polymerase-based groupings, these recombinations had occurred only in sapovirus genogroup II, which was more capable of recombination than other genogroups (10, 11). However, we identified the 6728/05/Maizuru/JP with a novel recombination between two distinct subtypes (subtype a and subtype b) within genogroup I genotype 1. This is the first report of the existence of acute gastroenteritis caused by intragenotype recombinant sapovirus. The results also provided evidence that the natural recombination occurs not only in sapovirus genogroup II but also in sapovirus genogroup I.

In this study, the 6728/05/Maizuru/JP shared the closest sequences of polymerase and capsid with the Mex11859/99 and the Manchester/93, respectively. Strain Mex11859/99 was detected in 1999-2000 in Mexico (6), whereas strain Manchester/93 was detected in 1993 in the United Kingdom (12). Quite possibly, the Mex11859/99 and the Manchester/93 were parental strains of the 6728/05/Maizuru/JP. However, the very distant geography of these strains did not provide evidence for where and when a recombination event might occur in nature. Even that recombination event in this sapovirus is not completely understood, but it can be potentially dangerous for host species, and it likely limits the virus control programs and has in addition major implications in viral vaccine design.

The capsid gene is predicted to be well suited for the genotype classification of circulating sapovirus strains (4, 6). In this study, the HU/5862/Osaka/JP was recognized to belong to two distinct sapovirus subtypes by capsid- and polymerase-based groupings. Therefore, in order to monitor the evolution of sapovirus in nature, we suggest that classification of sapovirus strains should rely not only on the capsid sequence but also on the polymerase sequence. Altogether, this novel recombinant sapovirus could greatly affect phylogenetic groupings and confuse molecular epidemiological studies.

The primary mechanism involved in recombination in RNA viruses is the copy-choice model in which recombination is known to depend on various immunological and intracellular constraints, (i) successful co-infection of the host and in a single cell by two parental strains, (ii) efficient replication of parental viral genomes with template switching, (iii) adaptation to different environments to be transmitted (9). Furthermore, the observation of circulating 6728/05/Maizuru/JP in the community underscored that this strain theoretically fulfilled all prerequisites for its recombination. However, it was unclear whether the child was infected with this recombinant sapovirus, or whether the recombinant sapovirus resulted from co-infection with 2 different viruses in this child. Using CLUSTAL X it was interesting that the 6728/05/Maizuru/JP, the Manchester/93, and reference sapovirus strains of GI/1 in GenBank shared the 42 nucleotide-conserved region (100% identity) including the last 21 nucleotides of polymerase and the first 21 nucleotides of capsid (Figure 2). This highly conserved region might represent the break and rejoin site for recombination during viral replication. In fact, RNA recombination often takes a major part leading to the emergence of novel viral agents (9). Therefore, this novel recombinant sapovirus in our study will have an epidemiological importance in causing diarrheal diseases that warns of the threat it poses in future. Further epidemiological studies should be conducted to determine whether this recombinant strain would become more predominant in Japan in the coming year.

In conclusion, RNA recombination plays a key role in virus evolution and it shapes a good deal of the virus diversity. Our results have described the genetic characterization of novel, naturally occurring recombinant sapovirus as well as increased the evidence for the worldwide distribution of recombinant sapovirus. This report is also the first indication of intragenotype recombination in sapovirus.

Acknowledgements

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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-

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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)

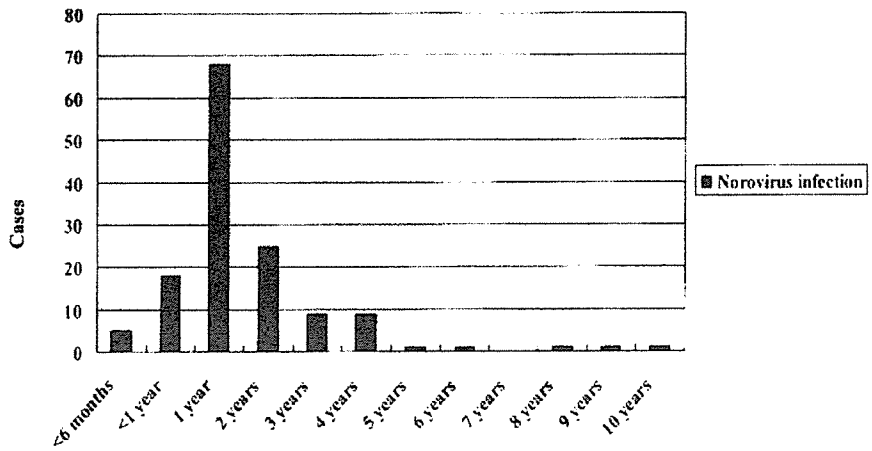

Figure 1: Age-related distribution of NoV infection among infants and children with acute gastroenteritis in four different regions (Maizuru, Tokyo, Sapporo, and Osaka) of Japan during the period of July 2004 to June 2005.

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 uncultivatable 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.

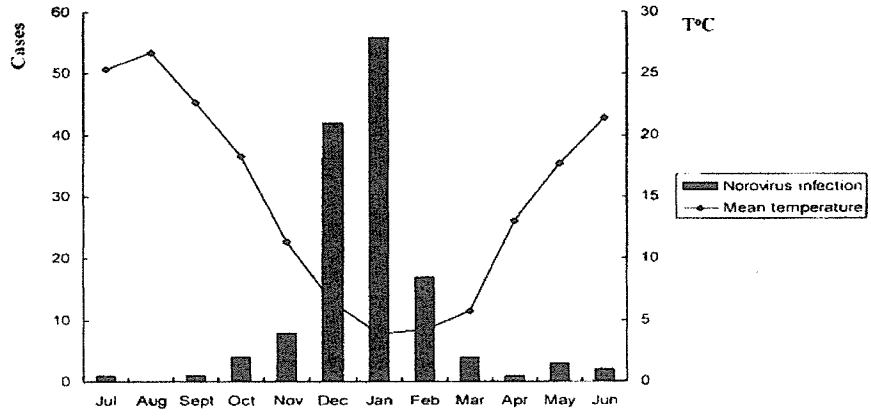

Figure 2: Seasonal variation of NoV infection among infants and children with acute gastroenteritis in four different regions (Maizuru, Tokyo, Sapporo, and Osaka) of Japan during the period of July 2004 to June 2005. The mean temperature in Japan is also shown.

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 (1µg/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 G1SKF (CTGCCCGAATTYGTAAATGA) and G1SKR (5'-CCAACCCARCCATTRTACA), and COG2F (CARGARBCNATGTTYAGRTGGATGAG) and G2SKR (CCRCCNGCATRHCCRTTRTACAT) [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

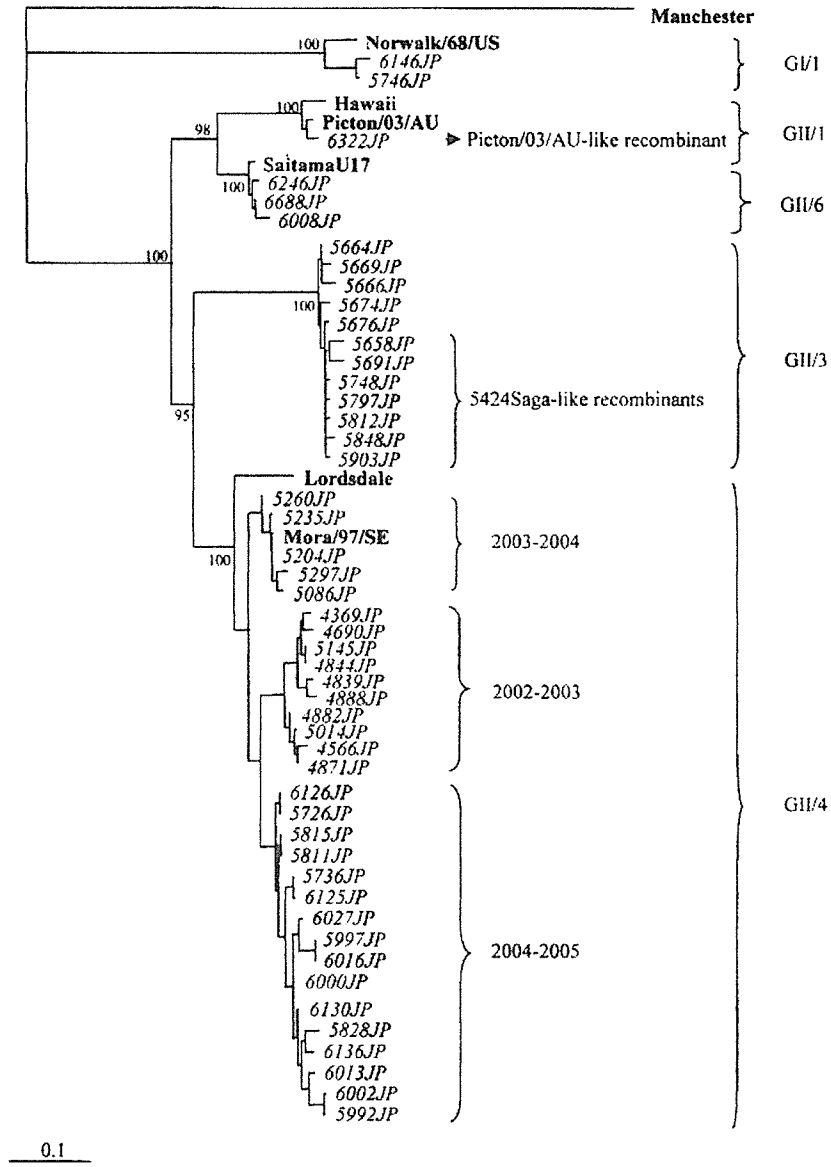

Figure 3: Phylogenetic tree of the nucleotide sequences of NoV. The tree was constructed from partial nucleotide sequences of the capsid region of NoV GII strains detected in Japan. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the 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, GII4, 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 co-infections between group A rotavirus and NoV GII, and another co-infection between adenovirus and NoV GII