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)

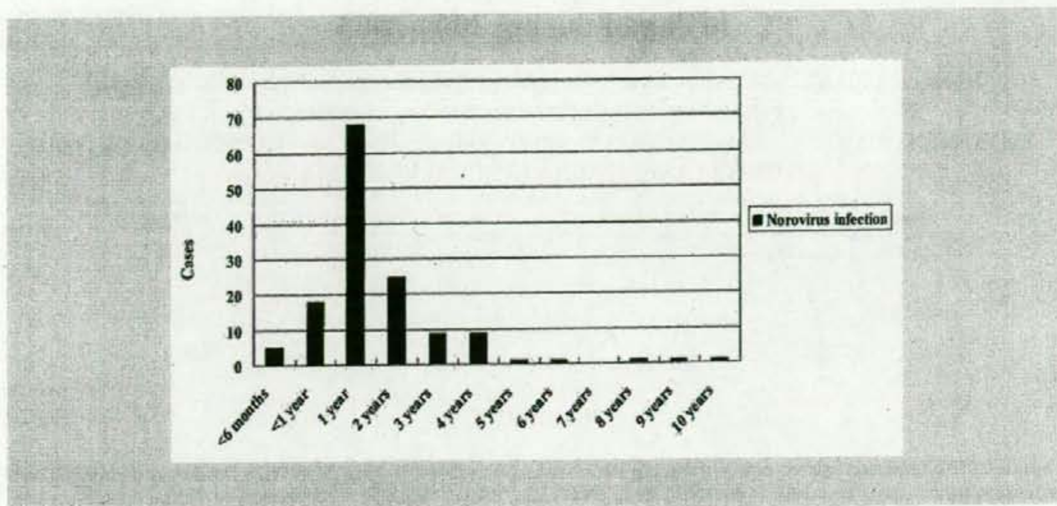

Figure 1: Age-related distribution of NoV infection among infants and children with acute gastroenteritis in four different regions (Maizuru, Tokyo, Sapporo, and Osaka) of Japan during the period of July 2004 to June 2005.

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.

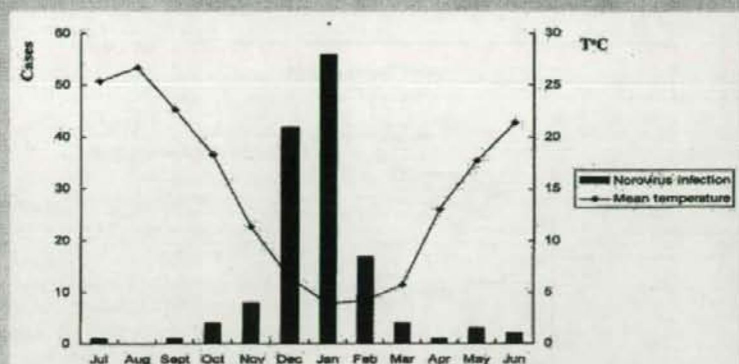

Figure 2: Seasonal variation of NoV infection among infants and children with acute gastroenteritis in four different regions (Maizuru, Tokyo, Sapporo, and Osaka) of Japan during the period of July 2004 to June 2005. The mean temperature in Japan is also shown.

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 (CTGCCCGAATTY-GTAAATGA) and G1SKR (5'-CCAACCCARCCATT-RTACA), and COG2F (CARGARBCNATGTTYAGRT-GGATGAG) and G2SKR (CCRCCNGCATRHCCRTT-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

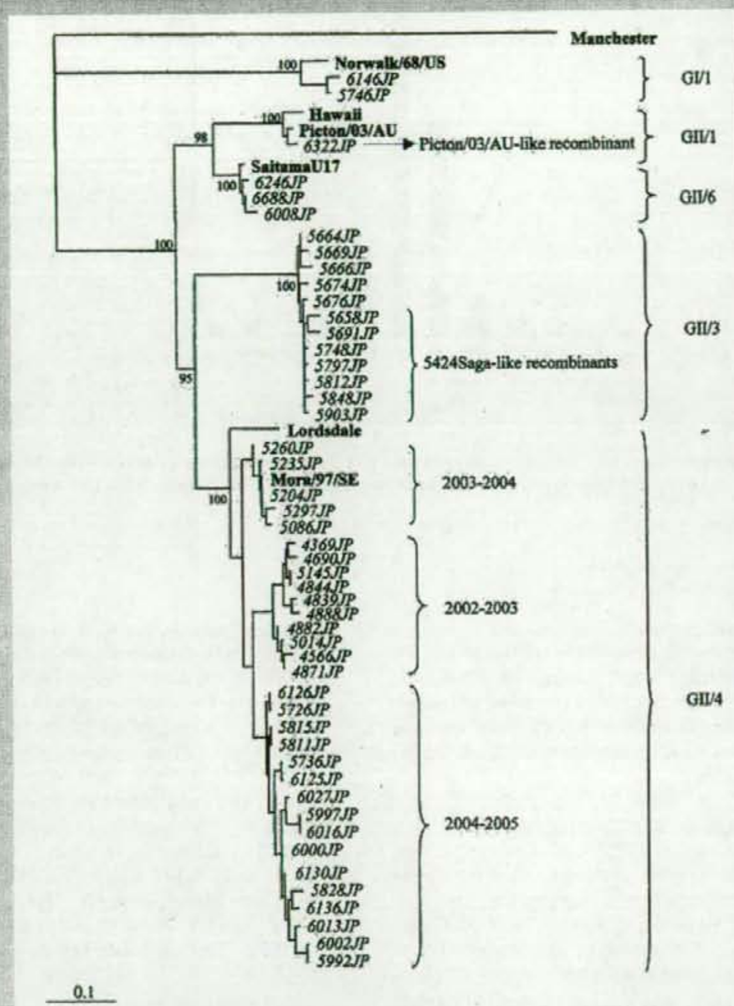

Figure 3: Phylogenetic tree of the nucleotide sequences of NoV. The tree was constructed from partial nucleotide sequences of the capsid region of NoV GII strains detected in Japan. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the 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, 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

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 recombinant. 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

This study was supported by Grants-in-Aid from the Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan.

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Clin. Lab. 2006;52:363-366
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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).

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

Manuscript accpted March 30, 2006

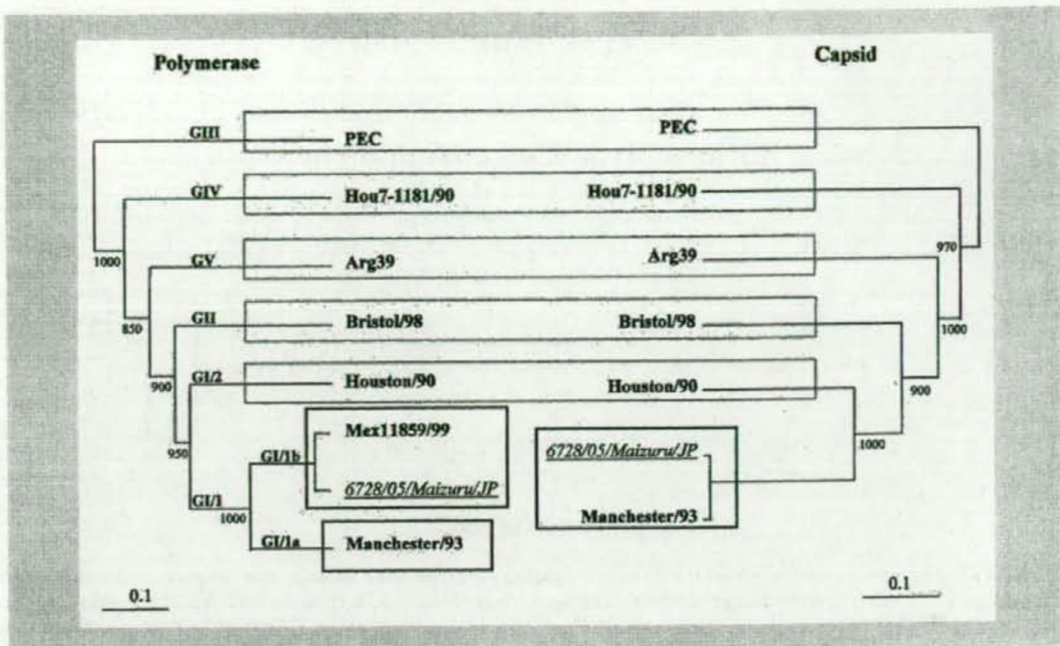

Figure 1: Observation of changes of sapovirus subtypes on the basis of phylogenetic trees of nucleotide sequences of the 6728/05/Maizuru/JP. The trees were constructed from partial nucleotide sequences of the capsid and polymerase regions of the 6728/05/Maizuru/JP. The 6728/05/Maizuru/JP is underlined 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
This study was supported by Grants-in-Aid from the Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan. This study was also supported by the Heiwa Nakajima Foundation, the Mishima-Kaiun Foundation and the Sumitomo Foundation in Japan.

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JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2006, p. 3784–3786
0095-1137/06/$08.00+0 doi:10.1128/JCM.01373-06
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Vol. 44, No. 10

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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Received 4 July 2006/Accepted 11 July 2006

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: attB1NVGI (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 attB1NVGII (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 inset 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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TABLE 1. Minimal detected concentrations of rVLPs by ELISA

VLP	Mean minimal rVLP concn (μg/ml) ± SD[a]	
	Theoretical[b]	Detected[c]
GI/1	0.00106 ± 0.00042 (3)	
GI/3	0.0368 ± 0.0084 (3)	
GI/4		8.33 ± 2.89 (3)
GI/8	0.118 ± 0.084 (3)	
GI/11	0.0752 ± 0.0273 (3)	
GII/1	0.456 ± 0.191 (4)	
GII/2	0.116 ± 0.012 (3)	
GII/3		6.00 ± 3.65 (5)
GII/4	0.00298 ± 0.00100 (3)	
GII/5	4.22 ± 3.26 (4)	
GII/6		10 (1)
GII/7	10 < (3)*	
GII/12	0.468 ± 0.393 (3)	
GII/13	10 < (3)*	
GII/14		3.33 (2)
GII/15		6.67 ± 4.71 (2)

[a] The values show three significant figures. Each assay was done in triplicate, and the assay for single rVLP was repeated three to six times. The number of samples is given in parentheses.

[b] The theoretical values were calculated from the absorbance given by the serial dilution of rVLPs. The calculations were performed by cubic logit-log analysis ($R^2 > 0.949$). *, these rVLPs (GII/7 and GII/13) could not be determined for values of <10 μg/ml in the assay.

[c] The rVLPs given in this column could not be used to calculate the theoretical values because a theoretical value of <10 μg/ml (used maximum concentration) could not be calculated in even one assay. In these cases, the minimal detected concentrations are given.

GI/3 were done with kits of the same lot number. In the manual, the cutoff value is calculated as an absorbance value of negative control plus 0.15. Values that are 10% above or below the cutoff value are considered to be in the gray zone and therefore need to be examined again. In view of this, the theoretical minimal detectable concentration of each rVLP was determined as a calculated value which gave an absorbance value that was 10% above the cutoff value in each assay. Each assay was conducted in triplicate, and the experiment for each rVLP was repeated three to six times.

Five hundred and three stool samples were collected from infants and children with AGE who visited six pediatric clinics in Sapporo, Tokyo, Maizuru, and Osaka, Japan, from July 2004 to March 2005. All of the stool samples were stored at −30°C until testing. Watery stool samples were diluted 1:2 with phosphate-buffered saline (PBS), and hard stool samples were suspended to 1:5 with PBS. The suspensions were clarified by centrifugation at 10,000 × *g* for 15 min. The supernatants were diluted to 1:3 with the sample dilution buffer of the kit and used for the assay. The positives or negatives of the samples were determined as mentioned above.

Ten percent stool suspensions of 503 samples were prepared with PBS from the same aliquots for ELISA, and viral RNA was extracted by the QIA amp viral RNA mini kit (QIAGEN, Tokyo, Japan). The detection of NoV (GI and GII), astrovirus, sapovirus, rotavirus, and adenovirus was performed by two sets of multiplex PCR (21, 22). NoV-negative samples were examined by using two sets of monoplex PCRs, for NoV GI and GII. Twenty samples were further assayed by seminested PCR using a primer set, which were G2SKF and G2SKR for NoV GII (9). The genotypes of NoV were determined according to the method of Phan et al. (16).

TABLE 2. Sensitivity, specificity, and agreement of ELISA and RT-PCR[a]

ELISA	Detection (no. of samples) by RT-PCR		
	+	−	Total
+	87	20[b]	107
−	27	369	396
Total	114	389	503

[a] Sensitivity = 76.3% (87/114); specificity = 94.9% (369/389); agreement = 90.7% (456/503).

[b] These samples were positive as determined by seminested PCR.

The minimal detectable sensitivity is indicated in Table 1. The kit could detect GI/1 and GII/4 rVLPs at concentrations of <0.01 μg/ml. rVLPs of GI/3, GI/8, GI/11, GII/1, GII/2, and GII/12 were detectable within a range between 0.04 and 1 μg/ml. On the other hand, rVLPs of GI/4, GII/3, GII/5, GII/6, GII/14, and GII/15 were detected at more than 1 μg/ml. In cases where the assays for GI/4, GII/3, GII/6, GII/14, and GII/15 could not be detected at concentrations of <10 μg/ml and theoretical detectable concentrations could not be calculated, minimal concentrations given by the assay have been indicated (Table 1). GII/6 rVLP could be detected once at the highest concentration, 10 μg/ml. Two rVLPs of GII/7 and GII/13 could not be detected at a concentration of <10 μg/ml.

NoV in stool samples collected from sporadic cases in Japan was examined using both the ELISA kit and the RT-PCR, and the kit was evaluated based on the RT-PCR (Table 2). The calculated percent sensitivity, specificity, and agreement were 76.3, 94.9, and 90.7%, respectively. Twenty samples were determined to be positive by the kit but negative by the RT-PCR. These samples became positive when tested by the seminested PCR using NoV GII-specific primer pair. A total of 27 samples were positive with the RT-PCR but negative with the kit. The genotypes of 134 positive stool samples recorded by the RT-PCR were identified by using the clustering determined by Kageyama et al. (6) (Table 3). The genotypes of kit-positive, PCR-positive samples were 1 GI/1, 3 GII/3, 82 GII/4, and 1 GII/6, and the sensitivities of GI/1, GII/3, GII/4, and GII/6 were 50, 23.1, 85.4, and 33.3%, respectively. The low sensitivities of GII/3 and GII/6 were comparable to the results for the rVLPs. RT-multiplex PCR detected four other species of viruses in 503 stool samples. These were 7 group A rotavirus, 27 adenovirus, 30 sapovirus, and 1 astrovirus samples, and the stool samples containing these viruses were determined to be negative by ELISA. Furthermore, multiplex-PCR indicated that 8 of 112 NoV GII-positive samples were mixed infected

TABLE 3. Genotypes of norovirus in positive stool samples as determined by RT-PCR

Genogroup/ genotype	No. of samples	Frequency (%)	No. of samples positive by ELISA (%)
GI/1	2	1.8	1 (50.0)
GII/3	13	11.4	3 (23.1)
GII/4	96	84.2	82 (85.4)
GII/6	3	2.6	1 (33.3)
Total	114	100.0	87

with other viruses (5 sapovirus, 2 group A rotavirus, and 1 adenovirus).

Some studies showed that the strains belonging to GII/4 cluster were most predominant not only in stool samples from sporadic cases involving infants and children but also from the outbreaks (8, 10, 12, 15, 16, 19). On the other hand, it was found that various genotypes of NoV strains were detected in the outbreak cases, and there were no predominant genotypes in outbreak strains (20). Furthermore, a change in the distribution of NoV genotypes in the sporadic cases and the emergence of recombinant viruses has been reported (7, 11, 17, 18).

The ELISA kit could detect two kinds of rVLPs (GI/1 and GII/4) with a high sensitivity. Meanwhile, the GII/3 and GII/6 rVLPs formed a group that was responsive at higher concentrations. A total of 23.1% of the stool samples containing GII/3 NoV, and 33.3% of the samples with the GII/6 genotype could be effectively examined by the kit. NoV genotypes with low reactivity levels in the stool samples could be detected by the kit in cases with a sufficient viral load. On the other hand, the genotypes of 20 samples, which were ELISA positive and seminested PCR positive, were 7 GII/3 and 13 GII/4. It would appear that these samples have a smaller viral load than monoplex PCR-positive stools. This suggests that there are other factors, such as inhibitors, that may cause the lower sensitivity of ELISA.

The sensitivity, specificity, and agreement of the kit were superior to those of the Denka and Dako kits (1). Dimitriadis and Marshall showed in 2005 that the RIDASCREEN ELISA kit could not be recommended for the study of stool samples in Australian outbreaks (2). In that report, the sensitivity and specificity of the kit were 71 and 47%, respectively, with the same cutoff calculations as our own. The difference between their sensitivity value and our own, which was 76.3%, was not large. On the other hand, the specificity was very different. In the present study, the specificity of the kit based on RT-PCR assay was 94.9%. There were the false-positive samples in their results. The reason for the difference in the specificities is unclear. We have been unable to obtain either the Denka kit or the Dako kit and have not been able to compare the RIDASCREEN kit with these kits using the same stool samples.

In conclusion, our results indicated that the kit could be a useful tool for sporadic diarrheal samples. However, it is quite possible to contain many kinds of genotypes in diarrheal samples derived from food-borne sources, and the particular kinds of genotypes found in such cases are not always the same as the genotypes found in sporadic cases. All in all, the reactivity for GII/3 and GII/6 needs to be improved in order to facilitate the detection of etiological agents in outbreaks.

This study was supported by Grants-in-Aid from the Ministry of Education and Sciences of Japan.

We thank T. Kaneshi, A. Yamamoto, S. Nishimura, S. Nakaya, and T. Nishimura for collecting stool samples.

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Infection, Genetics and Evolution 7 (2007) 247–253

Infection, Genetics and Evolution

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Genetic characterization of group A rotavirus strains circulating among children with acute gastroenteritis in Japan in 2004–2005

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Received 7 April 2006; received in revised form 10 October 2006; accepted 11 October 2006
Available online 15 November 2006

Abstract

A total of 752 fecal specimens collected from July 2004 to June 2005 from children with acute gastroenteritis in four localities in Japan (Maizuru, Tokyo, Sapporo, and Osaka) were screened for group A rotavirus by RT-PCR. It was found that 82 (10.9%) specimens were positive for group A rotavirus. The G-(VP7 genotypes) and P-(VP4 genotypes) types were further investigated. The P-types of 18 rotavirus strains, which could not be typed by RT-PCR, were determined by sequencing analysis. Of these, 94% (17/18) were P[8] with multiple point mutations at the VP4 primer-binding site. Another sample turned out to be a rare genotype P[9], which was closely related to feline rotavirus. The predominant genotype was G1P[8] (46.4%), followed by G3P[8] (32.9%) and G2P[4] (12.2%). A number of unusual combinations including, G1P[4] (1.2%), G2P[8] (1.2%), G3P[9] (1.2%), G1G3P[8] (1.2%), and G2G3P[8] (3.7%), were also detected. A new nomenclature of P[8] was proposed, in which worldwide rotavirus P[8] strains were classified into four sub-lineages, namely IA, IB, IIA, and IIB. A wide range of amino acid substitutions (up to 22) specific for P[8] lineages and sub-lineages were also identified. Interestingly, only short amino acid motifs located at positions 32–35, 121–135, and 195–236 of VP4 correctly defined the phylogenetic P[8] lineages and sub-lineages. Of note, at least two distinct clusters of rotavirus P[8] were co-circulating in the Japanese pediatric population studied.
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Keywords: Rotavirus; Genotype; Gastroenteritis; Japan

1. Introduction

Acute gastroenteritis has been demonstrated as a major cause of morbidity and mortality among infants and young children in both developed and developing countries (Murray and Lopez, 1997; Thapar and Sanderson, 2004). Among different enteropathogenic viruses, rotaviruses are recognized as the major etiologic agents of gastroenteritis in children and young animals (Parashar et al., 2003; Mulholland, 2004). Rotaviruses are classified into seven groups (A–G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with groups A–C rotaviruses. Of these, group A rotavirus is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide (Murray and Lopez, 1997; Mulholland, 2004). Group A rotavirus is known to have the highest prevalence and pathogenicity, causing an estimated nearly one million deaths every year, predominantly in developing countries. Recent studies suggest that as global deaths from childhood diarrhea have decreased during the past two decades, the proportion of diarrhea hospitalizations attributable to rotavirus may have increased (Parashar et al., 2006).

Rotavirus, which is a member of the family Reoviridae, possesses a genome of 11 dsRNA segments that are enclosed in a triple-layered capsid. According to the antigenic and genetic diversity of two of rotavirus's outer capsid proteins, VP7 and VP4, P genotypes and G genotypes have been defined, respectively. In group A rotavirus, at least 15 G genotypes have been recognized by neutralization assay and 25 P genotypes have been identified by hybridization or sequence analysis. The four predominant rotavirus genotypes G1P[8], G2P[4], G3P[8], and G4P[8], comprise nearly 83% of all the rotavirus infections in the world (Estes, 1996; Kapikian et al., 2001). Of these, G1 is

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doi:10.1016/j.meegid.2006.10.002

reported to be most common cause of acute gastroenteritis in most countries, followed by G2, G3, and G4 in various countries of Europe, North and South America, Africa, and Asia (Maneekarn and Ushijima, 2000; Kapikian et al., 2001; Santos et al., 2003; Okitsu-Negishi et al., 2004). Therefore, these genotypes are the targets for current vaccine development strategies. Unlike antimicrobial therapies that are effective against some bacterial and parasitic agents, no specific treatment for rotavirus infection is available; therefore, a rotavirus vaccine is sorely needed. The first human rotavirus vaccine, which was known as a human–animal tetravalent vaccine with limited genotypic cover (G1–G4), was licensed in the United States in August 1998. This vaccine was, however, withdrawn from use after an alleged association with cases of bowel intussusceptions (Mulholland, 2004). Due to genetic diversity in different parts of the world, knowledge of molecular epidemiology of rotavirus in circulation is important in the effort to develop a suitable and efficacious vaccine.

Therefore, the objectives of the present study were to: (1) determine the detection rate of rotavirus infections in children with acute gastroenteritis in four different localities in Japan in 2004–2005; (2) characterize the detected rotaviruses according to G- and P-types and (3) describe the genetic diversity among them.

2. Materials and methods

2.1. Fecal specimens

A total of 752 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics in four localities (Maizuru, Tokyo, Sapporo, and Osaka) in Japan from July 2004 to June 2005. Of these, 231 specimens were from Osaka, 51 from Sapporo, 24 from Tokyo, and 446 from Maizuru. The case definition of diarrhea used in this study was as at least three passings of unformed (loose and watery) stool a day. Acute gastroenteritis was defined as the occurrence of diarrhea and other symptoms such as vomiting, fever, and abdominal pain. The ages of the subjects ranged from 2 months to 15 years, with a median of 27 months. 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 group A rotavirus.

2.2. Extraction of viral genome

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

2.3. Reverse transcription

For reverse transcription (RT), 4 μl of extracted viral genome was added to 4 μl of a reagent mixture consisting of 5× first strand buffer (Invitrogen, Carlsbad, CA, USA), dNTPs (10 mM/μl) (Roche, Mannheim, Germany), DTT (Invitrogen), superscript reverse transcriptase III (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (Toyobo, Osaka, Japan), and MilliQ water. The total 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 (Phan et al., 2005).

2.4. Polymerase chain reaction (PCR)

Using PCR with specific primers as previously reported resulted in the identification of rotavirus. Primers Beg9 (5′-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3′) and VP7-1′ (5′-ACTGATCCTGTTGGCCATCCTTT-3′) was used to amplify VP7 of rotavirus and specifically generated a size of amplicon of 395 bp (Phan et al., 2005). PCR was carried out with 1 μl of cDNA in 10 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/μl), primers (33 μM), Taq DNA polymerase (5 U/μl) (Promega) and MilliQ water. 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.

2.5. Group A rotavirus G-typing

G-typing of group A rotavirus was preformed using the protocol from the method previously presented by Das et al. (1994). The full-length of the VP7 gene was reversely transcribed and then further amplified with Beg9 and End9 primers. The expected size of the PCR product generated from the full-length VP7 gene was 1062 bp in length. The second amplification was performed using the first PCR product as the template with G-type 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 G1–G4 and G9 VP7 genes, respectively. These primers specifically generated five different sizes of amplicons: 158, 224, 466, 403, and 110 bp for G1, G2, G3, G4, and G9, respectively. The samples whose G-type could not be identified by the first set of primers, described by Das et al. (1994) were then identified using another alternative set of type-specific primers previously reported by Gouvea et al. (1990).

2.6. Group A rotavirus P-typing

P-typing was conducted using the method modified from Gentsch et al. (1992). The RT-PCR was performed using Con2 and Con3 primers for the amplification of the partial VP4 gene. In the second amplification, a mixture of primers, 1T-1, 2T-1, 3T-1, 4T-1, 5T-1, ND2, and Con3 primers were utilized for the identification of P[8], P[4], P[6], P[9], P[10], and P[11] with six different sizes of amplicons of 346, 484, 268, 392, 584, and 123 bp, respectively.

2.7. Electrophoresis

PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr) for 20 min

then visualized under ultraviolet light, and the results were recorded by photography.

2.8. Nucleotide sequencing and phylogenetic analysis

The group A rotavirus isolate whose P-type could not be determined by RT-PCR method was then subjected to nucleotide sequence analysis. The nucleotide sequences of PCR products (DNA) positive for the rotavirus VP4 gene were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Their VP4 nucleotide sequences were compared to each other as well as to those of reference rotavirus strains available in GenBank by BLAST. Sequence analysis was performed using CLUSTAL X software (Version 1.6). The phylogenetic tree with 100 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). The sequences of group A rotavirus strains 6672/JP, 6226/Japan, and 6299/Japan, had been submitted to GenBank and had been assigned accession numbers DQ479964, DQ479965, and DQ479966, respectively. Reference group A rotavirus strains and their accession numbers used in this study were as follows: BP785/00/Hungary (AJ605315), VA70/USA (AJ540229), WA/USA (L34161), HOCHI/Japan (AB039943), ODELIA/Japan (AB039942), MW670/Malawi (AJ302146), OP530/Malawi (AJ302152), AI-75/Japan (AB008285), MW258/Malawi (AJ302143), OP511/Malawi (AJ302151), CH927B/China (AB008273), MO/Japan (AB008278), Kagawa/90-513/Japan (AB039944), OP601/Malawi (AJ302153), CU132P8/Thailand (DQ235955), DK V98-893/Denmark (AY509908), DK V00-2138/Denmark (AY509910), S8/Porcine/Brazil (AF052449), CU90P8/Thailand (DQ235978), TF101/Taiwan (AF183870), Hun9/Hungary (AJ605320), WH-1194/China (AY856445), and WH-624/China (AY856444).

3. Results

3.1. Molecular epidemiology of group A rotavirus infection

Rotavirus was detected in 82 out of 752 (10.9%) specimens tested. The rotavirus detection rates were different between the four localities: 7.4% in Maizuru, 3.9% in Sapporo, 4.2% in Tokyo, and 19.9% in Osaka. Rotavirus was identified throughout the 6-month period from December 2004 to June 2005. However, none of rotaviruses was detected from July to November 2004. The rotavirus incidence was found to be highest in April (30.5%), followed by March (26.8%) and May (22%). The lowest rotavirus detection rate occurred in December (1.2%).

3.2. Distribution of G- and P-types with re-emergence of G1

The distributions of G- and P-types of group A rotavirus during the study period from July 2004 to June 2005 are shown in Table 1. Three different G-types, G1–G3, were detected. Of these, G1 was the most prevalent genotype (37.6%), followed by G3 (34.1%) and G2 (13.4%). When examined for their P-types, P[4] (13.4%) and P[8] (64.6%) were identified. However, there were 18 rotavirus isolates (22%) whose P-types could not be determined by RT-PCR using specific primers previously reported in the literature.

Table 1
Distribution of different G-type and P-type of rotaviruses in Japan in 2004–2005

G-type/P-type	P[4]	P[8]	P[9]
G1	1 (1.2%)	38 (46.4%)	–
G2	10 (12.2%)	1 (1.2%)	–
G3	–	27 (32.9%)	1 (1.2%)
Mix G1G3	–	1 (1.2%)	–
Mix G2G3	–	3 (3.7%)	–

3.3. Nucleotide sequence analysis of P-nontypeable group A rotavirus isolates

Eighteen group A rotavirus isolates whose P-types could not initially be determined using the RT-PCR method, even though their VP4 genes were successfully amplified by RT-PCR. Therefore, their P-types were assigned based on nucleotide sequence analysis by direct sequencing of VP4 genes using the consensus Con3 as a sequencing primer. After sequence analysis, 94% (17/18) were P[8]. Fig. 1 revealed that these rotavirus P[8] isolates contained four point mutations at the VP4 primer-binding site. As many as 16 of 17 (94.1%) P[8] isolates approved by sequence analysis were highly homologous at the nucleotide level, ranging from 99% to 100%, and were highly homologous (98%) with the Malaysian P[8] strain WH-1194. On the other hand, another rotavirus P[8] isolate, the 6690/Japan strain, had a low homology (only 86%) at the nucleotide level with other rotavirus P[8] isolates detected in the study. The 6690/Japan strain shared the highest identity (99%) with the BP785/00, rotavirus P[8] strain from Hungary. Of note, another P-nontypeable isolate, the 6299/Japan, turned out to be P[9] by sequencing, which was a rare human P-type. Its VP4 nucleotide sequence shared greater homology with the feline rotavirus (100%) P[9] strain FRV-1 (accession number D14618) than with human P[9] reference strains, 95% for strain K8 (accession number D90260) and 94% for strain PA151 (accession number D14623).

nt 339 nt 356	Position of binding site
3'-GCACGTTATCCAAGTAGA-5'	Primer 1T1
******CGAT*C******	6672JP P[8]
******CGAT*C******	6226JP P[8]
T**C*C**C***	6690JP P[8]

Fig. 1. Alignment of the VP4 gene fragment of Japanese group A rotavirus isolates that were not typed by RT-PCR and the reverse complementary sequences of the original primer 1T-1. Residues that match primer 1T-1 are denoted by asterisks.

The frequencies of various combinations of the G- and P-types of rotavirus detected in this study were also investigated. G1P[8] was the most predominant combination (46.4%), followed by G3P[8] (32.9%), and G2P[4] (12.2%). A number of unusual combinations such as G1P[4], and G2P[8] were also detected.

3.4. A new nomenclature for group A rotavirus P[8] genotypes

In an attempt to understand the molecular basis of rotavirus genetic diversity within the P[8] genotype, partial sequences of the VP4 gene (VP8*) of P[8] isolates detected in this study and worldwide rotavirus reference P[8] strains were used for genetic analysis. Phylogenetic analysis identified two distinct lineages, I and II, which were further divided into sub-lineages IA and IB, IIA and IIB (Fig. 2). It should be noted that sub-lineage IA consisted of both human rotavirus P[8] strains and the porcine rotavirus S8 strain. The high nucleotide homology of rotavirus strains within each sub-lineage ranged from 95% to 100%, indicating less than 5% of genetic difference among them. The nucleotide sequence divergence between sub-lineage IA and sub-lineage IB (within lineage I) was 11–14%. In contrast, the sequence variation among strains between sub-lineage IIA and sub-lineage IIB (within lineage II) was considerably lower, ranging from 7% to 9%.

3.5. Alignment of the partial amino acid sequences of VP4

Direct inspection of the partial sequence alignment of VP4 revealed that there are two kinds of amino acid

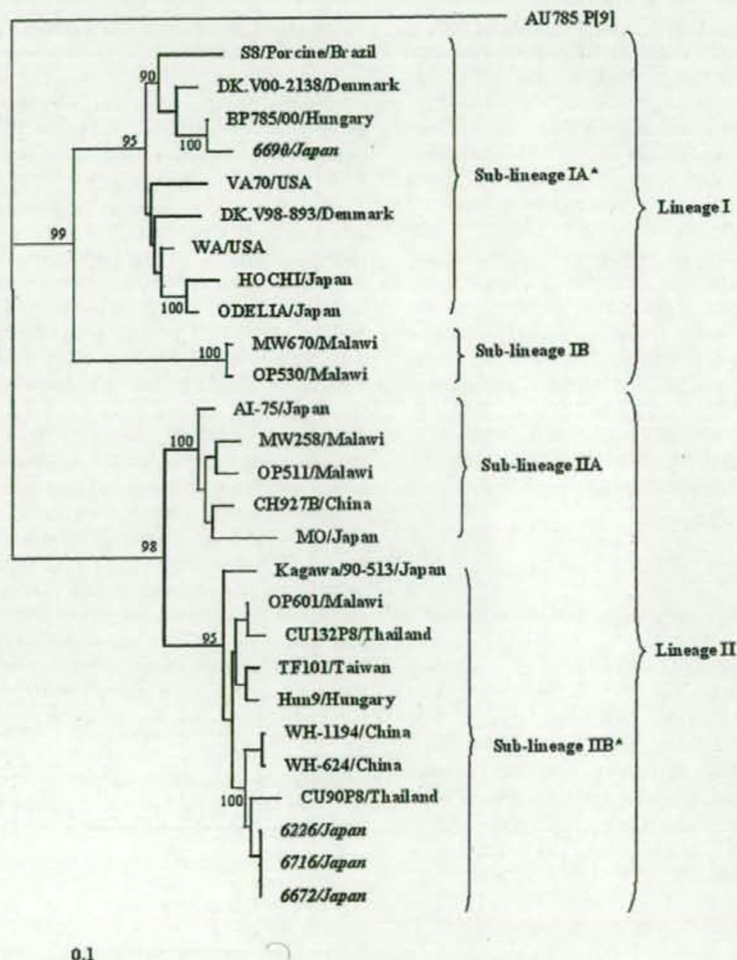

Fig. 2. Phylogenetic tree obtained from nucleotide sequences of the group A rotavirus P[8] VP8* gene. Reference group A rotavirus strains were selected from GenBank under the accession number indicated in the text. Japanese A rotavirus isolates detected are highlighted in italics. The AU785 P[9] strain 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. (*) Indicates the sub-lineage containing the rotavirus detected in this study.

Table 2

The amino acid substitutions from sequence data of the P[8] VP8* gene specific for lineages and sub-lineages. The amino acid substitutions highlighted in italics and in bold are specific for differentiation of lineages and sub-lineages, respectively. Shaded boxes indicate variable regions located at rotavirus VP8*. The phylogenetic lineage- and sub-lineage-defining sequences are indicated by a horizontal bar

Position			32	35	55	78	108	113	120	121	125	135	150	178	182	184	187	191	192	194	195	216	221	236
Amino acid			N	V	I	T	V	D	T	V	N	D	E	G	R	T	S	A	N	N	G	N	N	S
Lineage I	Sub-lineage IA	BP785/00	*	I	*	N	I	T	M	*I*	*S*	*N*	*	*	*	*	*	*	*	*	N	*	*	P
		S8/Porcine	*	I	*	N	I	N	M	*I*	*S*	*N*	*	*	*	*	*	*	*	*	N	*	*	P
		HOCHI	*	I	*	N	I	N	*	*I*	*S*	*N*	*	*	*	*	*	*	*	*	N	*	*	P
		ODELIA	*	I	*	N	I	N	*	*I*	*S*	*N*	*	*	*	*	*	*	*	*	N	*	*	P
		WA	*	I	*	N	I	N	*	*I*	*S*	*N*	*	*	*	*	*	*	*	*	N	*	*	P
		VA70	*	I	*	N	I	N	*	*I*	*S*	*N*	*	*	*	*	*	*	*	*	N	*	*	P
	Sub-lineage IB	MW670	D	*	V	K	*	*	*	*I*	*S*	*N*	*	K	G	V	G	S	D	T	S	I	Y	*
		OP530	D	*	V	K	*	*	*	*I*	*S*	*N*	*	K	G	V	G	S	D	T	S	I	Y	*
Lineage II	Sub-lineage IIA	MW258	*	*	*	I	*	S	*	*	*	*	*	*	*	*	*	*	*	*	D	*	*	*
		OP511	*	*	*	I	*	S	*	*	*	*	*	*	*	*	*	*	*	*	D	*	*	*
		CH927B	*	*	*	I	*	S	*	*	*	*	*	*	*	*	*	*	*	*	D	*	*	*
		MO	*	*	*	I	*	S	*	*	*	*	*	*	*	*	*	*	*	*	D	*	*	*
	Sub-lineage IIB	CU90P8	*	*	*	*	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		WH-1194	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		WH-624	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		TF101	*	*	*	*	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		Hun9	*	*	*	*	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		OP601	*	*	*	*	*	N	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		CU132P8	*	*	*	*	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		Kagawa315	*	*	*	*	*	N	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		6226/Japan	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		6716/Japan	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*
		6672/Japan	*	*	*	S	*	*	N	*	*	*	D	*	*	*	*	*	*	*	*	*	*	*

substitutions specific for lineages and sub-lineages (Table 2). For differentiation within lineages, three amino acid substitutions at positions 121, 125, and 135 of variable regions of VP8* were identified. Amino acid I, S, and N were specific for lineage I and amino acid V, N, and D were specific for lineage II. For differentiation within sub-lineages, up to 19 amino acid substitutions located at variable regions and conserved regions of VP8* were found. Of these, substitutions at 32 (D), 55 (V), 178 (K), 182 (G), 184 (V), 187 (G), 191 (S), 192 (D), 194 (T), 216 (I), and 221 (Y) were specific only for sub-lineage IB; substitutions at 35 (I), 108 (I), 113 (T or N), and 236 (P) for sub-lineage IA; substitution at 113 (S) for sub-lineage IIA. At positions 78 and 195, amino acid substitutions differed according to each sub-lineage, e.g., N for sub-lineage IA; K and S for sub-lineage IB.

Interestingly, only short signature sequences of VP4, which correctly defined the phylogenetic P[8] lineages and sub-lineages, were found. Three amino acids at positions 32, 35, and 55 formed an identification code of NII, DVV, and NVI for sub-lineages IA, IB and lineage II, respectively. Three amino acids at positions 121, 125, and 135 formed a code of ISN and VND for lineages I and II, respectively. Other amino acids at positions 195, 216, 221, and 236 also formed a code for sub-lineages, e.g., sub-lineage IA had a code of NNNP, and sub-lineage IB had a code of DNNS.

4. Discussion

In this study, the prevalence of rotavirus infection was 10.9% of children with acute gastroenteritis. This result was different from our previous report on rotavirus epidemiology in Japan from 2000 to 2003, with the detection rate ranging from 23% to 27% (Yoshinaga et al., 2006). This may result from the co-existence of multiple factors such as anti-rotavirus immunity in children, climate, water, and others, but direct evidence is lacking. In some reports, rotavirus was prevalent during the cold season, although several studies did not find a seasonal correlation (Nishio et al., 2000; Kang et al., 2002; Okitsu-Negishi et al., 2004). The findings in this study are in good agreement with the surveillance on pediatric cases of viral gastroenteritis in Japan, which demonstrated that the main peak of rotavirus infection was in the period of March and April (Zhou et al., 2003; Suzuki et al., 2005).

Extensive epidemiological studies of rotavirus infection worldwide including in Japan, which characterized rotavirus strains, indicated that G1 was the most prevalent genotype (Maneekarn and Ushijima, 2000; Kapikian et al., 2001; Santos et al., 2003; Zhou et al., 2003; Okitsu-Negishi et al., 2004). However, the emergence of new variant G2 and G3 was identified, and these strains became the leading genotypes in Japan in 2001–2004 (Yoshinaga et al., 2006; Phan et al., in press). At the same time, the prevalence of G1 rapidly dropped from 86% in 1998–1999 to 6% in 2002–2003 (Yoshinaga et al., 2006) and no G1 was found in 2003–2004 (Phan et al., in press). In this study, the changing pattern of the G-type distribution of rotavirus infection in children with acute gastroenteritis has been demonstrated. Of note, the G1 genotype re-emerged as the most prevalent with a high frequency (47.6%) compared to the lower frequency of G3 (34.1%) and G2 (13.4%), which were the second and third prevailing genotypes, respectively. The insufficient antibody protection from acquired viral immunity against G1 in a Japanese pediatric population due to the lack of a trigger of the previous G1 rotavirus infection during 2001–2004 was hypothesized. This hypothesis was in strong agreement with recent findings that the detection rate of G1 infection was very low during 2001–2004 (Yoshinaga et al., 2006; Phan et al., in press). Moreover, the common genotype G4 and the emerging genotype G9 were not detected in this epidemic season. Unlike the G-typing success of all rotaviruses, there were 18 rotavirus isolates whose P-types could not be determined by RT-PCR with specific primers. Therefore, their P-types were further approved by sequence analysis. After sequence analysis, 94% (17/18) had the closest relatives among rotavirus P[8] strains, demonstrating that our isolates belonged to P[8] genotype. These rotavirus P[8] isolates were found to contain four mismatches at the VP4 primer-binding site. Quite possibly, the initial failure in identifying a considerable fraction of the rotavirus P isolates in our study was due to those point mutations. Moreover, one P-nontypeable isolate turned out to be P[9], which is known to be a rare human P-type. Remarkably, this isolate shared the greatest homology (100%) of the VP4 gene nucleotide sequence with feline rotavirus strain FRV-1. This finding is additional evidence to support the notion that interspecies transmission of rotaviruses might be taking place in nature.

To gain further insights into the genetic variability within the P[8] genotype, VP8* of rotavirus P[8] strains were used for genetic analysis. The alignment of a partial amino acid sequence we compiled from a number of rotavirus P[8] strains detected in different parts of the world demonstrated two distinct lineages, I and II, which were further divided into sub-lineages IA and IB, IIA and IIB. Another interesting finding of this study was the discovery of a wide range (up to 22) of amino acid substitutions, which were specific for lineages and sub-lineages. These changes were exclusively present in only one or two lineages and/or sub-lineages, but they are absent in the other lineages and/or sub-lineages. Of these, as many as 14 amino acid positions were located at variable regions of VP8*. Interestingly, eight more positions were identified at the conserved sites of VP8*. At two positions, 78 and 195, amino acid substitutions consistently changed according to each sub-lineage. Consistent with a previous report (Maunula and von Bonsdorff, 1998), a short motif located in amino acids 121–135 of the VP4 variable region was found to differentiate rotavirus P[8] strains into phylogenetic lineages. The identification of two new amino acid motifs at positions 32–35 and 195–236 of VP4 conserved regions should be noted. These new signature motifs also correctly divided rotavirus P[8] strains not only into phylogenetic lineages but also into phylogenetic sub-lineages.

Based on the novel nomenclature of the P[8] genotype, at least two distinct rotavirus P[8] clusters (sub-lineages IA and IB) were co-circulating in the Japanese pediatric population studied. Sub-lineage IA contained both human rotavirus P[8] strains and the porcine rotavirus S8 strain from Brazil. This porcine rotavirus strain shared the high identities, ranging from 95% to 96%, at the nucleotide and the amino acid levels with other human rotavirus P[8] strains, including the 6690/Japan in this study. Taken together, in view of rotavirus evolution, a genomic relation might exist between human and porcine, and between human and feline strains.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Sciences and Technology and the Ministry of Health, Labor and Welfare, Japan. We are grateful to Drs Kunio Kaneshi, Yuichi Ueda, Shigekazu Nakaya, Shuichi Nishimura, Kumiko Sugita, Tadashi Nishimura, and Atsuko Yamamoto for fecal specimen collection.

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