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Changing Distribution of Norovirus Genotypes and Genetic Analysis of Recombinant GIIb Among Infants and Children With Diarrhea in Japan

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A total of 402 fecal specimens collected during July 2003–June 2004 from infants and children with acute gastroenteritis, encompassing five localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) of Japan, were tested for the presence of norovirus by RT-PCR. It was found that 58 (14.4%) fecal specimens were positive for norovirus. Norovirus infection was detected throughout the year with the highest prevalence in December. Norovirus GII was the most predominant genogroup (98.3%; 57 of 58). The genotypes detected in this study were GI/4, GII/2, GII/3, GII/4, and GII/6. Of these, NoV GII/3 (known as the Arg320 virus cluster) was the most predominant genotype (43.9%), followed by NoV GII/4 (the Lordsdale virus cluster; 35.1%) and others. Two norovirus strains clustered with a "new variant designated GIIb" and a "new variant of GII/4" were found circulating in Japan for the first time. It was interesting to note that NoV GIIb and NoV GII/3 appeared to be the recombinant strains and the recombination site was demonstrated at the overlap of ORF1 and ORF2. The majority (96%) of the dominant norovirus strains were identified as the recombination of GII/3 capsid and GII/12 polymerase. The recombination in the NoV GIIb capsid gene at the breakpoint located at P1 domain was also identified. Obviously, NoV GIIb isolate in Japan had double recombination. This is the first report demonstrating the existence of different "new variants" co-circulating in Japanese infants and children with acute gastroenteritis. ***J. Med. Virol. 78:971–978, 2006.***
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KEY WORDS: PCR; norovirus; recombination; Japan

INTRODUCTION

Norovirus (NoV) is recognized as a significant global enteropathogen, being a major cause of sporadic cases as well as of outbreaks of acute gastroenteritis in humans in various epidemiological settings, such as restaurants, schools, day-care centers, hospitals, nursing homes, and cruise ships [Chiba et al., 1979; McEvoy et al., 1996; Vinje et al., 1997; McIntyre et al., 2000]. The virus can be transmitted by food-borne, water-borne, air-borne, person-to-person spread by close contact and there might be some other unknown modes [Matson, 1994; Bon et al., 1999; Marks et al., 2000; Lopman et al., 2002; Oh et al., 2003]. 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 [Kageyama et al., 2004]. NoV is the distinct genus within the family *Caliciviridae*. The prototype strain of NoV is the Norwalk virus (Hu/NoV/Norwalk virus/1968/US), which was originally discovered from an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA in 1968. NoV possesses a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The NoV genome contains three open reading frames (ORFs). ORF1

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encodes the non-structural proteins, including the RNA-dependent RNA polymerase (RdRp) while 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, both known to infect humans. A recent study indicated that NoV GI and NoV GII could be classified into 14 and 17 genotypes, respectively [Kageyama et al., 2004]. The first naturally occurring recombinant NoV was the prototype Snow Mountain virus [Hardy et al., 1997]. Later several recombinant NoV strains causing sporadic cases and outbreaks of acute gastroenteritis were reported [Jiang et al., 1999a; Schreier et al., 2000; Katayama et al., 2002]. RNA recombination is one of the major driving forces of viral evolution [Worobey and Holmes, 1999]. To date, NoV is still uncultivable by standard culture methods with different cell lines. However, either VP1 alone or both VP1 and VP 2 could be expressed using recombinant baculovirus forming virus-like particles (VLPs) that are similar morphologically and antigenically to the native virion [Jiang et al., 1995]. Seroepidemiologic studies indicated a worldwide distribution of NoV. Moreover, it was found that serum antibody level to NoV was lowest in the first year of life and then rising after 2 years of age [Lopman et al., 2002; Dai et al., 2004; Peasey et al., 2004].

The objectives of this study were: to determine the incidence of NoV infections in infants and children with acute gastroenteritis in five different localities of Japan during 2003 and 2004; to characterize the genogroup and genotype of the detected NoV; and to describe the genetic diversity among them. Additionally, the age-related and seasonal distributions of NoV infection were determined.

MATERIALS AND METHODS

Fecal Specimens

A total of 402 fecal specimens were collected from infants and children with acute gastroenteritis, encompassing five different localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) of Japan during the period of July 2003–June 2004. Of these, 19 specimens were from Osaka, 22 from Sapporo, 22 from Tokyo, 45 from Saga, and 294 from Maizuru. The ages of the subjects were ranged from 2 months to 11 years with the median of 2.5 years (29 months). The majority (75%) of the affected children were aged less than 36 months and about half (54%) were male. The 10% fecal suspension was prepared in distilled water and clarified by centrifugation at 10,000*g* for 10 min. The supernatant was collected and stored at −30°C until use.

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.

Reverse Transcription (RT)

For reverse transcription (RT), 7.5 µl of extracted viral genome was added to the reaction mixture containing 2.05 µl of 5× first strand buffer (Invitrogen, Carlsbad, CA), 0.75 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 µl of 10 mM DTT (Invitrogen), 0.75 µl (200 U/µl) of superscript reverse transcriptase III (Invitrogen), 0.375 µl (1 µg/µl) of random primer (hexadeoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 µl (33 U/µl) of RNase inhibitor (Toyobo, Osaka, Japan), and 2.325 µl MilliQ water. The total volume of reaction mixture was 15 µl [Yan et al., 2003]. The RT step was carried out at 50°C for 1 hr, followed by 99°C for 5 min and then held at 4°C.

Polymerase Chain Reaction (PCR)

The NoV genogroups were identified by PCR method using specific primers as described [Yan et al., 2003]. Two pairs of specific primers G1SKF (CTGCCCGAATTYGTAAATGA) and G1SKR (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 capsid gene of NoV were used to detect NoV GI and NoV GII, respectively. These primers were specifically generated two different sizes of amplicons of 330 and 387 bp for NoV GI and NoV GII, respectively. The RNA polymerase gene of NoV was also amplified to identify the recombinant strain of NoV using the primers as described [Jiang et al., 1999b; White et al., 2002]. The PCR was carried out with 2.5 µl of cDNA in 22.5 µl of the reaction mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (2.5 mM/µl), primers (33 µM), Taq DNA polymerase (5 U/µl) (Promega, Madison, WI) and MilliQ water. The PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C. The full length of capsid and polymerase regions were amplified with a newly designed specific primer NVPOLR/A (GAT GAG GTT CTG ATG AGA) and the specific primers reported by Vinje et al. [2000] and Kawamoto et al. [2001]. The PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°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 PCR products (DNA) positive for NoV were determined using the BigDye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster

City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). Phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets were 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 the recombinant NoV sequences [Lole et al., 1999]. The nucleotide sequences of NoV strains 5424/03/Saga/JP and 5017/04/Maizuru/JP had been submitted to the DDBJ DNA/GenBank database and the assigned accession numbers were AB242256 and AB242257, respectively. Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Saitama T53GII/02/JP (AB112260), Girllington (AJ277606), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Alphatron (AF195847), Toronto (U02030), Seacroft (AJ277620), Leeds (AJ277608), Lordsdale (X86557), Idaho Falls/96/US (AY054299), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761), Saitama U1 (AB039775), Camberwell (AF145896), Snow Mountain (U70059), Paris Island/2003/USA (AY652979), Oberhausen 455/01/DE (AF539440), C14/2002/AU (AY845056), Herzberg 385/01/DE (AF539439), Arg320 (AF190817), VannesL169/2000/France (AY773210), Amsterdam (AF195848), White River/94/US (AF414423), Mexico (U22498), MD145 (AY032605), Mora/97/SE (AY081134), SaitamaT29GII/01/JP (AB112221), SaitamaKU80aGII/99/JP (AB058582), Mc37 (AY237415), Stockholm/IV4348/01/SE (AJ626633), and Pont de Roide 673/04/France (AY682549).

RESULTS

Epidemiology of Norovirus Infections

A total of 402 fecal specimens collected from infants and children with acute gastroenteritis from five different localities of Japan during July 2003 and June 2004 were examined for the presence of NoV. NoV was detected in 58 out of 402 (14.4%) specimens tested. The highest prevalence of NoV was found in infants and children with the age range of 12–23 months (36.2%). No case of NoV infection was identified among infants aged less than 6 months. It was also found that infants and children younger than 3 years old had a high rate of NoV infection (79.3%). NoV was detected throughout the period of 9 months starting from October 2003 to June 2004. However, none of NoV was detected from July to September 2003. The NoV incidence was found highest in December (27.5%), followed by November (19%), and January (12.1%). The lowest NoV detection rate fell into October (5.2%).

Nucleotide Sequence and Phylogenetic Analyses of NoV Genotypes

The partial nucleotide sequences of 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 58 NoV nucleotide sequences, including 1 of NoV GI and 57 of NoV GII were analyzed by phylogenetic grouping based on the recent NoV capsid region classification schemes described by Kageyama et al. [2004]. It was found that the NoV GI sequence (5226/04/Maizuru/JP) clustered into one distinct group with GI/4, which was represented by the Chiba/87/JP virus cluster. It was of interest to note that the 5226/04/Maizuru/JP strain showed the genetic relationship with the NoV Mie2001-U94/JP, which was previously isolated from oyster in Japan, with the nucleotide sequence identity to as high as 98%. Additionally, the 5226/04/Maizuru/JP strain revealed 90%–97% nucleotide sequence identities with those of other human NoV reference strains.

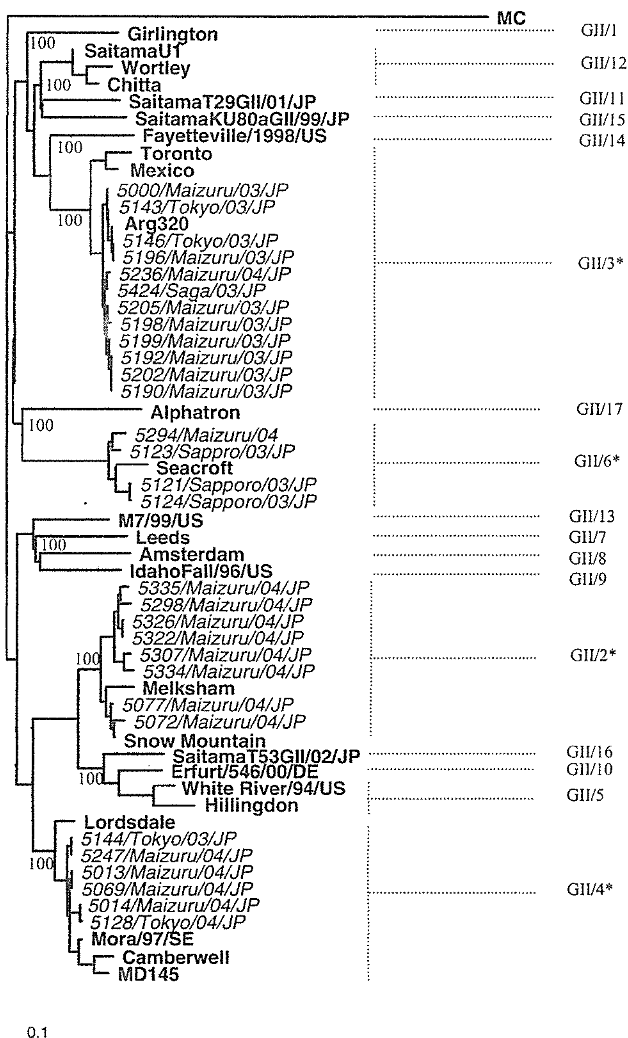

Fig. 1. Phylogenetic tree of nucleotide sequences of Japanese NoV GII. The tree was constructed from partial nucleotide sequences of capsid region of NoV GII isolates detected in Japan. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. MC 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 if 100% is given. *, Genotype contains Japanese NoV detected in the study.

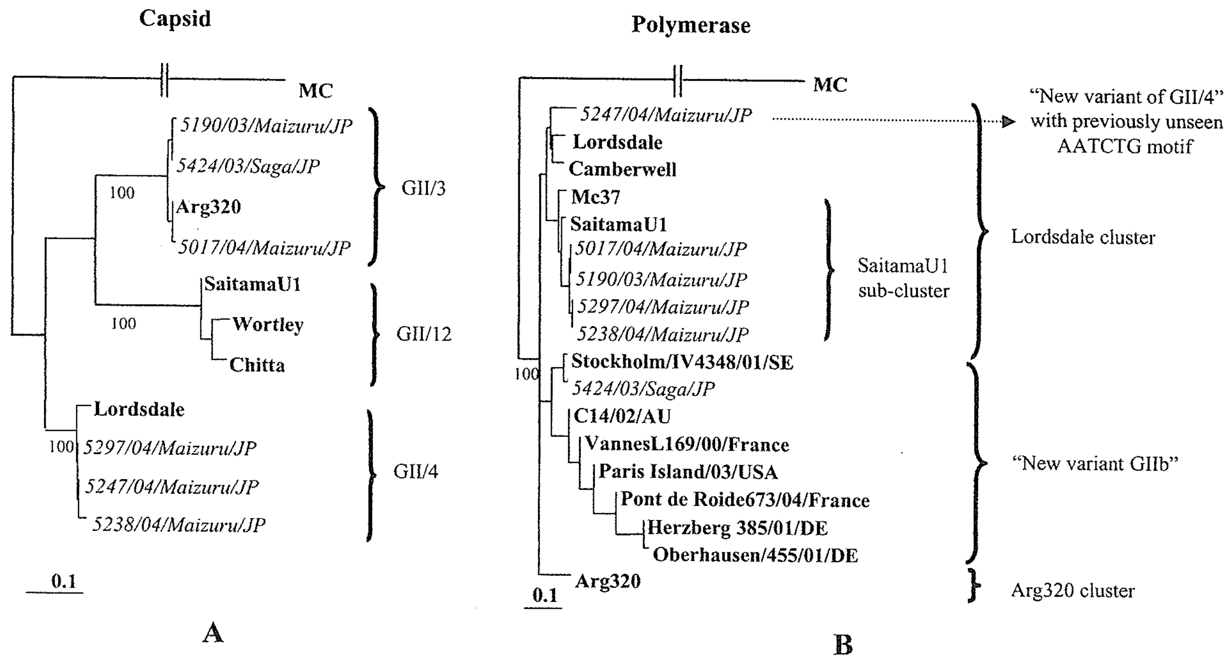

Fig. 2. Observation of changes of NoV genotypes on the basis of phylogenetic trees of nucleotide sequences. The trees were constructed from partial nucleotide sequences of capsid and polymerase regions of the Japanese representative isolates of NoV GII. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. MC 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 if 100% is given.

Of 57 NoV GII sequences, four distinct genotypes, GII/2, GII/3, GII4, and GII/6 had been identified (Fig. 1). Of these, the GII/3 (known as the Arg320 virus cluster) was the most predominant genotype with the prevalent rate of 43.9%, followed by 35.1% of GII/4 (the Lordsdale virus cluster), 14% of GII/2 (the Melksham virus cluster), and 7% of GII/6 (the Seacroft virus cluster). Considering the genotype distribution by localities, GII/3 was also the most predominant in all localities, except for Osaka where none of GII/3 was identified and only one GII/4 was detected in Osaka. The nucleotide sequence identities were ranged from 58% to 99% when NoV GII strains detected in this study were compared with those the reference strains previously registered in the DDBJ DNA/GenBank database.

Nucleotide Sequence and Genetic Analyses of NoV RNA Polymerase Gene

To verify the changing epidemiology of NoV genotypes, the RNA polymerase genes of all NoV GII/3 and

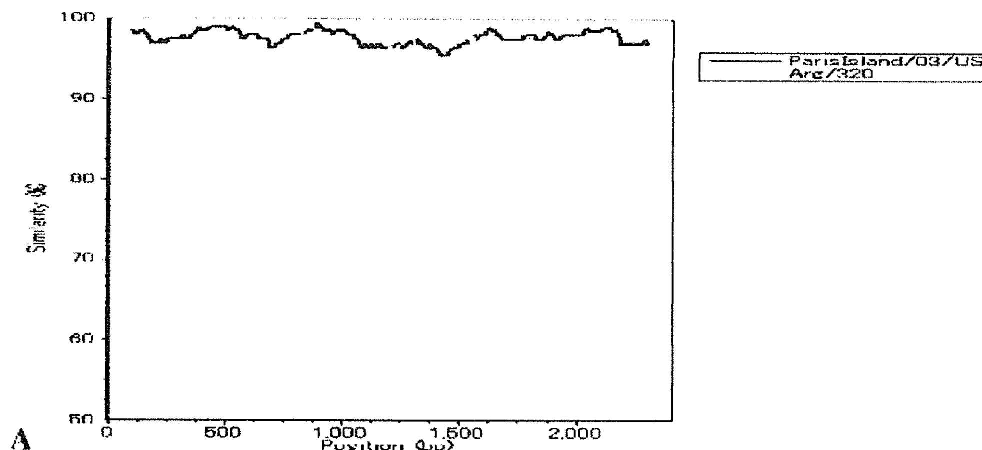

Fig. 3. Genetic characterization of recombinant NoV "new variant with polymerase GIIb." **A**: The Simplot analysis of the 5424/03/Saga/JP, the Paris Island/03/USA, and the Arg320. **B**: Evidence of recombination in NoV capsid gene.

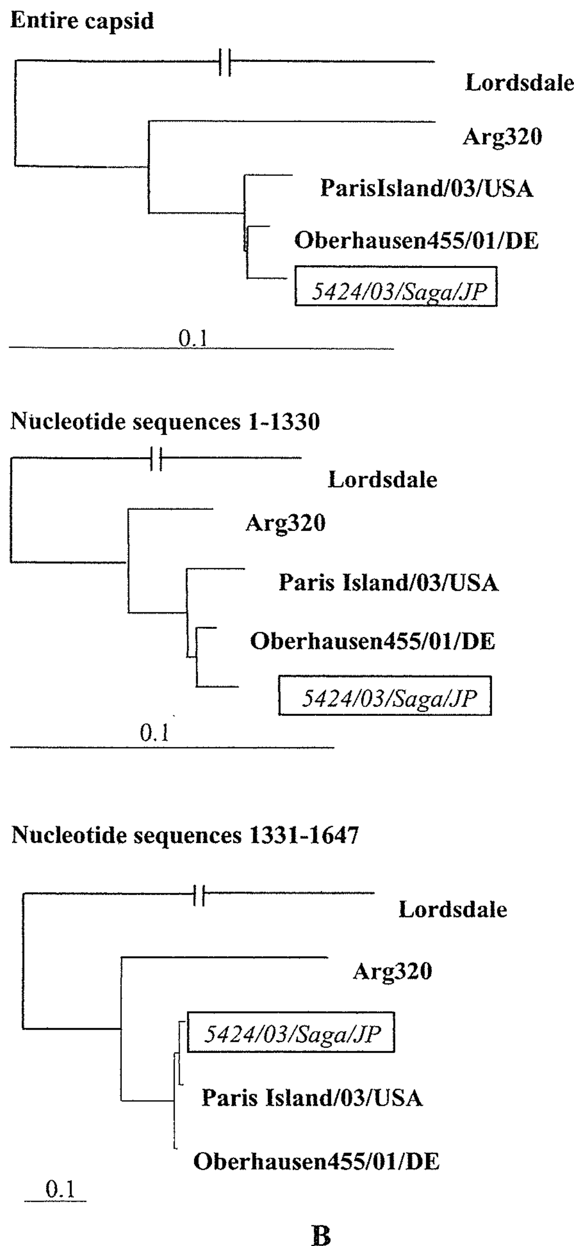

Fig. 3. *(Continued)*

NoV GII/4 were additionally amplified and sequenced. Of 25 NoV isolates with GII/3 capsid, 24 were classified into the SaitamaU1 sub-cluster (known as GII/12) but not into the Arg320 cluster when polymerase-based grouping was performed. The findings suggested that these 24 isolates were all recombinant viruses with GII/3 capsid and GII/12 polymerase. Interestingly, another NoV isolate, the 5424/03/Saga/JP, was grouped with NoV reference "new variants," which were designated as a GIIb in European countries (Fig. 2B). Taken together, the results indicated that the 5424/03/Saga/JP was also the recombinant strain.

In contrast, 20 NoV isolates belonging to GII/4 (the Lordsdale virus cluster), the genotype remained the same no matter the polymerase or capsid regions, was analyzed. Of these, 19 isolates shared significantly high identity of polymerase nucleotide sequences ranging from 98% to 100%. However, they shared only 93% sequence identity with those of the 5247/04/Maizuru/JP. It should be noted that the 5247/04/Maizuru/JP contained the previously unseen AATCTG motif starting at position 4,820 in the polymerase region (referring to the Norwalk virus, M87661). Obviously, this isolate was recognized as a "new variant of GII/4" according to the definition of Lopman et al. [2004]. Furthermore, as shown in Figure 2A, the majority (77.6%, 45 of 58) of NoV isolates were classified into GII/3 and GII/4 based on the partial capsid region, however, they were grouped into a SaitamaU1 sub-cluster based on the partial polymerase region (Fig. 2B).

Genetic Characterization of Recombinant Strain With GIIb Polymerase

To localize the potential recombination site and to understand a possible recombination mechanism of the "new variant" GIIb, the full-length nucleotide sequences of capsid and polymerase regions were determined and analyzed. When the nucleotide sequence of the 5424/03/Saga/JP was compared with those of the Arg320 and the Paris Island/03/US using the SimPlot software, region of genetic recombination was found between nucleotides 1,514 and 1,533 (the overlap of ORF1 and ORF2) (Fig. 3A). Up stream to this junction the nucleotide homology was notably different, and the SimPlot analysis showed a sudden drop in the nucleotide identity for the Arg320 but not for the 5424/03/Saga/JP and the Paris Island/03/US. The results demonstrated that the nucleotide sequences of capsid genes among these three strains were almost identical, but the polymerase sequences of the 5424/03/Saga/JP and the Paris Island/03/US were distinctly different from that of the Arg320.

Within the 5424/03/Saga/JP capsid sequence, the recombination at the breakpoint located at the beginning of P1 domain (position 1,330 nt in the capsid region) was identified. The capsid sequence of the recombinant 5424/03/Saga/JP showed alternate identities to the Oberhausen455/01/DE (nucleotides 1–1,330) and the Paris Island/03/USA (nucleotides 1,331–1,647) (Fig. 3B). The Oberhausen455/01/DE was detected in 2001 in Germany, whereas the Paris Island/03/USA was detected in 2003 in the United States. Quite possibly, the Paris Island/03/USA and the Oberhausen455/01/DE were putative parental strains of the 5424/03/Saga/JP. Taken together, the findings indicated that the 5424/03/Saga/JP had a double recombination.

Genetic Characterization of Recombinant Strain With SaitamaU1 Polymerase

As mentioned above, 24 isolates of GII/3 had high homology (98%–100%) at the nucleotide level of capsid

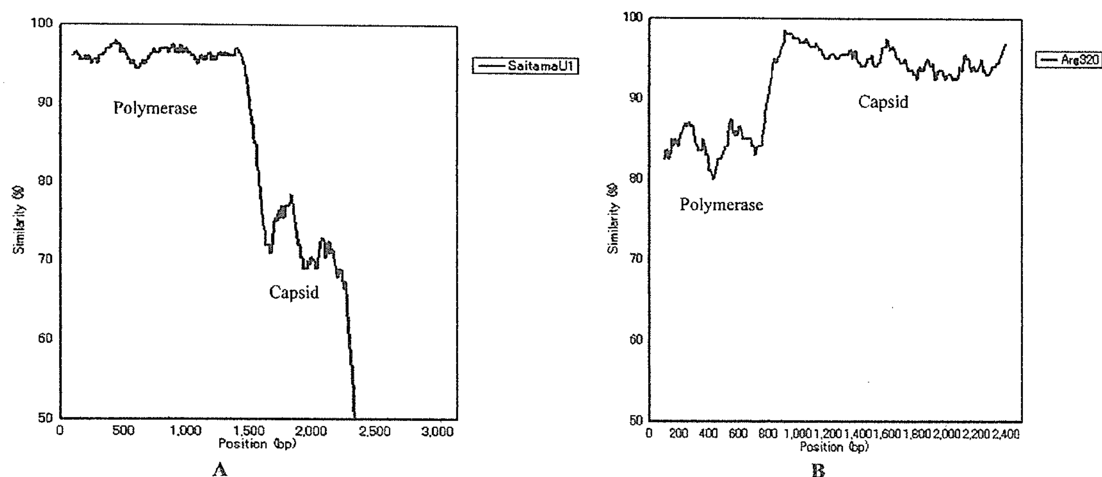

Fig. 4. Genetic characterization of recombinant virus with GII/3 capsid. A: The Simplot analysis of the NoV representative isolate, the 5017/04/Maizuru/JP, and the reference strain SaitamaU1. The high and low homologies with the polymerase and capsid regions among them, respectively, were found. B: The Simplot analysis of the 5017/04/Maizuru/JP and the reference strain Arg320. The low and high homologies with the polymerase and capsid regions among them, respectively, were found.

and polymerase. The findings demonstrated that they came from the same source of infection and represented the same strain. Furthermore, they were also suspected to be recombinant NoV based on their partial capsid and polymerase sequence. To further analyze this finding, the complete nucleotide sequences of the capsid and polymerase regions of one representative isolate, the 5017/04/Maizuru/JP, were determined. The 5017/04/Maizuru/JP shared a consistently low level of sequence identity (84%) in the RNA polymerase region but consistently high identity (95%) in the capsid region with the Arg320. In contrast, the 5017/04/Maizuru/JP shared consistently high level of sequence identity (96%) in the polymerase region and consistently low identity (70%) in the capsid region with those of the SaitamaU1. A recombinant site was also observed at the overlap of ORF1 and ORF2 (Fig. 4).

DISCUSSION

In this study, the prevalence of NoV infection among infants and children with acute gastroenteritis in five different localities of Japan was reported. Overall, the prevalence rate was 14.4% in all age groups of the subjects included in this study. However, the prevalence rate was increased up to 79.3% in infants and young children with the ages of less than 3 years old. These results were consistent with previous reports on NoV epidemiology worldwide in which the prevalence was ranged from 10% to 60% or more [Marks et al., 2000; Iritani et al., 2002; Lopman et al., 2002; Oh et al., 2003; Phan et al., 2004]. The findings suggested that approximately 14.4% of the etiologic agents of acute gastroenteritis cases in infants and children in Japan might be due to NoV and 85.6% might be responsible by other pathogens. The result also confirmed that NoV is one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. In some reports, NoV was prevalent in cold season, and several studies did not find a seasonal correlation [Vinje et al., 1997; Lopman et al., 2002; Phan et al., 2004]. The findings in this study are in agreement with the surveillance on pediatric cases of viral gastroenteritis in Japan, which demonstrated that the main peak of NoV infection was in the period of November, December, and January [Iritani et al., 2003; Inouye et al., 2000].

The results of this study showed that all Japanese NoV isolates belonged to two distinct genogroups, GI and GII, and these represented 1.7% and 98.3%, respectively. The results indicated that NoV GII was the dominant group causing acute gastroenteritis among Japanese pediatric population. It was interesting to note that the NoV GI 5226/04/Maizuru/JP, which was recovered from one 6-year-old-female patient with diarrhea, was closer genetically to the NoV Mie2001-U94/JP isolated from Japanese oyster than to human NoV reference strains available in the DDBJ DNA/GenBank database. This finding supported a view of possible NoV transmission to human through the contaminated oyster, which known as a reservoir of NoV. According to other reports published by different groups of investigators, NoV belonging to the Lordsdale cluster (GII/4) represented the most predominant genotype detected in sporadic gastroenteritis among infants and children not only in Japan but also in many other countries who run NoV surveillance [Chiba et al., 1979; McEvoy et al., 1996; Vinje et al., 1997; McIntyre et al., 2000]. However, it is surprising to note that in the present study NoV GII/3 was the most predominant, followed by NoV GII/4, NoV GII/2, and NoV GII/6. To verify this unusual observation, the polymerase regions of all NoV GII/3 and NoV GII/4 were further characterized. Remarkably, all NoV GII/3 except the 5424/04/Saga/JP were identified as the recombinant viruses that

related genetically to the SaitamaU1-like polymerase and the Arg320-like capsid. More interestingly, the SaitamaU1-like polymerase of NoV GII/3 was identical with those of NoV GII/4. The recombination of the NoV GII/4 polymerase and the Arg320-like capsid leading to an appearance of novel recombinant virus in the present study is postulated. Recently, NoV capsid protein was demonstrated to contain the determinants that are important for the immune recognition [Nilsson et al., 2003; Kirkwood, 2004]. Therefore, the emergence of recombinant virus with GII/3 capsid could be explained by a lack of acquired immunity for NoV GII/3 in Japanese infants and children. Interestingly, these recombinant strains suddenly appeared in a short period of 4 months (October 2003–January 2004) (data not shown). This sudden appearance and disappearance of strains might indicate that the virus appeared at the time that pediatric population lack antibody protection to these strains, and the virus disappeared by the time that the population began to acquire viral immunity. However, several studies reported that dominant strains could persist in one region over a number of years, which suggests that some other uncommon strains could be more virulent [Noel et al., 1999; Phan et al., 2004].

Another interesting finding of this study was the detection of "new variant with GIIb polymerase" 5424/03/Saga/JP in Japan. This isolate was isolated from a male patient with the age of 2 years old who developed a symptom of acute gastroenteritis in Saga, Japan. Surprisingly, based on the genetic analysis, this strain appeared to be an intratypic double recombinant. More interestingly, "new variant of GII/4 with unseen AATCTG motif" was also detected for the first time in a 2-year-old male patient with acute gastroenteritis in Maizuru, Japan in 2004. This motif was not present in any of the GII/4 sequences analyzed worldwide before 2002 from the food-borne viruses in European database and from the DDBJ DNA/GenBank database. This variant was first noted in Germany and the Netherlands in 2002 and become the predominant cause of NoV outbreaks throughout Europe [Lopman et al., 2004].

In conclusion, this is the first report on the existence of different "new variants" co-circulating in Japanese infants and children with acute gastroenteritis. This is also the first, description to the best of our knowledge, of the emergence and the importance of a novel recombinant virus causing acute gastroenteritis in Japan and warns of the threat it poses. Further epidemiologic studies should be conducted to determine whether this recombinant strain continues to be dominant in Japan in the coming year.

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Molecular Characterization of a Rare G3P[3] Human Rotavirus Reassortant Strain Reveals Evidence for Multiple Human-Animal Interspecies Transmissions

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An unusual strain of human rotavirus G3P[3] (CMH222), bearing simian-like *VP7* and caprine-like *VP4* genes, was isolated from a 2-year-old child patient during the epidemiological survey of rotavirus in Chiang Mai, Thailand in 2000–2001. The rotavirus strain was characterized by molecular analysis of its *VP4, VP6, VP7,* and *NSP4* gene segments. The VP4 sequence of CMH222 shared the greatest homology with those of caprine P[3] (GRV strain) at 90.6% nucleotide and 96.4% amino acid sequence identities. Interestingly, the VP7 sequence revealed highest identity with those of simian G3 rotavirus (RRV strain) at 88% nucleotide and 98.1% amino acid sequence identities. In contrast, percent sequence identities of both the *VP4* and *VP7* genes were lower when compared with those of human rotavirus G3P[3] reference strains (Ro1845 and HCR3). Analyses of VP6 and NSP4 sequences showed a close relationship with simian VP6 SG I and caprine NSP4 genotype C, respectively. Phylogenetic analysis of *VP4, VP6, VP7,* and *NSP4* genes of CMH222 revealed a common evolutionary lineage with simian and caprine rotavirus strains. These findings strongly suggest multiple interspecies transmission events of rotavirus strains among caprine, simian, and human in nature and provide convincing evidence that evolution of human rotaviruses is tightly intermingled with the evolution of animal rotaviruses. ***J. Med. Virol. 78:986–994, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: VP4; VP6; VP7; NSP4; G3P[3] genotype; Thailand

INTRODUCTION

Group A rotavirus is the most important etiologic agent of gastroenteritis and severe diarrhea in infants and young children, and in young animals of a wide variety of species [Estes, 2001]. Rotavirus is the member of the *Reoviridae* family. The rotavirus genome consists of 11 segments of double-stranded RNA. The two outer capsid proteins, VP4 and VP7, allow classification into P and G genotypes, respectively. To date, at least 15 G and 26 P genotypes have been isolated globally, with various G and P combinations [Rao et al., 2000; Estes, 2001; McNeal et al., 2005; Rahman et al., 2005; Martella et al., 2006]. The inner capsid protein VP6 bears the subgroup (SG) specificities that allows the classification of group A rotavirus into SG I, SG II, SG I + II, and SG non-I + II based on the reactivity with SG specific monoclonal antibodies (MAbs) [Greenberg et al., 1983a,b]. The nonstructural glycoprotein, NSP4, plays an important role in rotavirus morphogenesis, pathogenesis, and enterotoxic activity. Sequence analyses of the *NSP4* gene revealed the presence of at least five distinct NSP4 genotypes among human and animal rotaviruses, termed A–E genotypes [Horie et al., 1997; Ciarlet et al., 2000; Mori et al., 2002a,b].

There are a number of reports of atypical rotavirus strains isolated from humans and animals that share genetic and antigenic features of virus strains from heterologous species. In many cases, genetic analysis by hybridization has clearly demonstrated the genetic relatedness of gene segments from rotavirus strains isolated from different species. Together with the

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observation that some rotavirus strains appear to be transmitted to a different species as a whole genome constellation, these data suggest that interspecies transmission may occur frequently in nature [Nakagomi et al., 1990; Iizuka et al., 1994; Fujiwara and Nakagomi, 1997; Nakagomi and Nakagomi, 2002]. The factors that promote interspecies transmission of animal rotaviruses to human or vice versa are poorly understood. It could be possible that close contact between animals and humans may augment interspecies infections, and genetic reassortment during co-infection with rotavirus strains from different animal species may result in the generation of progeny viruses with novel or atypical genotypes [Palombo, 2002].

Rotavirus G3 strains are the only G genotype for which a broad host range has been described. Genotype G3 strains have been detected in several host species including humans, monkeys, rabbits, pigs, birds, cats, dogs, horses, mice, cows, and lambs [Gentsch et al., 1996, 2005; Estes, 2001; Martella et al., 2001b, 2003a; Lee et al., 2003; McNeal et al., 2005]. The P[3] rotavirus genotype has also frequently been isolated from animal origins, for example, goat, monkey, cat, dog, buffalo [Mackow et al., 1988; Taniguchi et al., 1994; Martella et al., 2001a,b, 2003b; Lee et al., 2003].

Currently, rotavirus strains bearing the P[3] genotype have been detected only in two human infections. The first strain (HCR3) was isolated from a child with diarrhea in Philadelphia, USA in 1984 [Li et al., 1993a]. Sequence information from some of the genome segments indicated that the human rotavirus strain HCR3 was more closely related to feline and canine rotavirus strains than to human strains. The second P[3] strain (Ro1845) was isolated in 1985 from an Israeli child with diarrhea, and was shown by RNA–RNA hybridization to be genetically related to canine and feline rotavirus strains [Aboudy et al., 1988; Nakagomi et al., 1990]. Both of these strains (HCR3 and Ro1845) displayed the G3P[3] genotype combination [Nakagomi et al., 1993; Li et al., 1993a, 1994].

In the present study, a third human rotavirus strain that displayed G3P[3] genotype (strain CMH222), which was isolated from a child hospitalized with severe diarrhea in Chiang Mai province, Thailand is described. Sequence and phylogenetic analyses revealed that *VP7* and *VP6* genes of CMH222 were closely related to those of simian while *VP4* and *NSP4* genes were closely related to those of caprine rotavirus.

MATERIALS AND METHODS

Rotavirus Antigen Detection

Rotavirus strain CMH222 was isolated in 2001 from a 2-year-old child admitted with severe diarrhea at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai province, Thailand [Khamrin et al., 2006]. Group A rotavirus antigen was detected by ELISA using polyclonal antibody against group A rotavirus as described previously [Hasegawa et al., 1987].

RNA Extraction, RT-PCR and Multiplex-PCR for G and P Genotyping

The G and P genotypes of CMH222 were determined by reverse transcription-polymerase chain reaction (RT-PCR) and multiplex-PCR. Viral dsRNA was extracted from 10% fecal supernatant using the QIAamp viral RNA Mini kit (QIAGEN, Inc., Hilden, Germany). Viral dsRNA was denatured in 50% of dimethyl-sulfoxide at 95°C for 5 min. The RT-PCR was carried out with a OneStep RT-PCR kit (QIAGEN). For PCR amplification of the *VP7* gene, a 1,062 bp fragment was generated using Beg9 (forward) and End9 (reverse) primers [Gouvea et al., 1990]. For PCR amplification of the *VP4* gene, a 876 bp fragment was generated using Con3 as a forward primer and Con2 reverse primer [Gentsch et al., 1992]. The reverse transcription reaction was carried out at 50°C for 30 min, followed by PCR activation at 95°C for 15 min and then further amplified for 30 cycles under the following condition: 94°C for 1 min, 55°C (for G genotyping) or 40°C (for P genotyping) for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The multiplex PCR for identification of genotypes G1–G6 and G8–G11 was conducted with three different pools of typing primer sets as previously described [Gouvea et al., 1990, 1994a; Das et al., 1994; Winiarczyk et al., 2002]. For P genotype identification, PCR was carried out by using pools of P genotype specific primers for P[1], P[4]–P[11], P[14] [Gentsch et al., 1992; Gouvea et al., 1994b; Mphahlele et al., 1999; Winiarczyk et al., 2002].

As the multiplex PCR using several sets of genotype-specific primers could not identify the P genotype of CMH222 strain, a new reverse primer, VP4-3R, was designed and used in combination with Con3 (forward primer) for amplification of 2,303 bp of *VP4* gene. P genotype was further identified by sequence analysis of this PCR fragment. The sequences of the primers for amplification and sequencing of *VP7* and *VP4* genes are shown in Table I.

Amplification of *VP6* and *NSP4* Genes

The full-length of *VP6* gene was amplified by primer pairs VP6-5F and VP6-3R, which were slightly modified from the original designated VP6 specific primers described by Shen et al. [1994]. *NSP4* full-length gene was amplified by NSP4-1a and NSP4-2b primer pairs [Kudo et al., 2001]. The sequences of primers used for amplification and sequencing of *VP6* and *NSP4* genes are shown in Table I.

Sequence and Phylogenetic Analyses

The PCR amplicons were purified with a QIAquick PCR purification kit (QIAGEN, Inc., Hilden, Germany) and sequenced in both directions using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated sequencer (ABI 3100; Perkin Elmer-Applied Biosystems, Inc.). The Con3, VP4F400, VP4F778,

TABLE I. Oligonucleotide Primers Used for the Amplification and Sequencing of *VP7*, *VP4*, *VP6*, and *NSP4* Genes

Primer	Gene	Sequence 5′–3′	Sense	Position	Reference
Beg9	*VP7*	GGCTTTAAAAGAGAGAATTTCCGTCTGG	+	1–28	Gouvea et al. [1990]
End9	*VP7*	GGTCACATCATACAATTCTAATCTAAG	−	1062–1036	Gouvea et al. [1990]
Con3	*VP4*	TGGCTTCGCTCATTTATAGACA	+	11–32	Gentsch et al. [1992]
Con2	*VP4*	ATTTCGGACCATTTATAACC	−	887–868	Gentsch et al. [1992]
VP4F400	*VP4*	GCTAACACTTCACAAACGC	+	400–418	This study
VP4F778	*VP4*	TCTAAAACGTCATTATGGAAAG	+	778–799	This study
VP4R1881	*VP4*	CATTTCTTTTAGCCTCAATCTTC	−	1859–1881	This study
VP4-3R	*VP4*	CAATTCTRTTHCGAATTATTGGRTT	−	2311–2287	This study
VP6-5F	*VP6*	GGCTTTTAAACGAAGTCTTC	+	1–20	Shen et al. [1994]
VP6-3R	*VP6*	GGTCACATCCTCTCACTA	−	1356–1339	Shen et al. [1994]
NSP4-1a	*NSP4*	GGCTTTTAAAAGTTCTGTTCCG	+	1–22	Kudo et al. [2001]
NSP4-2b	*NSP4*	GGTCACATTAAGACCGTTCC	−	750–731	Kudo et al. [2001]

VP4R1881, and VP4-3R primers (for *VP4*), VP6-5F and VP6-3R primers (for *VP6*), Beg9 and End9 primers (for *VP7*), and NSP4-1a and NSP4-2b primers (for *NSP4*) were used as sequencing primers (Table I). The nucleotide and deduced amino acid sequences of *VP4*, *VP6*, *VP7*, and *NSP4* genes were compared with those of reference strains available in the NCBI (National Center for Biotechnology Information) GenBank database using BLAST (Basic Local Alignment Search Tool) program [Altschul et al., 1990].

Nucleotide Sequence Accession Number

The nucleotide sequence of VP4, VP6, VP7, and NSP4 of CMH222 strain were deposited in GenBank under the accession number DQ288661, DQ288659, AY707792, and DQ288660, respectively.

RESULTS

G and P Genotyping of Human Rotavirus Strain CMH222

Between May 2000 and December 2001, a total of 315 fecal specimens were collected from infants and young children with acute gastroenteritis in Chiang Mai province, Thailand, as part of an epidemiological study of Group A rotaviruses. All fecal specimens were tested for the presence of group A rotavirus by ELISA, and then G and P genotypes were determined by using primers specific for *VP7* and *VP4* genes in RT-PCR and multiplex-PCR assays. Group A rotavirus was detected in 34% (107 of 315) of the fecal specimens collected during the study period. One rotavirus-positive stool specimen in this collection (CMH222), genotyped as G3 using the multiplex-PCR. However, the P genotype could not be determined using the existing P-specific primer sets, despite the generation of a first round 876 bp VP4 amplicon. Therefore, further molecular characterization of CMH222 was performed by nucleotide sequencing of the *VP4*, *VP6*, *VP7*, and *NSP4* genes.

Analysis of Nucleotide Sequence of *VP4* Gene

As the P genotype of CHM222 could not be identified by multiplex-PCR using P-type specific primers, the 2,303 bp fragment of *VP4* gene was amplified with the consensus forward primer Con3 and VP4-3R reverse primer. The nearly full-length of nucleotide and deduced amino acid sequence of this *VP4* gene was determined and compared with those of established reference strains, P[1]–P[26], available in the GenBank database (Table II). The VP4 sequence of CMH222 was closely related to all P[3] genotype strains analyzed thus far (78.4%–90.6% on nucleotide and 84.3%–96.4% on amino acid levels), and showed the highest identity to P[3] caprine rotavirus strain GRV (G3P[3]) at 90.6% on nucleotide and 96.4% on amino acid levels. A phylogenetic tree that included the VP4 sequences of all P[3] rotaviruses recognized to date from both human and non-human origins was constructed (Fig. 1). The result of phylogenetic analysis confirmed that CMH222 strain clustered with all P[3] genotypes. In addition, two major lineages were found among P[3] rotavirus strains. The P[3] sequence of strain CMH222 clustered in a lineage with caprine (GRV), simian (RRV) and buffalo (10733) rotavirus strains, and was most closely related to the P[3] sequence of GRV. The second lineage consisted of P[3] sequences from strains including human (Ro1845, HCR3), canine (RV198/95, K9, CU-1, RV52/96), and feline (FRV64, Cat97) P[3] sources.

Analysis of Nucleotide Sequence of *VP7* Gene

As shown in Table II and Figure 1, the *VP4* gene of CMH222 appeared to be closely related to rotaviruses of the animal origin. Therefore, the *VP7*, *VP6*, and *NSP4* genes were characterized by nucleotide sequencing to investigate whether the interspecies transmission could be demonstrated in other genes of strain CMH222. The complete nucleotide (1,062 bp) and deduced amino acid sequences of the *VP7* gene of the CMH222 strain were compared with VP7 sequences of the existing G1–G15 and also with several G3 strains of human and animal origins (Table III). Sequence comparison showed that the VP7 of CMH222 strain was most closely related to all G3 rotaviruses (80.8%–88.0% on nucleotide and 92.9%–98.1% on amino acid levels). Although CMH222 was identified as a G3, a common human genotype, VP7 sequence analysis demonstrated clearly that CMH222 was more closely related to a simian rotavirus strain (RRV) at

TABLE II. Comparison of the Nucleotide and Amino Acid Sequence Identities of the Genome Segment Encoding VP4 Protein of CMH222 Strain With Those of 26 Known P Genotypes[a]

			Similarity (%)	
Strain	Species	P genotype	Nucleotide	Amino acid
A5	Bovine	P[1]	74.7	83.3
SA11	Simian	P[2]	76.0	83.9
Ro1845	Human	P[3]	81.1	89.9
HCR3	Human	P[3]	80.5	88.9
RRV	Simian	P[3]	82.8	92.0
FRV64	Feline	P[3]	81.4	90.3
K9	Canine	P[3]	80.9	89.8
CU-1	Canine	P[3]	80.5	88.9
Cat97	Feline	P[3]	80.4	88.9
GRV	Caprine	P[3]	90.6	96.4
10733	Buffalo	P[3]	82.9	90.1
RV52/96	Canine	P[3]	78.4	86.4
RV198/95	Canine	P[3]	78.7	84.3
L26	Human	P[4]	69.3	70.4
UK	Bovine	P[5]	70.3	75.9
Gottfried	Porcine	P[6]	69.0	72.7
OSU	Porcine	P[7]	73.4	80.9
KU	Human	P[8]	68.5	70.1
K8	Human	P[9]	67.8	69.0
69M	Human	P[10]	76.7	85.6
B223	Bovine	P[11]	58.5	58.1
H-2	Equine	P[12]	74.9	80.9
MDR-13	Porcine	P[13]	73.5	77.7
PA169	Human	P[14]	67.7	70.3
Lp14	Ovine	P[15]	75.5	82.6
EW	Murine	P[16]	68.9	77.4
993/83	Bovine	P[17]	62.4	61.5
L338	Equine	P[18]	74.6	78.2
Mc323	Human	P[19]	71.6	74.7
EHP	Murine	P[20]	70.9	81.5
Hg18	Bovine	P[21]	74.0	77.8
160/01	Lapine	P[22]	66.6	61.8
A34	Porcine	P[23]	71.7	76.5
TUCH	Simian	P[24]	75.6	84.1
Dhaka6	Human	P[25]	66.6	67.4
134/04-15	Porcine	P[26]	73.8	80.9

[a]The GenBank accession numbers of the following strains are given in parentheses: A5 (D13395), SA11 (M23188), Ro1845 (D14726), HCR3 (L19712), RRV (M18736), FRV64 (D14723), K9 (D14725), CU-1 (D13401), Cat97 (D13402), GRV (AB055967), 10733 (AY281359), RV52/96 (AF339844), RV198/95 (AF339843), L26 (M58292), UK (M21014), Gottfried (M33516), OSU (X13190), KU (M21014), K8 (D90260), 69M (M60600), B223 (D13394), H-2 (L04638), MDR-13 (L07886), PA169 (D14724), Lp14 (L11599), EW (U08429), 993/83 (D16352), L338 (D13399), Mc323 (D38052), EHP (U08424), Hg18 (AF237665), 160/01 (AF526376), A34 (AY174094), TUCH (AY596189), Dhaka6 (AY773004), 134/04-15 (DQ061053).

88.0% on the nucleotide and 98.1% on the amino acid levels. Comparison between the VP7 sequences of CMH222 and human genotype G3 strains (B4106, HCR3, AU-1, TK28) only revealed nucleotide and amino acid identities ranging from 80.8% to 86.4% and 92.9% to 96.0%, respectively. Phylogenetic analyses confirmed that the CMH222 strain formed a cluster with G3 rotavirus reference strains and showed the closest lineage with the G3 simian strain RRV sharing more than 98.0% amino acid identity (Fig. 2). Taken together, analysis of *VP4* and *VP7* genes revealed that CMH222 belonged to G3P[3] genotype.

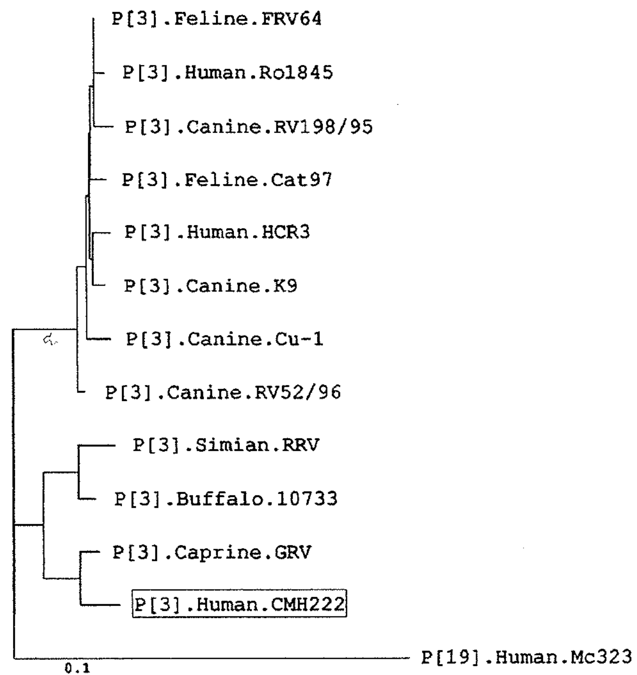

Fig. 1. Phylogenetic analysis of the deduced amino acid sequence derived from *VP4* gene of the CMH222 human rotavirus strain and other P[3] rotavirus genotypes recognized to date. The tree was generated based on the neighbor-joining method using ClustalX program.

Analysis of Nucleotide Sequence of *VP6* Gene

Within group A rotavirus, four subgroups have been identified based on reactivities with subgroup-specific MAbs that recognize epitopes within the VP6 protein. These include SG I, SG II, SG I + II, and SG non-I + II. Utilizing an ELISA-based subgroup assay, CMH222 did not react with MAbs specific for both SG I and SG II (data not shown). Therefore, based on the subgroup ELISA results, CMH222 was assigned as SG non-I + II specificity. Generally, human rotaviruses carry either SG I or SG II VP6 specificity [Iturriza Gomara et al., 2002]. It was of interest to determine whether the *VP6* gene of CMH222 might be of animal origins similar to the results observed for the *VP4* and *VP7* genes. The nucleotide and deduced amino acid sequences of CMH222 were compared with those of four representative established subgroups. The fundamental characteristics of *VP6* gene of CMH222 was found to be similar to the sequence of SG I rotavirus strains reported previously. Strain CMH222 displayed the greatest VP6 amino acid sequence identity with SG I simian strain TUCH (96.2%) whereas lower amino acid homology, ranging from 90.9% to 93.9%, was observed with strains displaying SG II, SG I + II and SG non-I + II specificities (data not shown). Phylogenetic analysis of the *VP6* gene also clustered CMH222 in the same branch with the simian TUCH but separated from other SG I rotaviruses (Fig. 3). The results suggest that VP6 of human CMH222 strain shared a common ancestor with VP6 of simian TUCH strain.

TABLE III. Comparison of the Nucleotide and Amino Acid Sequence Identities of the Genome Segment Encoding VP7 Protein of CMH222 Strain With Those of 15 Known G Genotypes[a]

Strain	Species	G genotype	Similarity (%)	
			Nucleotide	Amino acid
KU	Human	G1	77.5	83.4
S2	Human	G2	75.0	74.2
RV52/96	Canine	G3	87.7	97.5
J63	Bovine	G3	87.4	96.6
JE75	Equine	G3	87.1	95.3
HO-5	Equine	G3	87.0	95.0
RV198/95	Canine	G3	85.6	96.0
GRV	Caprine	G3	87.9	96.6
RRV	Simian	G3	88.0	98.1
JE29	Equine	G3	87.2	95.7
K9	Canine	G3	85.4	95.0
B4106	Human	G3	85.6	96.0
HCR3	Human	G3	86.4	96.0
AU-1	Human	G3	80.9	92.9
TK28	Human	G3	80.8	93.5
Hochi	Human	G4	75.5	77.9
OSU	Porcine	G5	79.1	86.1
NCDV	Bovine	G6	76.2	84.9
CH2	Chicken	G7	62.8	60.0
B37	Human	G8	76.2	81.9
116E	Human	G9	79.6	85.5
61A	Bovine	G10	78.3	85.2
YM	Porcine	G11	79.4	88.6
L26	Human	G12	77.5	81.2
L338	Equine	G13	77.8	83.4
CH3	Equine	G14	82.9	85.8
Hg18	Bovine	G15	74.1	79.4

[a]The GenBank accession numbers of the following strains are given in parentheses: KU (D16343), S2 (M11164), RV52/96 (AF271090), J63 (AF386914), JE75 (AB046466), HO-5 (AB046464), RV198/95 (AF271089), GRV (AB056650), RRV (AF295303), JE29 (AB046465), K9 (U97199), B4106 (AY456382), HCR3 (L21666), AU-1 (D86271), TK28 (D86283), Hochi (AB012078), OSU (X04613), NCDV (M63266), CH2 (X56784), B37 (J04334), 116E (L14072), 61A (X53403), YM (M23194), L26 (M58290), L338 (D13549), CH3 (D25229), Hg18 (AF237666).

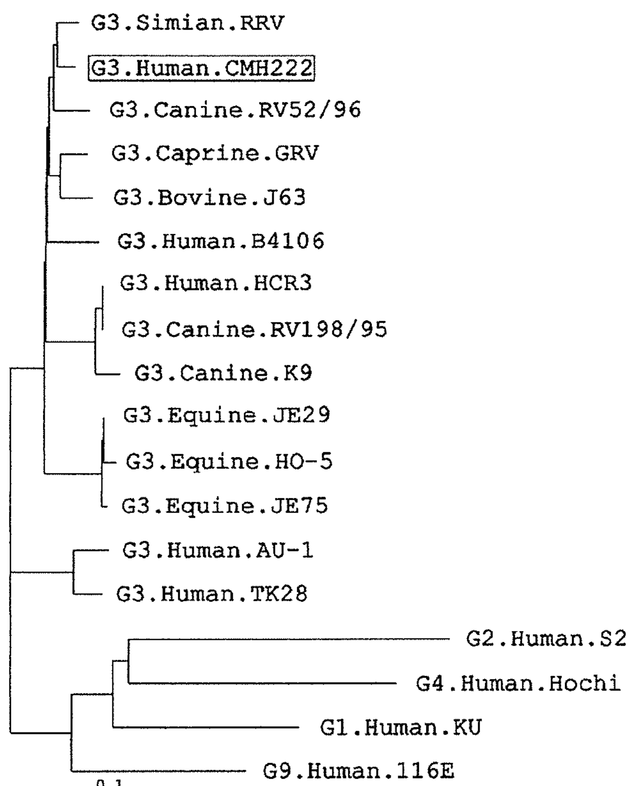

Fig. 2. Phylogenetic analysis of the deduced amino acid sequence derived from *VP7* gene of the CMH222 human rotavirus strain and other G3 rotavirus genotypes. The tree was generated based on the neighbor-joining method using ClustalX program.

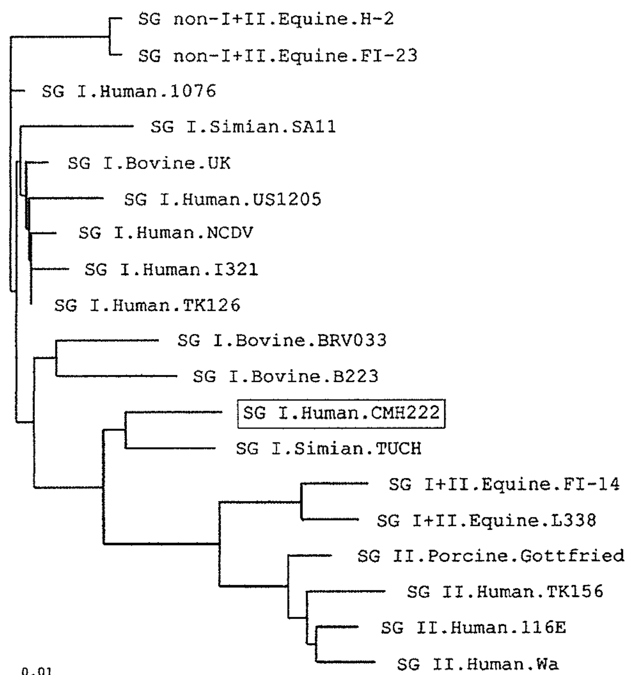

Fig. 3. Phylogenetic analysis of the deduced amino acid sequence derived from *VP6* gene of the CMH222 human rotavirus strain and other rotavirus subgroups. The tree was generated based on the neighbor-joining method using ClustalX program.

Analysis of Nucleotide Sequence of *NSP4* Gene

Sequence analysis of *NSP4* gene of CMH222 strain revealed the greatest similarity with caprine strain GRV genotype C at 98.8% amino acid identity. In contrast, non-genotype C showed only 29.7%–86.2% amino acid identity (data not shown). Phylogenetic analysis also confirmed that the *NSP4* gene of human CMH222 rotavirus strain clustered with the NSP4 genotype C caprine GRV and feline FRV-384 rotavirus strains (Fig. 4).

DISCUSSION

Rotavirus interspecies transmission strains have been described frequently in the literature, and there are an increasing number of reports of atypical rotaviruses that are apparently derived from transmission between humans and animals [Isegawa et al., 1992; Nakagomi et al., 1993; Li et al., 1993a,b]. During two-years surveillance of rotavirus infection in children admitted to hospital with diarrhea in Chiang Mai, Thailand, CMH222, was detected, which appeared to be

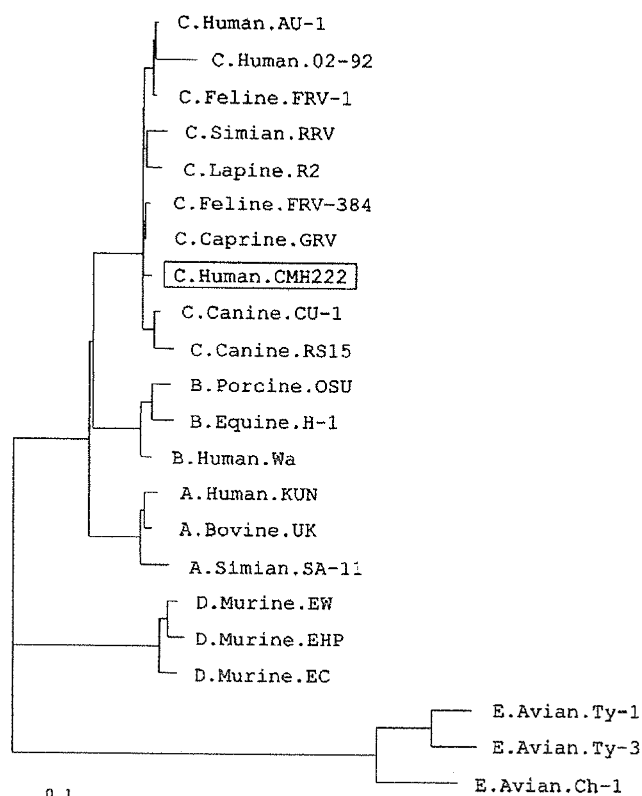

Fig. 4. Phylogenetic analysis of the deduced amino acid sequence derived from *NSP4* gene of the CMH222 human rotavirus strain and other NSP4 rotavirus genotypes. The tree was generated based on the neighbor-joining method using ClustalX program.

related to rotavirus strains of animal origin. Strain CMH222 was identified as genotype G3 and found in combination with a rare P[3] genotype. Sequence analysis of the *VP7* and *VP4* genes revealed a close relationship to simian *VP7* and caprine *VP4* genes, respectively. In addition, sequence analysis of the genes encoding VP6 and NSP4 also revealed close relationships with rotavirus strains of animal origin. Rotavirus strains with a G3 genotype and P[3] genotype are usually found in association with animal host species, for example, feline, canine, simian, and caprine [Mackow et al., 1988; Taniguchi et al., 1994; Martella et al., 2001a,b; Lee et al., 2003]. In contrast, detection of G3P[3] in humans is uncommon and only two strains of human G3P[3], HCR3 [Li et al., 1993a] and Ro1845 [Aboudy et al., 1988] have been reported. The detection of G3P[3] in humans was presumably due to the interspecies transmission of the virus from animals to humans [Nakagomi et al., 1990, 1992, 1993; Li et al., 1993a,b; Fujiwara and Nakagomi, 1997; Nakagomi and Nakagomi, 2000].

Rotavirus strains sharing >89% VP4 amino acid sequence identities are considered to belong to the same P genotype, while those sharing VP4 amino acid sequence identities <89% belong to different genotypes [Gorziglia et al., 1990; Estes, 2001]. Amino acid sequence of *VP4* gene of strain CMH222 displayed the highest identity to a caprine GRV P[3] rotavirus VP4 sequence with amino acid homology of 96.4%, indicating that strain CMH222 belonged to a caprine-like P[3] genotype. The non-P[3] rotavirus strains showed only 58.9%–84.7% amino acid identities with CMH222. In addition, it was noticed that simian rotavirus strain RRV shared a 92.0% amino acid homology in the VP4 sequence with CMH222. Previously, the study of relationships among G3P[3] rotavirus by phylogenetic analysis of *VP4* gene revealed two major lineages within P[3] genotype. Lineage one consisted of two human P[3] strains (HCR3 and Ro1845) together with rotavirus strains from feline and canine animal sources, while the simian rotavirus strain RRV formed a lineage distinct from these P[3] rotaviruses [Santos et al., 1998]. As shown in Figure 1, CMH222 strain was the only human P[3] that clustered within the same lineage of caprine GRV, buffalo, and simian strains, but distinct from those of feline, canine, and human P[3] rotaviruses. Our data analysis has consistently confirmed the hypothesis that the *VP4* gene of P[3] genotypes probably has two major lineages.

Characterization of the *VP7* gene by multiplex-PCR identified CMH222 as genotype G3, one of the most common genotypes found in humans [Gentsch et al., 1996, 2005]. Although the G3 genotype is shared by rotaviruses from a broad spectrum of mammalian species, including human, it has been possible to identify species-specific sequences in the *VP7* gene as well as to show a species-specific segregation of the VP7 by phylogenetic analysis [Nishikawa et al., 1989; Ciarlet et al., 1995; Martella et al., 2001b]. Assessment of the CMH222 VP7 sequence revealed the highest identity to the VP7 sequence of genotype G3 simian strain RRV and only limited identity to human G3 strains. Further evidence to support the simian origin of CMH222 was the fact that both RRV and CMH222 displayed G3P[3] genotypes.

Based on phylogenetic analysis, the *VP7* gene of genotype G3 rotavirus strains could be splited into two major lineages (Fig. 2). Human G3 rotaviruses clustered into one lineage while all non-human G3 rotaviruses tended to cluster in a separate lineage, with the exception of two human rotavirus strains, B4106 and HCR3 [De Leener et al., 2004]. It was interesting to note that despite the isolation of CMH222 from a human source, the strain clustered in a lineage with the non-human G3 strains and within the same branch as simian rotavirus strain RRV. This finding suggests that the *VP7* gene of human G3 strain CMH222 probably emerge from a common ancestor to the *VP7* gene of RRV.

It is now well known that the major inner capsid protein VP6 contains domains which specify the subgroup antigens [Greenberg et al., 1983a; Taniguchi et al., 1984]. Subgroup specificity has been defined according to the presence or absence of two distinct epitopes reactive with one, both, or neither of the MAbs [Greenberg et al., 1983b]. The epitopes recognized by the subgroup-specific MAbs are thought to be conformational and therefore present only in the

trimeric form of protein [Gorziglia et al., 1985; Sabara et al., 1987]. Strain CMH222 was assigned initially SG non-I + II specificity based on the subgroup ELISA assay. However, sequencing of the *VP6* gene gave contradictory results and CMH222 was found to have SG I specificity. The *VP6* gene of CMH222 was found to have the highest amino acid and nucleotide sequence identities with the *VP6* gene of SG I simian strain TUCH. In addition, phylogenetic analysis revealed that the *VP6* genes of these strains may have emerged from the same ancestor.

Subgroup specificities have a broad correlation with G serotypes in humans, that is, SG I specificity is usually associated with serotype G2, G8, and G9 strains, while SG II specificity is more commonly associated with G1, G3, G4, and G9 strains. Molecular characterization of the *VP6* gene has shown that SG I and SG II epitopes are typically found in human rotavirus strains, and SG I, SG I + II, and SG non-I + II epitopes are typically in rotavirus strains of non-human origin [Gorziglia et al., 1988; Ito et al., 1997; Tang et al., 1997; Iturriza Gomara et al., 2002]. Additionally, among human rotaviruses, SG I specificity is associated with short electropherotype and SG II with long electropherotype. On the other hand, long electropherotype with SG I specificity is usually found in animal rotaviruses [Kapikian et al., 2001]. The detection of a human G3 strain (CMH222) with the VP7 sequence closely related to G3 simian strain RRV and carried SG I specificity may be indicative of an animal strain causing infection in a human subject. Unfortunately, the electropherotype of CMH222 could not be detected by PAGE even though its genome has been successfully amplified by RT-PCR.

It was unclear why CMH222 did not react with SG I specific MAb. It is more likely that the accumulation of point mutations may lead to amino acid changes on the epitopes and, therefore, could not be recognized by SG-specific MAbs. However, low concentration of the virus in the sample cannot be discounted. It has been suggested that an Ala residue at position 172 may play a significant contribution to the formation of SG I specific epitope [Lopez et al., 1994; Tang et al., 1997]. Indeed, the VP6 amino acid sequence of the CMH222 strain contained a unique change at amino acid 172 from Ala to Gln. An alternative possibility is that the addition of a nucleotide in the *VP6* gene may results in a frame-shift mutation that lead to the change of SG specificity. In this regard, the VP6 deduced amino acid sequence of CMH222 was compared with the reference strains, no frame-shift mutation was observed.

Although five rotavirus NSP4 genotypes have been identified to date [Cunliffe et al., 1997; Horie et al., 1997; Ciarlet et al., 2000; Mori et al., 2002a], the diversity of the NSP4 encoding genes among human rotaviruses has been mainly restricted to genotypes A or B, and a few genotype C. Conversely, all five NSP4 genotypes (A–E) have been identified in rotaviruses of animal origins. To our knowledge, only two strains of human rotaviruses, AU-1 and 09/92, have been reported to belong to NSP4 genotype C. In our analysis, the CMH222 strain also belonged to the NSP4 genotype C and formed phylogenetic cluster with other NSP4 genotype C rotavirus of animal origins. Again, this finding suggests that the NSP4 of the CMH222 strain may have originated from genotype C of animal origins by interspecies transmission events.

In conclusion, molecular genetic analyses provide strong evidence that CMH222 might be the reassortant, which resulted from multiple interspecies transmission events that lead to reassortment of the viruses of human, caprine, and simian origins.

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Existence of Multiple Genotypes Associated With Acute Gastroenteritis During 6-Year Survey of Norovirus Infection in Japan

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Norovirus (NoV) is recognized as one of the most common causative agent of diarrheal disease in young children worldwide. The current study was undertaken to determine the distribution of NoV genotypes in Japan. A total of 3,864 fecal specimens from children with acute gastroenteritis in five regions (Tokyo, Maizuru, Saga, Sapporo, and Osaka) of Japan from July 1995 to June 2001 were collected and then tested for the presence of NoV by RT-PCR. Three hundred sixty four were found to be positive for NoV, accounting for 11%. The highest prevalence of NoV infection was in November, December, and January as the early winter months in Japan. NoV was subjected to be further characterized to sequencing analysis. All NoVs belonged to two different genogroups I and II and these represented 3% and 97%, respectively. This finding indicated that NoV genogroup II was the dominant group causing acute gastroenteritis in Japan. Interestingly, NoV strains were classified into 16 distinct genotypes including genogroup II genotype 9 that was firstly identified in Japan. Of these, NoV genogroup II genotypes 3 and 4 dominated over other genotypes and became the leading strains in Japanese pediatric population. In conclusion, diarrhea due to NoV infection is still a health burden in Japan. This report also stresses the great genetic diversity as well as the importance of NoV causing the diarrhea in Japan. ***J. Med. Virol. 78:1318–1324, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: norovirus; genotype; diversity; Japan

INTRODUCTION

Norovirus (NoV) is in the family *Caliciviridae* and contains a single-stranded positive-sense RNA genome, approximately 7.7 kb in size. The NoV genome composes of three open reading frames (ORFs). ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase, ORF 2 encodes the capsid protein, and ORF3 a small capsid protein. To date, NoV can be genetically divided into three genogroups (GI, GII, and GIII) based on genome sequence. Of these, NoV GI and GII are known to infect humans and NoV GIII infects animals including bovine and murine. NoV cannot be cultivated in cell culture or experimental animal models. Detection of NoV has relied mainly on RT-PCR using specific primers with the binding sites at the polymerase region or the capsid region [Katayama et al., 2002]. For the genetic classification of NoV, the polymerase region or the capsid region has been used independently. Recently, genetic classification of NoV has described at least 14 and 17 different genotypes for NoV GI and GII, respectively [Kageyama et al., 2004] in which strain Alphatron belongs to NoV genogroup II genotype 17. This capsid region-based classification appeared to distinguish successfully the antigenicity determined by both antigen and antibody ELISA with recombinant virus-like particle [Kobayashi et al., 2000a,b]. Hardy et al. [1997] reported a naturally, occurring recombinant in NoV, then several NoV strains have been described as recombinants and the recombination site were found at the junction of ORF1 and ORF2 [Jiang et al., 1999; Hansman et al., 2004].

Norovirus has been reported as one of the major causative agents of non-bacterial gastroenteritis in all

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age groups [Inouye et al., 2000; Lopman et al., 2002]. NoV is highly infectious and associated with food-borne and water-borne outbreaks of acute gastroenteritis worldwide in different epidemiologic settings such as hospitals, hotels, schools, cruise ship, and restaurants [Inouye et al., 2000; Billgren et al., 2002; Kageyama et al., 2004]. However, the diarrheal illness due to NoV is usually mild and self-limiting. Global outbreaks of gastroenteritis have been caused previously by different strains of NoV GI and II. Since a study reported by Noel et al. [1999] found the "95/96-US" strain which is grouped into genogoup II genotype 4 (GII/4, known as a Lordsdale cluster) having a global distribution, an unusual increase in the number of NoV outbreaks was reported in Europe and the United States [Lopman et al., 2004; Vipond et al., 2004]. Even NoV infection has a great impact on people in both developing and developed countries; and effective anti-NoV drugs have not been developed. Molecular epidemiology of NoV infection is needed in order to successfully control and prevent illnesses caused by NoV.

The objectives of this study were: to determine the incidence of NoV infections in children with acute gastroenteritis in five different regions of Japan from 1995 to 2001; 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 determined.

MATERIALS AND METHODS

Fecal Specimens

A total of 3,864 fecal specimens were collected from children with acute gastroenteritis in Sapporo, Tokyo, Maizuru, Osaka, and Saga of Japan from July 1995 to June 2001. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000*g* for 10 min. The supernatants were collected and stored at −30°C until use for the detection of NoV.

Extraction of Viral Genome

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

Reverse Transcription (RT)

For RT, 7.5 µl of extracted viral genome was added with a reagent mixture consisting of 2.05 µl of 5× First strand buffer (Invitrogen, Carlsbad, CA), 0.75 µl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 µl of 10 mM DTT (Invitrogen), 0.75 µl (200 U/µl) of superscript reverse transcriptase III (Invitrogen), 0.375 µl (1 µg/µl) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 µl (33 U/µl) of RNase Inhibitor (Toyobo, Osaka, Japan), and 2.325 µl MilliQ water. The total of the reaction mixture was 15 µl [Yan et al., 2003]. RT step was carried out at 50°C for 1 hr, followed by 99°C for 5 min and then held at 4°C.

Polymerase Chain Reaction (PCR)

Using PCR with specific primers as previously reported resulted in the identification of two genogroups of NoV [Yan et al., 2003]. Two pairs of specific primers G1SKF (CTGCCCGAATTYGTAAATGA) and G1SKR (CCAACCCARCCATTRTACA), and COG2F (CARGAR BCNATGTTYAGRTGGATGAG) and G2SKR (CCRCC NGCATRHCCRTTRTACAT) [where B is C, G, or T; H is A, C, or T; N is any base; R is A or G; and Y is C or T] that amplify capsid gene of NoV were used to detect NoV GI and GII, respectively. These primers were generated specifically for two different sizes of amplicons of 330 bp and 387 bp for NoV GI and NoV GII, respectively. PCR was carried out with 2.5 µl of cDNA in 22.5 µl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI), 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 sec, 55°C for 30 sec, 72°C for 60 sec, 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 then visualized under ultraviolet (UV) light, and 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., Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). 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 Kimura's two-parameter method (PHYLIP). Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Toronto (U02030), Lordsdale (X86557), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761), Saitama U1 (AB039775), Camberwell (AF145896), Snow (U70059), Arg320 (AF190817), Mexico (U22498), MD145 (AY032605), Mora/97/SE (AY081134), Saitama-KU80aGII/99/JP (AB058582), Bristol (X76716), SaitamaU16 (AB039778), SaitamaU17 (AB039779), WUG1 (AB081723), Chiba (AB022679), Birmingham (AJ277612), and Saitama KU8/99/JP (AB058547).

TABLE I. Distribution of NoV Infection Among Children by Age Group From 1995 to 2001

	0 m	6 m	1 y	2 y	3 y	4 y	5 y	6 y	7 y	8 y	9 y	10 y	ND	Total
1995/1996	1	4	11	7	2	3	1	0	0	0	0	0	6	35
1996/1997	2	6	16	5	2	0	0	0	0	0	0	0	0	31
1997/1998	7	14	27	8	3	1	0	0	0	0	1	1	9	71
1998/1999	3	9	24	9	2	2	3	0	1	0	0	3	4	60
1999/2000	2	14	30	15	9	2	2	3	1	1	2	2	13	96
2000/2001	0	18	21	13	3	2	2	4	3	2	0	2	1	71
Total	15	65	129	57	21	10	8	7	5	3	3	8	33	364

Note: m, month; y, year; ND, not determined.

RESULTS

Epidemiology of NoV Infection

A total of 3,314 fecal specimens collected from children with acute gastroenteritis in Sapporo, Tokyo, Maizuru, Osaka, and Saga of Japan during July 1995 and June 2001 were examined for NoV. In the pediatric population, the lowest age was 0 month and the highest was 10 years. Of 3,314 fecal specimens tested, 364 were detected to be positive for NoV and this represented 11%. Table I showed that the highest NoV infection was in the 1-year old group (35.4%; 129 of 364). The NoV infection was identified among children aged less than 6 months (4.1%; 15 of 364). It was also found that children younger than 3 years had a high rate of NoV infection (73.1%, 266 of 364).

Seasonal Variation of NoV Infection

The NoV detection rate was analyzed between July 1995 and June 2001. Figure 1 shows that NoV was detected continuously for 10 months (September to June). No NoV was found in both July and August. The highest prevalence of NoV infection was in December (41.5%; 151 of 364), followed by January and November with 15.7% (57 of 364) and 13% (47 of 364), respectively. The lowest NoV detection rate was in October (0.3%; 1 of 364).

Distribution of NoV G-Types

The nucleotide sequence of the 5′ ends of the NoV capsid gene was determined by direct sequencing with the amplified fragments. This region has been shown to be suitable for genotyping. All NoV sequences were analyzed by phylogenetics and grouped using the NoV capsid region classification scheme of Kageyama et al. [2004]. In the present study, all of the NoV sequences were classified into two distinct genogroupes I and II and these represented 3% (11 cases) and 97% (353 cases), respectively. The NoV GI sequences clustered into four genotypes (GI/3, GI/4, GI/8, and GI/11), accounting for 27.3% (3 of 11), 54.5% (6 of 11), 9.1% (1 of 11), and 9.1% (1 of 11), respectively. In NoV GII, genotype 4 was dominant every year, from 41.9% (1996–1997) to 80% (1995–1996) followed by genotype 3 as second predominant strain, ranging from 19.1% (1999–2000) to 38% (1997–1998) (Table II). Moreover, many other NoV genotypes including GII/1, GII/2, GII/5, GII/6, GII/9, GII/10, GII/12, GII/13, GII/14, and GII/15 were found

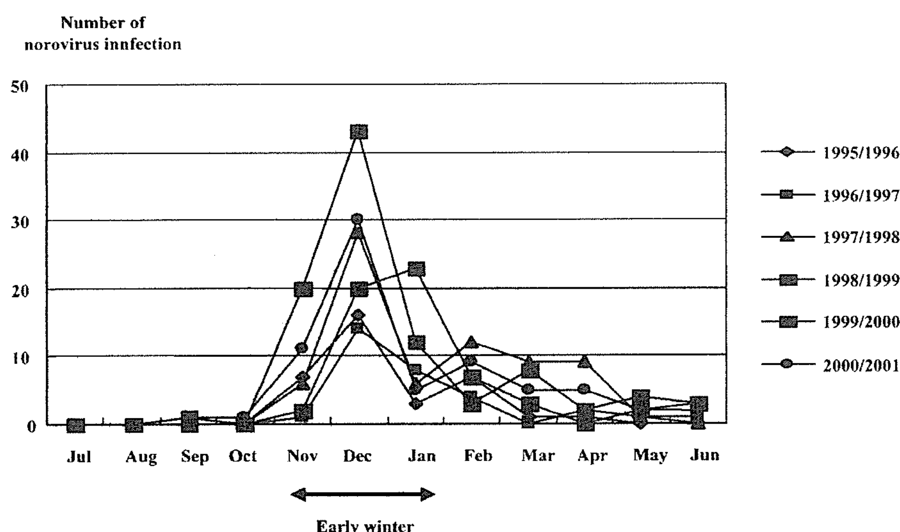

Fig. 1. Seasonal pattern of NoV detected among children with acute gastroenteritis during 6-year survey of NoV infection in Sapporo, Tokyo, Maizuru, Osaka, and Saga of Japan during July 1995 and June 2001. The cold season was also indicated.