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.

ACKNOWLEDGMENTS

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.

REFERENCES

Bon F, Fascia P, Dauvergne M, Tenenbaum D, Planson H, Petion AM, Pothier P, Kohli E. 1999. Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France. J Clin Microbiol 37:3055–3058.

Chiba S, Sakuma Y, Kogasaka R, Akihara M, Horino K, Nakao T, Fukui S. 1979. An outbreak of gastroenteritis associated with calicivirus in an infant home. J Med Virol 4:249–254.

Dai YC, Nie J, Zhang XF, Li ZF, Bai Y, Zeng ZR, Yu SY, Farkas T, Jiang X. 2004. Seroprevalence of antibodies against noroviruses among students in a Chinese military medical university. J Clin Microbiol 42:4615–4619.

Hardy ME, Kramer SF, Treanor JJ, Estes MK. 1997. Human calicivirus genogroup II capsid sequence diversity revealed by analyses of the prototype Snow Mountain agent. Arch Virol 142: 1469–1479.

Inouye S, Yamashita K, Yamadera S, Yoshikawa M, Kato N, Okabe N. 2000. Surveillance of viral gastroenteritis in Japan: Pediatric cases and outbreak incidents. J Infect Dis 181:270–274.

Iritani N, Seto Y, Kubo H, Haruki K, Ayata M, Ogura H. 2002. Prevalence of "Norwalk-like virus" infections in outbreaks of acute nonbacterial gastroenteritis observed during the 1999–2000 season in Osaka City, Japan. J Med Virol 66:131–138.

Iritani N, Seto Y, Kubo H, Murakami T, Haruki K, Ayata M, Ogura H. 2003. Prevalence of Norwalk-like virus infections in cases of viral gastroenteritis among children in Osaka City, Japan. J Clin Microbiol 41:1756–1759.

Jiang X, Matson DO, Ruiz-Palacios GM, Hu J, Treanor J, Pickering LK. 1995. Expression, self-assembly, and antigenicity of a snow mountain agent-like calicivirus capsid protein. J Clin Microbiol 33:1452–1455.

Jiang X, Espul C, Zhong WM, Cuello H, Matson DO. 1999a. Characterization of a novel human calicivirus that may be a naturally occurring recombinant. Arch Virol 144:2377–2387.

Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO. 1999b. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J Virol Methods 83:145–154.

Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, Takai R, Oka T, Takeda N, Katayama K. 2004. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. J Clin Microbiol 42:2988–2995.

Katayama K, Shirato-Horikoshi H, Kojima S, Kageyama T, Oka T, Hoshino F, Fukushi S, Shinohara M, Uchida K, Suzuki Y, Gojobori T, Takeda N. 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology 299:225–239.

Kawamoto H, Yamazaki K, Utagawa E, Ohyama T. 2001. Nucleotide sequence analysis and development of consensus primers of RT-PCR for detection of Norwalk-like viruses prevailing in Japan. J Med Virol 64:569–576.

Kirkwood C. 2004. Viral gastroenteritis in Europe: A new norovirus variant? Lancet 363:671–672.

Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC. 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73:152–160.

Lopman BA, Brown DW, Koopmans M. 2002. Human caliciviruses in Europe. J Clin Virol 24:137–160.

Lopman B, Vennema H, Kohli E, Pothier P, Sanchez A, Negredo A, Buesa J, Schreier E, Reacher M, Brown D, Gray J, Iturriza M, Gallimore C, Bottiger B, Hedlund KO, Torven M, von Bonsdorff CH, Maunula L, Poljsak-Prijatelj M, Zimsek J, Reuter G, Szucs G, Melegh B, Svennson L, van Duijnhoven Y, Koopmans M. 2004. Increase in viral gastroenteritis outbreaks in Europe and epidemic spread of new norovirus variant. Lancet 363:682–688.

Marks PJ, Vipond IB, Carlisle D, Deakin D, Fey RE, Caul EO. 2000. Evidence for airborne transmission of Norwalk-like (NLV) in a hotel restaurant. Epidemiol Infect 120:481–487.

Matson DO. 1994. Viral gastroenteritis in day-care settings: Epidemiology and new developments. Pediatrics 94:999–1001.

McEvoy M, Blake W, Brown D, Green J, Cartwright R. 1996. An outbreak of viral gastroenteritis on a cruise ship. Commun Dis Rep CDR Rev 6:188–192.

McIntyre L, Vallaster L, Kurzac C, Fung J, McNabb A, Lee MK, Daly P, Petric M, Isaac-Renton J. 2000. Gastrointestinal outbreaks associated with Norwalk virus in restaurants in Vancouver, British Columbia. Can Commun Dis Rep 28:197–203.

Nilsson M, Hedlund KO, Thorhagen M, Larson G, Johansen K, Ekspong A, Svensson L. 2003. Evolution of human calicivirus RNA in vivo: Accumulation of mutations in the protruding P2 domain of the capsid leads to structural changes and possibly a new phenotype. J Virol 77:13117–13124.

Noel JS, Fankhauser RL, Ando T, Monroe SS, Glass RI. 1999. Identification of a distinct common strain of "Norwalk-like viruses" having a global distribution. J Infect Dis 179:1334–1344.

Oh DY, Gaedicke G, Schreier E. 2003. Viral agents of acute gastroenteritis in German children: Prevalence and molecular diversity. J Med Virol 71:82–93.

Peasey AE, Ruiz-Palacios GM, Quigley M, Newsholme W, Martinez J, Rosales G, Jiang X, Blumenthal UJ. 2004. Seroepidemiology and risk factors for sporadic norovirus/Mexico strain. J Infect Dis 189: 2027–2036.

Phan TG, Okame M, Nguyen TA, Maneekarn N, Nishio O, Okitsu S, Ushijima H. 2004. Human astrovirus, norovirus (GI, GII), and sapovirus infections in Pakistani children with diarrhea. J Med Virol 73:256–261.

Schreier E, Doring F, Kunkel U. 2000. Molecular epidemiology of outbreaks of gastroenteritis associated with small round structured viruses in Germany in 1997/98. Arch Virol 145:443–453.

Vinje J, Altena SA, Koopmans MP. 1997. The incidence and genetic variability of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. J Infect Dis 176:1374–1378.

Vinje J, Green J, Lewis DC, Gallimore CI, Brown DW, Koopmans MP. 2000. Genetic polymorphism across regions of the three open reading frames of "Norwalk-like viruses". Arch Virol 145:223–241.

White PA, Hansman GS, Li A, Dable J, Isaacs M, Ferson M, McIver CJ, Rawlinson WD. 2002. Norwalk-like virus 95/96-US strain is a major cause of gastroenteritis outbreaks in Australia. J Med Virol 68:113–118.

Worobey M, Holmes EC. 1999. Evolutionary aspects of recombination in RNA viruses. J Gen Virol 80:2535–2543.

Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H. 2003. Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods 14:37–44.

Clin. Lab. 2006;52:247-253

ORIGINAL ARTICLE

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

TUNG GIA PHAN[1], FUMIHIRO YAGYU[1], VLADIMIR KOZLOV[2], ALEXEI KOZLOV[2]
SHOKO OKITSU[1], WERNER E.G. MÜLLER[3], HIROSHI USHIJIMA[1]

[1]*Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[2]*Department of Pediatrics, Mother and Child Healthcare Institute, Far-Eastern State Medical University, Khabarovsk, Russia*
[3]*Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität Mainz, Germany*

SUMMARY

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

KEY WORD

norovirus, recombination, Eastern Russia

INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality of children due to gastroenteritis is greater in developing than in the developed countries [1]. It has been well established that virtually every child becomes infected with a rotavirus at least once by 3 years of age [2]. Rotaviruses are classified into seven groups (A to G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with group A, B and C rotaviruses. Of these, group A rotavirus is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [3]. Apart from group A rotavirus as the most common cause of gastroenteritis, norovirus is also considered to be a global enteropathogen. This virus is associated with sporadic cases and outbreaks of acute gastroenteritis in such settings as kindergartens, schools, nursing homes for the elderly, and among military recruits [4, 5]. The transmission routes of this virus are classified into foodborne, airborne, person-to-person spread and perhaps by some other unknown modes [4, 6]. However, norovirus is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make norovirus a major public health concern [7].

Norovirus (NoV, formerly known as "Norwalk-like virus") is the distinct genus within the family *Caliciviridae*. Norovirus contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The norovirus genome contains three ORFs (ORF1, 2 and

Manuscript accepted January 9, 2006

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

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

MATERIALS AND METHODS

Fecal specimens

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

Extraction of viral genome

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

Reverse transcription (RT)

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

Polymerase chain reaction (PCR)

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

Group A rotavirus serotyping

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

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

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

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

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

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

Electrophoresis

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

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of the PCR products (DNA) positive for diarrheal virus were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). A phylogenetic tree with 1,000 bootstrap replicates of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3) was used to compare recombinant norovirus sequences. The nucleotide sequence data of norovirus strain HU/78/04/RU had been submitted to the DDBJ DNA/GenBank database and had been assigned accession number AB242258. Reference norovirus strains and accession numbers used in this study were as follows: Manchester (X86560), Melksham (X81879), Toronto24/91/CA (U02030), Lordsdale (X86557), Camberwell (AF145896), MD145 (AY032605), Hawaii (U07611), Mora/97/SE (AY081134), and Bristol (X86557).

RESULTS

Epidemiology of viral infections

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

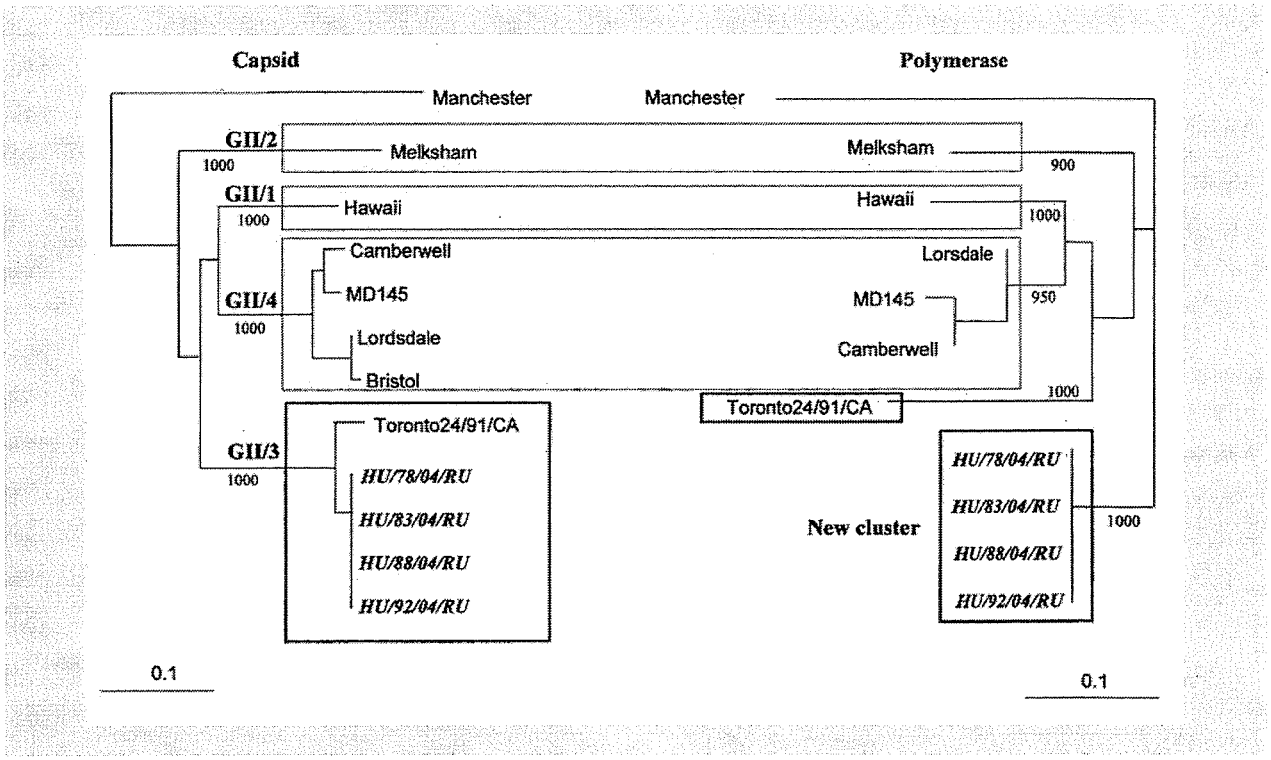

Figure 1: Observation of changes of norovirus genotypes on the basis of phylogenetic trees of nucleotide sequences. The trees were constructed from partial nucleotide sequences of capsid and polymerase regions of Russian isolates of norovirus genogroup II. Reference strains of norovirus were selected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

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

Clinical manifestations

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

Detection of norovirus infection

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

Nucleotide sequencing and phylogenetic analysis of norovirus genotype

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

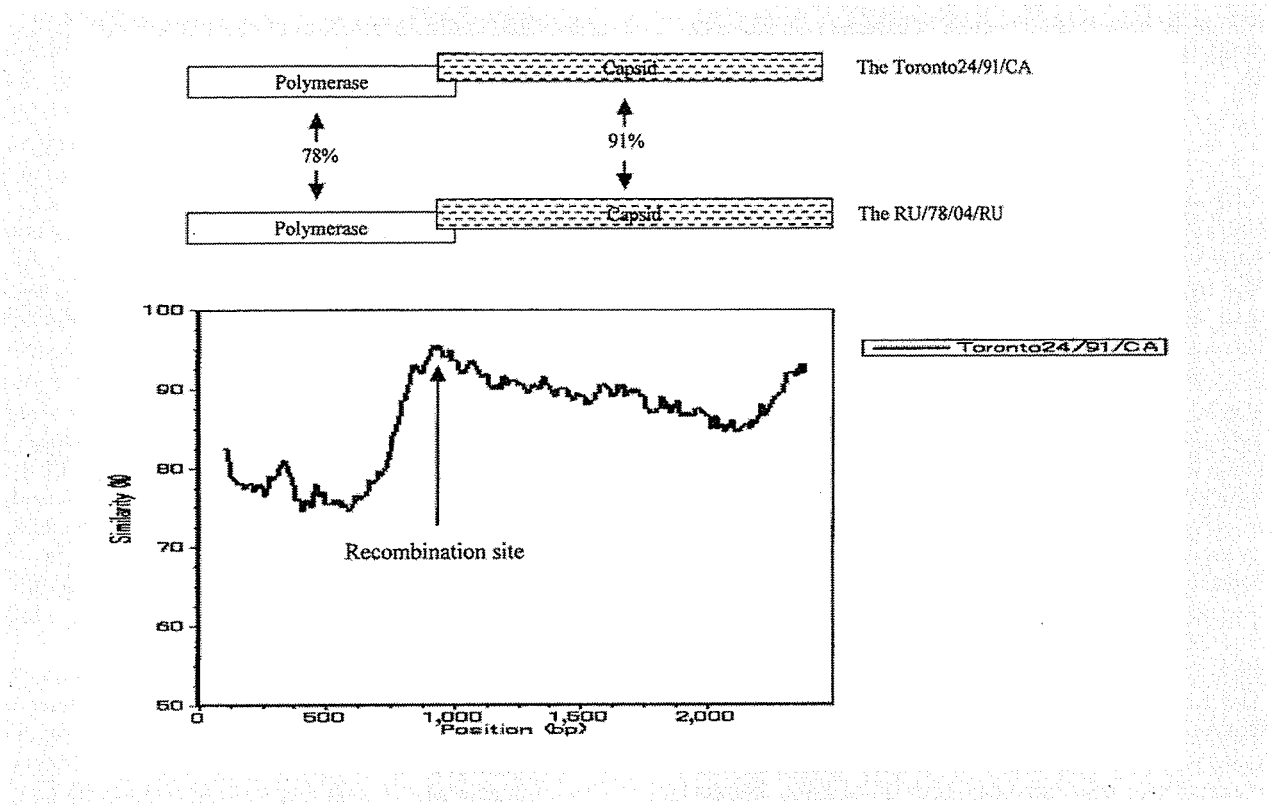

Figure 2: Genetic characterization of recombinant norovirus: the Simplot analysis of strains HU/78/04/RU and the Toronto24/91/CA. The HU/78/04/RU was used as query strain and the Toronto24/91/CA was used as reference strain. Low and high homologies with polymerase and capsid regions among them were found.

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

Nucleotide sequencing and genetic analysis of norovirus polymerase

To further analyze the genetic characteristics of noroviruses from Eastern Russia, the partial polymerase regions of all NoV with GII/3 capsid were additionally amplified and sequenced. Interestingly, all norovirus isolates were not classified into any previously published norovirus cluster, even with the Toronto24/91/CA when polymerase-based grouping was performed (Figure 1). The nucleotide levels of the polymerase region of these norovirus isolates had low homologies, ranging from 57% to 78%, with other norovirus reference strains. Taken together, the norovirus isolates in Eastern Russia represented a novel cluster based on polymerase sequences. It was found that the HU/78/04/RU shared a low level of sequence identity (78%) in the RNA polymerase region and a high level of nucleotide identity (91%) in the capsid region with the Toronto24/91/CA. Therefore, these 4 norovirus isolates were expected to be recombinants with GII/3 capsid and novel polymerase.

Recombination in norovirus ORF1/ORF2 overlap

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

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

DISCUSSION

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

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

RNA recombination is a mechanism for virus evolution [20]. There is now a fairly rich literature documenting recombination in norovirus. The first naturally occurring recombinant norovirus was the prototype Snow Mountain virus in the US [21]. Later, several recombinant noroviruses causing sporadic cases and outbreaks of acute gastroenteritis were reported worldwide, such as the Thai isolate Mc37 [22], the Japanese isolate Saitama U1 [15], the Australian isolate Sydney C14/02/AU [23], the German isolate BRA/2.2/98/DEU [24], and the Arg302 from Argentina [25] but no recombinant norovirus from Russia. In this study, the capsid sequences of the HU/78/04/RU and the Toronto24/91/CA were highly identical, but the polymerase sequence of the HU/78/04/RU was distinctly different from that of the Toronto24/91/CA. Furthermore, an apparent site of genetic recombination of this norovirus was found in the ORF1/ORF2 overlap. This pattern of genetic characterization of the HU/78/04/RU implied a naturally occurring recombinant norovirus with GII/7 capsid and novel polymerase and would be the first recombinant norovirus identified in Russia. The phylogenetic analysis of the polymerase and capsid sequences of the four norovirus isolates in the present study demonstrated only one recombinant NoV GII sequence. Moreover, they had high homology (100%) at the nucleotide level and the amino acid level. It was indicative that they came from the same source of norovirus infection. However, in order to monitor the quality of extraction and RT-PCR, Hu/NoVGII/Maizuru/5188 and MilliQ water were used as positive and negative controls, respectively. To avoid false positive results of the experiments, instructions to prevent cross-contamination were strictly followed [26]. Therefore, contamination leading to the detection of these NoV isolates was unlikely.

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

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

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

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

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan and by Fund Grant of the Alliance for Global Sustainability supported by the University of Tokyo.

References

1. Murray CJ, Lopez AD. Mortality by cause for eight regions of the world: Global burden of disease study. Lancet 1997; 349: 1269-76
2. Mulholland EK. Global control of rotavirus disease. Adv Exp Med Biol 2004; 549: 161-8
3. Bridger JC. Non-group A rotavirus. In: Kapikian AZ (ed) Viral infection of the gastrointestinal tract. New York: Marcel Dekker, 1994: 369-408
4. Lopman BA, Brown DW, Koopmans M. Human caliciviruses in Europe. J Clin Virol 2002; 24: 137-60
5. Noel JS, Fankhauser RL, Ando T, Monroe SS, Glass RI. Identification of a distinct common strain of "Norwalk-like viruses" having a global distribution. J Infect Dis 1999; 179: 1334-44
6. McIntyre L, Vallaster L, Kurzac C, et al. Gastrointestinal outbreaks associated with Norwalk virus in restaurants in Vancouver, British Columbia. Can Commun Dis Rep 2000; 28: 197-203
7. Kageyama T, Shinohara M, Uchida K, et al. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to norovirus in Japan. J Clin Micro-biol 2004; 42: 2988-95
8. Jiang X, Matson DO, Ruiz-Palacios GM, Hu J, Treanor J, Pickering LK. Expression, self-assembly, and antigenicity of a snow mountain agent-like calicivirus capsid protein. J Clin Microbiol 1995; 33: 1452-5
9. Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. J Virol 1972; 10: 1075-81
10. Dai YC, Nie J, Zhang XF, et al. Seroprevalence of antibodies against noroviruses among students in a Chinese military medical university. J Clin Microbiol 2004; 42: 4615-9
11. Peasey AE, Ruiz-Palacios GM, Quigley M, et al. Seroepidemiology and risk factors for sporadic norovirus/Mexico strain. J Infect Dis 2004; 189: 2027-36
12. Jiang X, Matson DO, Velazquez FR, et al. Study of Norwalk-related viruses in Mexican children. J Med Virol 1995; 47: 309-16
13. Phan TG, Nguyen TA, Yan H, et al. Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in Eastern Russia. Clin Lab 2005; 51: 429-35
14. Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J Virol Methods 1999; 83: 145-54
15. Katayama K, Shirato-Horikoshi H, Kojima S, et al. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology 2002; 299: 225-39
16. Das S, Varghese V, Chaudhuri S, et al. Genetic variability of human rotavirus strains isolated from Eastern and Northern India. J Med Virol 2004; 72: 156-61
17. Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H. Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods 2003; 14:37-44
18. Yan H, Tuan AN, Phan TG,Okitsu S, Yan L, Ushijima H. Development of RT-multiplex PCR assay for detection of adenovirus, group A and C rotaviruses in diarrheal fecal specimens from children in China. Kansenshogaku Zasshi 2004; 78: 699-709
19. Phan TG, Nguyen TA, Yan H, Okitsu S, Ushijima H. Novel RT-multiplex PCR for enteroviruses, hepatitis A and E viruses and influenza A virus among infants and children with diarrhea in Vietnam. Arch Virol 2005, 150:1175-85
20. Worobey M, Holmes EC. Evolutionary aspects of recombination in RNA viruses. J Gen Virol 1999; 80: 2535-43
21. Jiang X, Espul C, Zhong WM, Cuello H, Matson DO. Characterization of a novel human calicivirus that may be a naturally occurring recombinant. Arch Virol 1999; 144: 2377-87
22. Hansman GS, Katayama K, Maneekarn N, et al. Genetic diver-sity of norovirus and sapovirus in hospitalized infants with spo-radic cases of acute gastroenteritis in Chiang Mai, Thailand. J Clin Microbiol 2004; 42: 1305-7
23. Bull RA, Hansman GS, Clancy LE, Tanaka MM, Rawlinson WD, White PA. Norovirus recombination in ORF1/ORF2 over-lap. Emerg Infect Dis 2005; 11: 1079-85
24. Schreier E, Doring F, Kunkel U. Molecular epidemiology of outbreaks of gastroenteritis associated with small round structured viruses in Germany in 1997/98. Arch Virol 2000; 145: 443-53
25. Jiang X, Espul C, Zhong WM, Cuello H, Matson DO. Characterization of a novel human calicivirus that may be a naturally occurring recombinant. Arch Virol 1999; 144: 2377-87
26. Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1989; 339: 237-38
27. Lai MM. RNA recombination in animal and plant viruses. Microbiol Rev 1992; 56: 61-79
28. Kirkwood C. Viral gastroenteritis in Europe: a new norovirus variant? Lancet 2004; 363: 671-2

Correspondence: Hiroshi Ushijima
Mailing address: Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033
Phone: +81-3-5841-3590
Fax: +81-3-5841-3629
Email: ushijima@m.u-tokyo.ac.jp

Novel Recombinant Norovirus in China

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

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

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

To eliminate the possibility of coinfection with 2 different NoV genotypes, to localize the potential recombination site, and to clarify a possible recombination mechanism, the ORF1/ORF2 overlap and flanking polymerase and capsid regions of 146/Kunming/04/China was amplified with primers Yuri22F and GIISKR to produce a 1,158-bp amplicon (*3*,*4*). When the sequence of 146/Kunming/04/China was compared with that of Saitama U4 by using SimPlot, a recombination site was found at the ORF1/ORF2 overlap. Before this junction, 146/Kunming/04/China and Saitama U4 were homologous. After the ORF1/ORF2 overlap, however, the homology was notably different. SimPlot showed a sudden drop in the nucleotide identity for 146/Kunming/04/China. ClustalX showed that 146/Kunming/04/China shared a high identity (93%) in the polymerase region and a low identity (78%) in the capsid region with Saitama U4. In contrast, high identity (95%) in the capsid region was found between 146/Kunming/04/China and Leeds/90/UK. Since Leeds/90/UK polymerase was not available in GenBank, the polymerase homology between 146/Kunming/04/China and Leeds/90/UK was unknown. Polymerase of 146/Kunming/04/China was almost

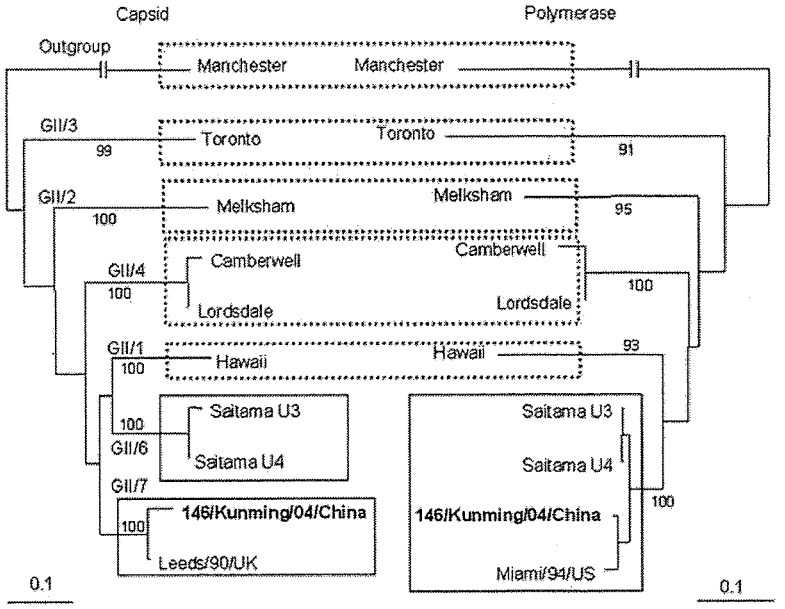

Figure. Changes in norovirus (NoV) genotypes on the basis of phylogentic trees of nucleotide sequences of 146/Kunming/04/China. Trees were constructed from partial nucleotide sequences of capsid and polymerase regions of 146/Kunming/04/China. 146/Kunming/04/China is **boldface**. Dashed boxes indicate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

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

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

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

The NoV capsid is predicted to be well suited for genotype classification (*10*). In this study, 146/Kunming/04/China belonged to 2 distinct genotypes, 7 and 6, by capsid- and polymerase-based groupings, respectively. Moreover, the recent demonstration of recombination in an increasing number of NoVs suggests that it is a more widespread event than was previously realized. Consequently, the phylogenetic classification of NoV on the basis of on capsid sequence is questionable. We suggest that classification of NoV strains should rely on not only capsid sequence but also polymerase sequence.

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

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, Japan.

Tung Gia Phan,* Hainian Yan,* Yan Li,† Shoko Okitsu,* Werner E.G. Müller,‡ and Hiroshi Ushijima*

*The University of Tokyo, Tokyo, Japan; †Kunming Medical College, Kunming, People's Republic of China; and ‡Universität Mainz, Mainz, Germany

References

1. Lopman BA, Brown DW, Koopmans M. Human caliciviruses in Europe. J Clin Virol. 2002;24:137–60.
2. Jiang X, Matson DO, Ruiz-Palacios GM, Hu J, Treanor J, Pickering LK. Expression, self-assembly, and antigenicity of a Snow Mountain agent-like calicivirus capsid protein. J Clin Microbiol. 1995;33:1452–5.
3. Phan TG, Nguyen TA, Yan H, Yagyu F, Kozlov V, Kozlov A, et al. Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in Eastern Russia. Clin Lab. 2005;51:429–35.
4. Saito H, Saito S, Kamada K, Harata S, Sato H, Morita M, et al. Application of RT-PCR designed from the sequence of the local SRSV strain to the screening in viral gastroenteritis outbreaks. Microbiol Immunol. 1998;42:439–46.
5. Lai MM. RNA recombination in animal and plant viruses. Microbiol Rev. 1992;56:61–79.
6. Bull RA, Hansman GS, Clancy LE, Tanaka MM, Rawlinson WD, White PA. Norovirus recombination in ORF1/ORF2 overlap. Emerg Infect Dis. 2005;11:1079–85.
7. Katayama K, Shirato-Horikoshi H, Kojima S, Kageyama T, Oka T, Hoshino F, et al. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology. 2002;299:225–39.
8. Green J, Vinje J, Gallimore CI, Koopmans M, Hale A, Brown DW, et al. Capsid protein diversity among Norwalk-like viruses. Virus Genes. 2000;20:227–36.
9. Worobey M, Holmes EC. Evolutionary aspects of recombination in RNA viruses. J Gen Virol. 1999;80:2535–43.
10. Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, et al. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to norovirus in Japan. J Clin Microbiol. 2004;42:2988–95.

Address for correspondence: Hiroshi Ushijima, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; email: ushijima@m.u-tokyo.ac.jp

Instructions for Emerging Infectious Diseases Authors

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

closely related to *E. coli*, as was previously observed in Tanzania (*9*). This finding is also the first report of CTX-M-3 in sub-Saharan Africa.

Multidrug resistance profiles involving non–β-lactam antimicrobial drugs coselected these ESBL-producing isolates. We suggest that the misuse of antimicrobial drugs in the Central African Republic and the migratory flux of regional populations could result in emergence and selection of these ESBL phenotypes in the community. We could not establish a relationship between the different strains isolated in hospitalized and ambulatory patients. Because of the implications for treating such infections, particularly in developing countries, the spread of ESBL-producing *Enterobacteriaceae* merits close surveillance in the Central African Republic.

This work was financed by grants from Institut Pasteur de Bangui, Faculté de Médecine Pierre et Marie Curie, Université Pierre et Marie Curie (Paris VI), and the European Community, contract LSHM-CT 2003-503335.

Thierry Frank,* Guillaume Arlet,†‡ Valerie Gautier,† Antoine Talarmin,* and Raymond Bercion*

*Institut Pasteur de Bangui, Bangui, Central African Republic; †Université Pierre et Marie Curie (Paris VI), Paris, France; and ‡Hôpital Tenon AP-HP, Paris, France

References

1. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. Clin Microbiol Rev. 2005;18:657–86.
2. Bonnet R. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother. 2004;48: 1–14.
3. Jarlier V, Nicolas MH, Fournier G, Phillipon A. Extended broad–spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev Infect Dis. 1988;10:867–78.
4. Eckert C, Gautier V, Saladin-Allard M, Hidri N, Verdet C, Ould-Hocine Z, et al. Dissemination of CTX-M–type β-lactamases among clinical isolates of *Enterobacteriaceae* in Paris, France. Antimicrob Agents Chemother. 2004;48:1249–54.
5. Pitout JDD, Thomson KS, Hanson ND, Ehrhardt AF, Moland ES, Sanders CC. β-lactamases responsible for resistance to expanded-spectrum cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* isolates recovered in South Africa. Antimicrob Agents Chemother. 1998;42:1350–4.
6. Kariuki S, Corkill JE, Revathi G, Musoke R, Hart CA. Molecular characterization of a novel plasmid-encoded cefotaximase (CTX-M-12) found in clinical isolates from Kenya. Antimicrob Agents Chemother. 2001;45:2141–3.
7. Weill FX, Perrier-Gros-Claude JD, Demartin M, Coignard S, Grimont P. Characterization of extended–spectrum β-lactamase (CTX-M-15) producing strains of *Salmonella enterica* isolated in France and Senegal. FEMS Microbiol Lett. 2004;238:353–8.
8. Gangoue-Pieboji J, Miriagou V, Vourli S, Tzelepi E, Ngassam P, Tzouvelekis LS. Emergence of CTX-M-15-producing enterobacteria in Cameroon and characterization of a $bla_{CTX-M-15}$–carrying element. Antimicrob Agents Chemother. 2005; 49:441–3.
9. Blomberg B, Jureen R, Manji KP, Tamim BS, Mwakagile DSM, Urassa WK, et al. High rate of fatal cases of pediatric septicemia caused by gram-negative bacteria with extended-spectrum beta-lactamases in Dar es Salaam, Tanzania. J Clin Microbiol. 2005;43:745–9.
10. Soge OO, Queenan AM, Ojo KK, Adeniyi BA, Roberts MC. CTX-M-15 extended-spectrum β-lactamase from Nigerian *Klebsiella pneumoniae*. J Antimicrob Chemother. Epub 2005 Nov 30.

Address for correspondence: Guillaume Arlet, Service de Bactériologie-Hygiène, Hôpital Tenon, AP-HP, rue de la Chine, 75970 Paris CEDEX 20, France; email: guillaume.arlet@tnn.ap-hop-paris.fr

Novel Recombinant Sapovirus, Japan

To the Editor: *Sapovirus* is the distinct genus within the family *Caliciviridae*; these viruses cause sporadic cases and outbreaks of gastroenteritis in humans worldwide (*1*). The sapovirus genome contains 2 open reading frames (ORFs). ORF1 encodes nonstructural and capsid proteins while ORF2 encodes a small protein (*2*). Sapovirus has a typical "Star of David" configuration by electron microscopic examination. The prototype sapovirus is the Sapporo virus (Hu/SaV/Sapporo virus/1977/ JP), which was originally discovered from an outbreak in a home for infants in Sapporo, Japan, in 1977 (*3*). Sapovirus is divided into 5 genogroups, among which only genogroups I, II, IV, and V are known to infect humans (*4*).

A fecal specimen was collected from a 1-year-old boy with acute gastroenteritis in Osaka, Japan, in March 2005. The viral genome was extracted by using a QIAamp kit (Quigen, Hilden, Germany). By using multiplex reverse transcription–polymerase chain reaction (RT-PCR), 2 groups of diarrheal viruses were identified. The first group included astrovirus, norovirus, and sapovirus; the second group included rotavirus and adenovirus (*5*). Sapovirus polymerase region was also amplified to identify recombinant sapovirus by using primers P290 and P289 (*6*). To eliminate the possibility of co-infection of 2 different sapovirus genotypes, to localize the potential recombination site, and to understand a possible recombination mechanism of recombinant sapovirus, flanking polymerase and capsid regions, with their junction of HU/5862/Osaka/JP, were amplified with primers P290 and SLV5749 to produce a 1,162-bp product (*5,6*). Products were directly sequenced, and capsid- and polymerase-based phylogenetic trees showed recombinant sapovirus.

The fecal specimen was positive for sapovirus. HU/5862/Osaka/JP clustered into the genogroup I genotype 8 (GI/8 the 8/DCC/Tokyo/JP/44 cluster) (Figure) by using the recent sapovirus capsid region classification (*7*). HU/5862/Osaka/JP with GI/8 capsid was classified into GI/1 (the Sapporo/82 cluster) when polymerase-based grouping was performed. When the sequence of HU/5862/Osaka/JP was compared with that of Sapporo/82 by using SimPlot Version 1.3 (available from http://sray.med.som.jhmi.edu/SCRoftware/simplot), the recombination site was identified at the polymerase-capsid junction. Before this junction, sequences of HU/5862/Osaka/JP and Sapporo/82 were highly homologous. However, homology between them was notably different after the junction, with a sudden drop in the identity for HU/5862/Osaka/JP. By using ClustalX, HU/5862/Osaka/JP shared a 96% identity in polymerase sequence and an 85% identity in capsid sequence with Sapporo/82. In contrast, homology was 99% in the capsid region between HU/5862/Osaka/JP and 8/DCC/Tokyo/JP/44. Since a polymerase sequence of 8/DCC/Tokyo/JP/44 was not available in GenBank because of the unsuccessful amplification, homology in the polymerase region between HU/5862/Osaka/JP and 8/DCC/Tokyo/JP/44 was unknown.

Altogether, the findings underscored that HU/5862/Osaka/JP represented a novel, naturally occurring, recombinant sapovirus with GI/8 capsid and GI/1 polymerase. To determine whether the child was infected with this novel recombinant sapovirus or whether the novel recombinant sapovirus resulted from co-infection with 2 different viruses, Svppo (Sapporo/82-specific primer), Svdcc (8/DCC/Tokyo/JP/44-specific primer), and SLV5749 were used to amplify the capsid region (*5*). However, no amplicon was found. These negative results indicate no co-infection in this child.

Even though many molecular epidemiologic studies on sapovirus infection have been performed worldwide, reports documenting recombination in sapovirus are still limited. The first recombinant sapovirus identified was the Thai isolate Mc10 or the Japanese isolate C12 (*8*); the Japanese isolate Ehime1107 and the SW278 isolate from Sweden were identified later (*9*). Recombination occurred only in sapovirus genogroup II, which is more capable of recombination than other genogroups (*8,9*). In this study, we identified HU/5862/Osaka/JP with a novel recombination between 2 distinct genotypes within genogroup I. This is the first report of acute gastroenteritis caused by recombinant sapovirus genogroup I. The findings underscore that natural recombination occurs not only in sapovirus genogroup II but also in genogroup I.

In recent studies of sapovirus recombination, evidence for the location of the recombination event is lacking because of the distant geographic relationship of parent and progeny strains. HU/5862/Osaka/JP shared the closest sequences of polymerase and capsid with Sapporo/82 and 8/DCC/Tokyo/JP/44, respectively. Sapporo/82 was first isolated in 1982, and 8/DCC/Tokyo/JP/44 was isolated in 2000, both in Japan. Possibly, Sapporo/82 and 8/DCC/Tokyo/JP/44 were parental strains of HU/5862/Osaka/JP, and the event leading to the novel recombination might have occurred in Japan.

The capsid region was used for genotype classification of sapovirus (*7*). When capsid-based grouping was performed, HU/5862/Osaka/JP distinctly belonged to genotype 8. When polymerase-based grouping was

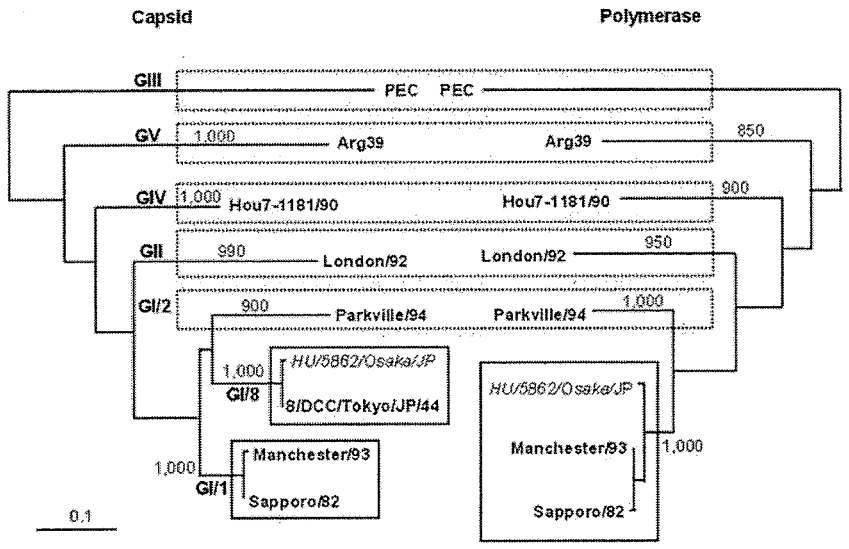

Figure. Changing genotypes of sapovirus on the basis of phylogenetic trees. Trees were constructed from partial amino acid sequences of capsid and polymerase of HU/5862/Osaka/JP highlighted in *italics*. Phylogenetic tree with 1,000 bootstrap resamples of the nucleotide alignment datasets was generated by using the neighbor-joining method with ClustalX. The genetic distance was calculated by using Kimura 2-parameter method (PHYLIP). The scale indicates amino acid substitutions per position. The numbers in branches indicate bootstrap values. Porcine enteric calicivirus was used as an outgroup strain for phylogenetic analysis. The nucleotide sequence data of sapovirus strain HU/5862/Osaka/JP has been submitted to GenBank and has been assigned accession no. DQ318530. Reference sapovirus strains and accession nos. used in this study were as follows: PEC (AF182760), London/92 (U95645), Arg39 (AY289803), Parkville/94 (U73124), Manchester/93 (X86560), Sapporo/82 (U65427), Hou7-1181/90 (AF435814), and 8/DCC/Tokyo/Japan/44 (AB236377).

performed, HU/5862/Osaka/JP distinctly belonged to genotype 1. Therefore, sapovirus classification based on capsid sequence is questionable. We suggest that sapovirus classification should rely not only on capsid sequence but also on polymerase sequence.

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

Tung Gia Phan,* Shoko Okitsu,* Werner E.G. Müller,† Hideki Kohno,‡ and Hiroshi Ushijima*

*University of Tokyo, Tokyo, Japan; †Institut für Physiologische Chemie, Mainz, Germany; and ‡Nihon University, Chiba, Japan

References

1. Lopman BA, Brown DW, Koopmans M. Human caliciviruses in Europe. J Clin Virol. 2002;24:137–60.
2. Lambden PR, Caul EO, Ashley CR, Clarke IN. Human enteric caliciviruses are genetically distinct from small round structured viruses. Lancet. 1994;343:666–7.
3. Chiba S, Sakuma Y, Kogasaka R, Akihara M, Horino K, Nakao T, et al. An outbreak of gastroenteritis associated with calicivirus in an infant home. J Med Virol. 1979;4:249–54.
4. Farkas T, Zhong WM, Jing Y, Huang PW, Espinosa SM, Martinez N, et al. Genetic diversity among sapoviruses. Arch Virol. 2004;149:1309–23.
5. Phan TG, Nguyen TA, Yan H, Yagyu F, Kozlov V, Kozlov A, et al. Development of a novel protocol for RT-multiplex PCR to detect diarrheal viruses among infants and children with acute gastroenteritis in eastern Russia. Clin Lab. 2005;51:429–35.
6. Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO. Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. J Virol Methods. 1999;83:145–54.
7. Akihara S, Phan TG, Nguyen TA, Yagyu F, Okitsu S, Muller WE, et al. Identification of sapovirus infection among Japanese infants in a day care center. J Med Virol. 2005;77:595–601.
8. Katayama K, Miyoshi T, Uchino K, Oka T, Tanaka T, Takeda N, et al. Novel recombinant sapovirus. Emerg Infect Dis. 2004;10:1874–6.
9. Hansman GS, Takeda N, Oka T, Oseto M, Hedlund KO, Katayama K. Intergenogroup recombination in sapoviruses. Emerg Infect Dis. 2005;11:1916–20.

Address for correspondence: Hiroshi Ushijima, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033; email: ushijima@m.u-tokyo.ac.jp

Postmortem Confirmation of Human Rabies Source

To the Editor: Rabies is a fatal encephalitis caused by a neurotropic RNA virus of the family *Rhabdoviridae*, genus *Lyssavirus*. The predominant rabies virus reservoir hosts are bats and carnivores. Among these, rabid dogs represent a substantial public health problem, particularly in developing countries (*1*).

Laboratory diagnosis of rabies is essential to guide control programs, epidemiologic surveys, and prophylactic measures (*2*). Among the laboratory tests recommended by the World Health Organization (WHO), the fluorescent antibody test (FAT) is the accepted standard for rabies diagnosis (*1*). Although rabies virus antigens can be detected in decomposed samples, FAT is less effective when such samples are tested. In those cases, polymerase chain reaction (PCR) can provide better results (*3*). Since the degree of decomposition at which FAT starts to become ineffective is unknown (*4*), when smears from decomposed samples are made for FAT, a suspension of the same brain tissues should be made in the appropriate diluents for the mouse inoculation test (MIT), cell culture, or reverse transcription–polymerase chain reaction (RT-PCR) (*2*). However, if all test results are negative, rabies cannot be ruled out because of the condition of the sample.

On February 28, in the city of Carbonita, Minas Gerais State, in southeastern Brazil, a 62-year-old man was bitten by a bat on the right ankle. Approximately 50 days later, his leg began to feel numb, and he experienced a continuous headache, pain at the site of the bite, convulsions, frequent urge to clear his throat, hiccups, nausea, difficulty in swallowing, dry lips, slightly elevated body temperature (37°C–37.5°C), paralysis of superior and inferior left limbs, shaking, and hallucinations. On May 4, 16 days after clinical manifestations began, the patient died; the cause of death was registered as a cerebral vascular accident. One month later, the body was exhumed to obtain a sample from the central nervous system (CNS), which was sent to Instituto Pasteur, São Paulo, registered as sample 5341 M/04 and tested by FAT, MIT, and RT-PCR.

In total, 8 smears were prepared from the sample to be analyzed by FAT according to the method of Dean et al. (*5*) with fluorescein isothiocyanate–labeled polyclonal antinucleocapsid antibodies. MIT was carried out as described by Koprowski (*6*) with 7 mice. For RT-PCR, RNA was extracted from the CNS sample with TRIzol, according to the manufacturer's instructions (Invitrogen, Rockville, MD, USA). RT-PCR was carried out with modifications as described by Orciari et al. (*7*), with primers 504 (sense) and 304 (antisense), aiming at the amplification of a 249-bp fragment of rabies virus nucleoprotein (N) gene, by using Superscript II (Invitrogen) and Taq DNA-polymerase (Invitrogen).

Fluorescent inclusions were observed in 6 of the 8 slides prepared for the FAT. The RT-PCR of the RNA

Journal of Medical Virology 78:986–994 (2006)

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

Pattara Khamrin,[1] Niwat Maneekarn,[2] Supatra Peerakome,[2] Fumihiro Yagyu,[1] Shoko Okitsu,[1] and Hiroshi Ushijima[1]*

[1]*Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, the University of Tokyo, Japan*
[2]*Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*

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 non-structural 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

Grant sponsor: Japan Society for the Promotion of Science (JSPS); Grant sponsor: Endowment Fund for Medical Research, Faculty of Medicine, Chiang Mai University, Thailand.

*Correspondence to: Hiroshi Ushijima, MD, PhD, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, the University of Tokyo, 7-3-1 Hongo Bunkyu-ku, Tokyo 113-0033, Japan.
E-mail: ushijima@m.u-tokyo.ac.jp

Accepted 28 March 2006
DOI 10.1002/jmv.20651
Published online in Wiley InterScience (www.interscience.wiley.com)

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.

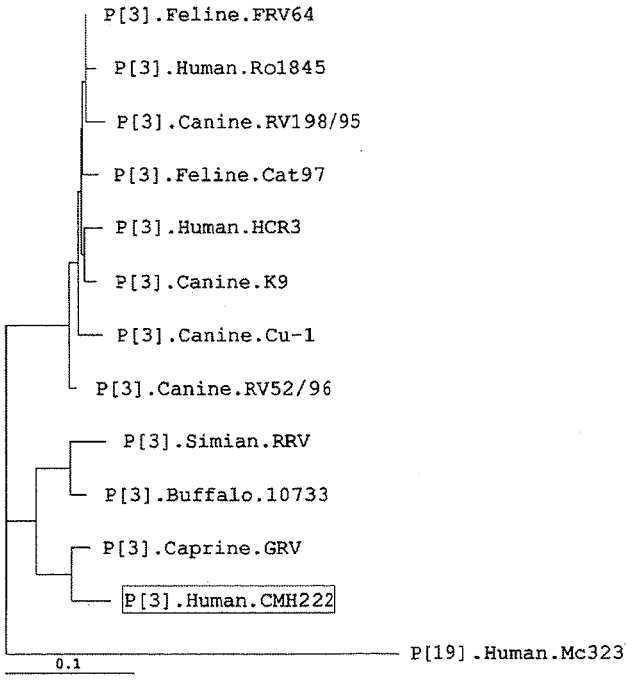

Fig. 1. Phylogenetic analysis of the deduced amino acid sequence derived from *VP4* gene of the CMH222 human rotavirus strain and other P[3] rotavirus genotypes recognized to date. The tree was generated based on the neighbor-joining method using ClustalX program.

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).

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

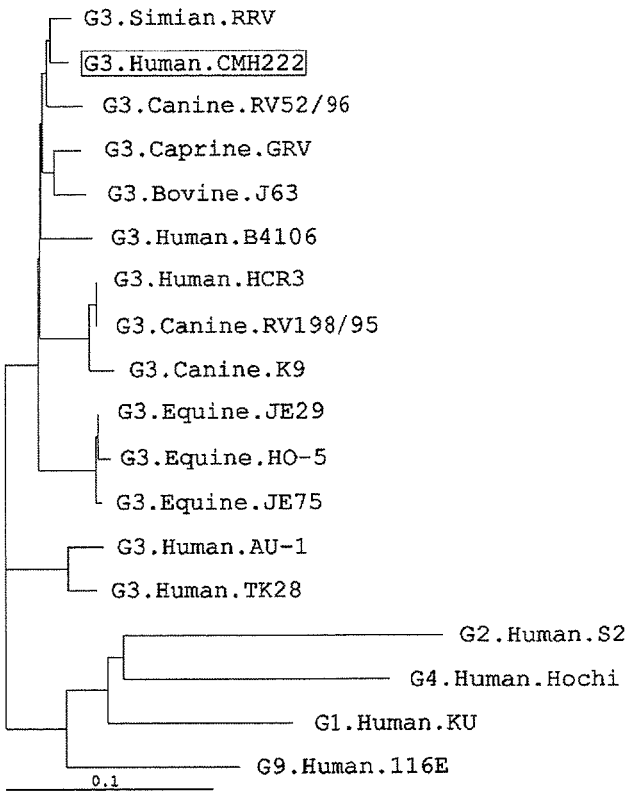

Fig. 2. Phylogenetic analysis of the deduced amino acid sequence derived from *VP7* gene of the CMH222 human rotavirus strain and other G3 rotavirus genotypes. The tree was generated based on the neighbor-joining method using ClustalX program.

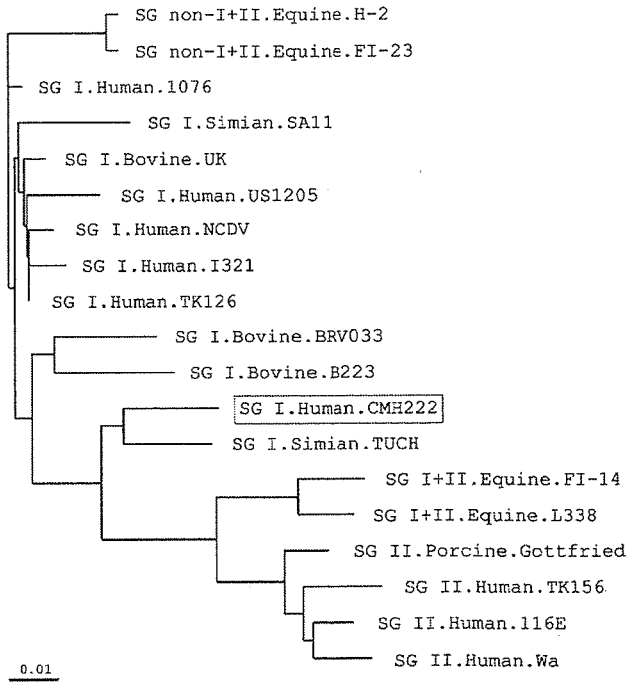

Fig. 3. Phylogenetic analysis of the deduced amino acid sequence derived from *VP6* gene of the CMH222 human rotavirus strain and other rotavirus subgroups. The tree was generated based on the neighbor-joining method using ClustalX program.

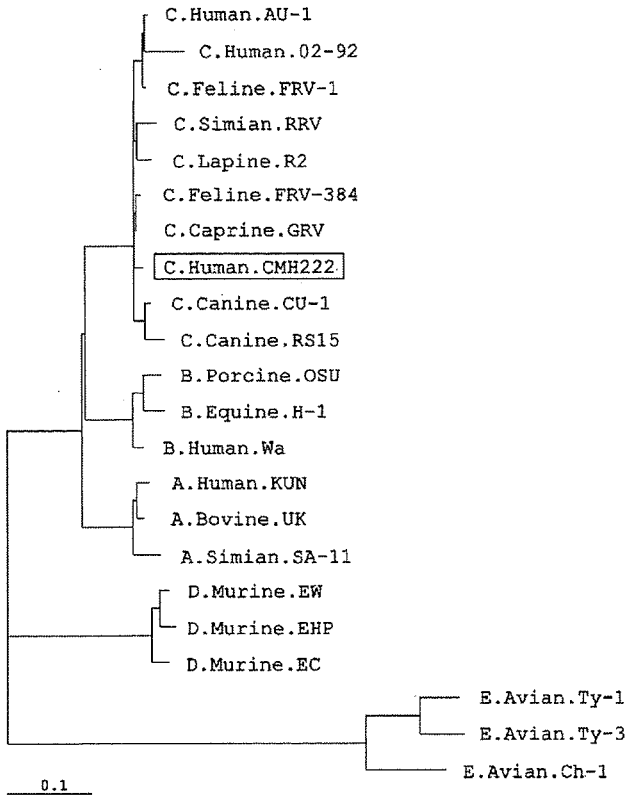

Fig. 4. Phylogenetic analysis of the deduced amino acid sequence derived from *NSP4* gene of the CMH222 human rotavirus strain and other NSP4 rotavirus genotypes. The tree was generated based on the neighbor-joining method using ClustalX program.

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