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Genetic analysis of noroviruses associated with fatalities in healthcare facilities

Brief Report

M. Okada[1], **T. Tanaka**[2], **M. Oseto**[3], **N. Takeda**[4], and **K. Shinozaki**[1]

[1]Division of Virology, Chiba Prefectural Institute of Public Health, Chiba, Japan
[2]Sakai City Institute of Public Health, Sakai, Osaka, Japan
[3]Microbiology Division, Ehime Prefecture Institute of Public Health and Environmental Science, Matsuyama, Ehime, Japan
[4]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

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Summary. Norovirus outbreaks occurred in 236 healthcare facilities for the elderly in Japan during the winter of 2004–2005. Three norovirus strains associated with three fatal clinical courses were isolated from geographically separate facilities and genetically analyzed along with three strains from non-fatal cases in the same season. All six isolates were classified as the GII-4 genotype. No new variant strains like those observed in Europe in 2002 and 2004 were found in fatal cases, and the three outbreaks were deemed to have been caused by genetically close conventional norovirus GII-4 strains.

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Norovirus (NoV) is a leading cause of acute gastroenteritis in humans and animals [10, 14], causing worldwide outbreaks in various epidemiological settings including hospitals, nursing homes, schools and restaurants [4, 8, 9, 15]. Transmission of NoV occurs via the faecal-oral route, food-borne route, person-to-person contact, and environmental contamination, and infection occurs in all age groups [4, 8, 9]. Human NoV is divided into two genogroups, genogroup I (GI) and GII [1], which are further classified into 15 and 18 genotypes, respectively, based on the capsid protein [12]. The GII-4 genotype, represented by Lordsdale virus isolated in the United Kingdom in 1993 [13], is a dominant genotype worldwide [7, 11, 12].

Although NoV causes relatively mild gastroenteritis in healthy individuals [10] with few fatal cases, elderly and immunocompromised patients can suffer from severe gastroenteritis, sometimes resulting in death [2, 8]. Fatality rates

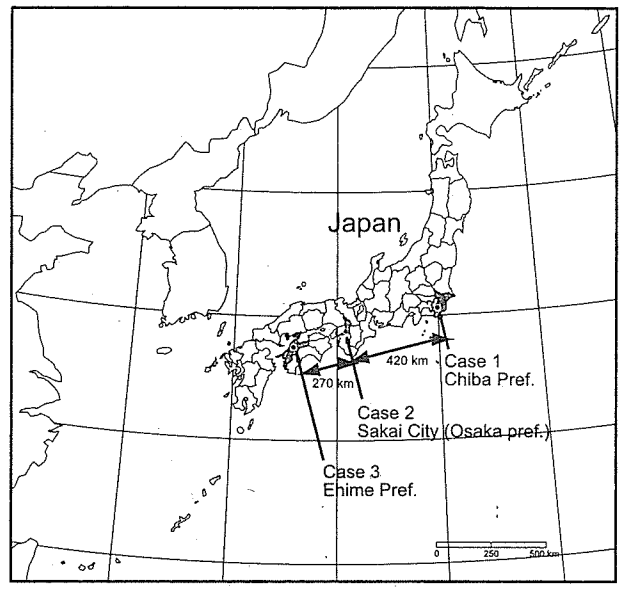

Fig. 1. Geographic relationships between three independent NoV outbreaks in Japan analyzed in this study. The geographic locations of the three outbreaks are shown in the map

associated with NoV outbreaks are reportedly 0.075 and 0.087% in England, Wales and the United States, respectively [8, 10]. In England and Wales, 43 fatal cases were observed in 38 outbreaks in hospitals and residential care facilities between 1992 and 2000. Recently, five fatal cases (fatality rate: 2.0%) associated with a large-scale gastroenteritis outbreak in nursing homes in Israel were also reported [2].

NoV outbreaks occurred in 236 healthcare facilities for the elderly in Japan in 2004–2005 with 12 fatal cases reported in six prefectures (http://www.mhlw.go.jp/houdou/2005/01/h0112-3.html). NoV samples were obtained from three independent fatal cases from geographically separate facilities in Chiba, Sakai and Ehime, respectively. The geographic locations of the three outbreaks analyzed in this study are shown in Fig. 1, and the epidemiological findings of the above three outbreaks are summarized in Table 1. Three NoV strains, Chiba/04-1050/2005 (Chiba/04-1050), Sakai/04-179/2005 (Sakai/04-179) and Ehime/05-30/2005 (Ehime/05-30), from these three fatal cases were analyzed. RNA extraction and RT-PCR targeting the 5′ end of open reading frame (ORF) 2 followed by genetic analysis were performed as described previously [12]. Comparisons of the nucleotide sequences demonstrated that these three strains had high nucleotide identities (approximately 99%), and these strains were classified into genotype GII-4 (data not shown). For further genome analysis, the NoV genome was amplified as three separate overlapping segments. The amplified products were directly sequenced as previously described [12], and the complete nucleotide sequence of Chiba/04-1050 and nearly complete nucleotide sequences minus the 5′ terminus of Sakai/04-179 and Ehime/05-30 were determined. Nucleotide sequences determined in this study were submitted to DDBJ with accession numbers AB220921 to AB220926.

Table 1. Summary of the three independent NoV outbreaks in healthcare facilities for the elderly analyzed in this study

	Case 1	Case 2	Case 3
Location Facility	Chiba Prefecture special nursing home for the elderly	Sakai City (Osaka Prefecture) hospital and healthcare facility for the elderly	Ehime Prefecture healthcare facility for the elderly
Total number of individuals			
residents	78	484 (in total)	97
workers	60		69
Affected individuals			
residents	43 (55.1%)	68 (14.0)%	35 (36.1)%
workers	20 (33.3%)		15 (21.7)%
Duration period	1st to 16th of January, 2005	3rd to 28th of January, 2005	2nd to 15th of January, 2005
Major symptoms	diarrhea, vomiting, fever, abdominal pain	diarrhea, vomiting, fever, abdominal pain	diarrhea, vomiting, fever
Number of death	1	1	1
Fatality rate	1.59%	1.47%	2.0%
NV testing methods	RT-PCR, electron microscopy	RT-PCR	RT-PCR, electron microscopy
Tested samples			
residents	10 stools & 1 vomitus	9 stools	12 stools & 2 vomitus
workers	3 stools	ND	2 stools
Positivity for NV[a]			
residents	7 [5] stools & 1[0] vomitus	7 stools	9 [6] & 2 [2] vomitus
workers	0 [0] stools	ND	2 [0] stools
Rate of positive samples[a]	57.1 [35.7]%	77.8%	81.3 [50.0]%
Enteric bacterial pathogen	not detected	not detected	not detected
Fatal cases			
age, sex	82 years, female	95 years, female	90 years, male
onset	8th January	9th January	7th January
death	10th January	17th January	10th January
Cause of death	suffocation as a result of vomiting	septicemia	Acute bleeding in the gastrointestinal tract
Sample ID	Chiba/04-1050/2005	Sakai/04-179/2005	Ehime/05-30/2005
Source of NV detection	stool on 9th January	stool on 17th January	vomitus on 10th January
NV genotype (ORF2)	GII-4	GII-4	GII-4

[a]Values in brackets show positivity with electron microscopy; other value show positivity with RT-PCR

Chiba/04-1050 was composed of 7,559 nucleotides without a poly-A tail, while the other two strains from Sakai and Ehime comprised 7,533 nucleotides lacking the 5′ terminus. The average nucleotide identities among the three strains were 99.2% in ORF 1, 98.6% in ORF 2, and 98.8% in ORF 3. In addition, 10, 4

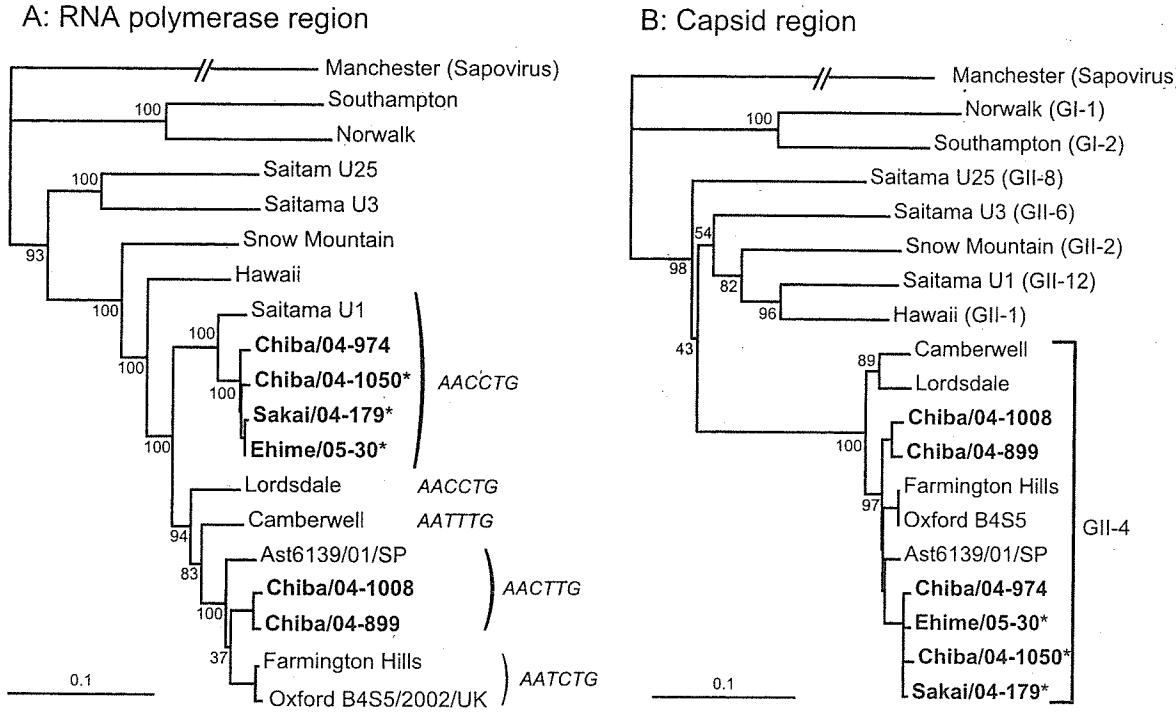

Fig. 2. Phylogenetic trees were constructed using the neighbor-joining method based on part of the RdRp region corresponding to 4307–5017 (**A**) and the capsid region corresponding to 5085–5509 (**B**) of Lordsdale virus. A sapovirus, Manchester strain, was used as the out-group. The six strains examined are shown in bold. Three strains from fatal cases are shown by asterisks, in which the complete and nearly complete genomes were amplified by RT-PCR with the following primers: NV5END (GAATGAAGATG GCGTCTAACGACG) and NV2690R (TGAGACCTTTGCTTGAGAAGGCTGT) for the 5′ genome region, NV2570F (CCAAAACCCAAAGATGATGAGGAGT) and NV5550R (GGTAAGGGGATCAACACAGGTTCCA) for the central region, and G2F1 and dT25VN [(T)25V(A/G/C)N(A/G/C/T)] for the 3′ genome region [12]. The 5′ end of the genome was amplified with the 5′ RACE Amplification System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Nucleotide sequences characterized as GII-4 variants reported by Lopman et al. [7] are also indicated in italic on the tree (A). Reference strains were Manchester virus (X86560), Norwalk virus (M87661), Southampton virus (L07418), Snow Mountain virus (AY134748), Lordsdale virus (X86557), Camberwell virus (AF145896), Farmington Hills (AY502023), Ast6139/01/SP (AJ583672), Oxford B4S5 (AY587984), Saitama U25 (AB067543), SaitamaU3 (AB039776) and Saitama U1 (AB039775)

and 6 amino acid substitutions were identified in each ORF. Phylogenetic trees based on the partial RNA-dependent RNA polymerase (RdRp) region (ORF1) and partial capsid region (ORF2) are shown in Fig. 2A and B. Phylogenetic analysis based on ORF2 indicated that the three strains were genetically close and clustered together with known GII-4 strains (Fig. 2B). These strains were also clustered with GII-4 strains when the RdRp region was compared (Fig. 2A). The Saitama

U1 strain is a recombinant strain between a GII-4-like (ORF 1) and GII-12 (ORF 2) strain [5]. These results clearly indicate that the three strains isolated from fatal cases were genetically close and indistinguishable from known GII-4 strains.

To further investigate the RdRp and capsid regions, an additional three strains detected in Chiba prefecture in the same season, Chiba/04-899/2004 (Chiba/04-899; outbreak in a nursery school), Chiba/04-974/2004 (Chiba/04-974, sporadic gastroenteritis patient) and Chiba/04-1008/2004 (Chiba/04-1008, outbreak in a healthcare facility) were similarly analyzed. Based on the capsid protein, these three strains were also grouped into GII-4 and shown to be closely related to the three strains from the fatal cases (Fig. 2B). When the RdRp region was compared, the three fatal case strains and Chiba/04-974 were closely related and grouped into a cluster including the Saitama U1 strain (Fig. 2A). In contrast, Chiba/04-899 and Chiba/04-1008 were closely related to other GII-4 strains including Lordsdale virus. Therefore, the six strains analyzed in this study were deemed conventional GII-4 strains widely circulating in this season. In addition, at least two genetically distinct GII-4 strains with different ORF1 sequences were shown to be co-circulating at the same time in Chiba prefecture.

Lopman et al. reported an increase in NoV-associated gastroenteritis in European countries due to emergence of new genetic variants of the GII-4 strain [7]. GII-4 strains detected before 2002 have an "AACTTG" sequence in the RdRp region while those detected in 2002 (new variants) show "AATCTG" [7]. Intermediate sequences have also been observed [7, 13]. Of the six GII-4 strains analyzed in this study, the three fatal strains and Chiba/040974 showed an intermediate sequence, "AACCTG" (Fig. 2A). The other two strains, Chiba/040899 and Chiba/041008, had 10 nucleotide substitutions in the RdRp region, which were observed in GII-4 2004 variant strains [6]. Therefore, no new variant strains like those isolated in 2002 and 2004 were identified in fatal cases in this study.

Previous studies have described the GII-4 genotype as the dominant genotype of NoV-associated gastroenteritis worldwide [7, 12, 15]. Furthermore, GII-4 strains are mainly detected in outbreaks in healthcare facilities such as nursing homes and hospitals [4, 9]. Lopman et al. reported that outbreaks in healthcare facilities showed a higher death rate and prolonged duration when compared to other outbreak settings [8]. Recently, fatal cases associated with GII-4 NoV outbreaks in nursing homes have been reported in Israel [2]. We detected genetically similar GII-4 strains from three independent outbreaks in geographically isolated healthcare facilities in Japan. Sequence analysis comparisons with an additional three strains from Chiba prefecture clearly indicated that these strains were not specific to these outbreaks. Although NoV infection is not likely the principal cause of death in most cases, NoV-associated outbreaks occurring in healthcare facilities for the elderly might constitute an additional burden. As neither common food nor food stuff was identified in the three fatal cases presented here, person-to-person transmission by either direct contact with stool or vomitus or through the caregiver was considered the most likely mode of transmission. These findings

suggest that we need to pay more attention to the activity of NoV, especially that of the GII-4 genotype, in outbreaks in healthcare facilities.

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Author's address: Mineyuki Okada, Division of Virology, Chiba Prefectural Institute of Public Health, 666-2, Nitona-Cho, Chuo-Ku, Chiba-Shi, Chiba 260-8715, Japan; e-mail: m.okd4@mc.pref.chiba.jp

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ORIGINAL ARTICLE

Genetic variability in the sapovirus capsid protein

Mineyuki Okada · Yasutaka Yamashita · Mitsuaki Oseto · Tomoko Ogawa · Ikuo Kaiho · Kuniko Shinozaki

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Abstract *Sapovirus* (SV), which causes gastroenteritis in humans, is composed of genetically divergent viruses classified into 5 genogroups. In this study, 2.2-kb nucleotide sequences of the 3′ terminus of the genome of 15 SV strains detected in Japan were determined. The 15 SV strains could be classified into four genogroups (GI, GII, GIV and GV), and in two of these, GI and GII, 10 genotypes were identified. The amino acid sequences of the central variable region of the capsid protein showed less than 81% identity when strains belonging to different genotypes were compared. It was therefore supposed that antigenic variety exists between different genotypes. These results will be useful for further genetic and antigenic analyses of SV.

M. Okada (✉) · T. Ogawa · I. Kaiho · K. Shinozaki
Division of Virology, Chiba Prefectural Institute of Public Health, 666-2, Nitona-cho, Chuo-ku, Chiba-shi, Chiba 260-8715, Japan
e-mail: m.okd4@mc.pref.chiba.jp

T. Ogawa
e-mail: t.ogw11@mb.pref.chiba.jp

I. Kaiho
e-mail: i.kih@ma.pref.chiba.jb

K. Shinozaki
e-mail: k.shnzk4@mb.pref.chiba.jb

Y. Yamashita · M. Oseto
Microbiology Division, Ehime Prefecture institute of Public Health and Environmental Science, Ehime 790-0003, Japan
e-mail: yamashita-yasutaka@pref.ehime.jp

M. Oseto
e-mail: oseto-mitsuaki@pref.ehime.jp

Keywords Sapovirus · Capsid · Phylogenetic analysis · Genetic divergence

Introduction

Sapovirus (SV) is a member of the family *Caliciviridae*. Caliciviruses contain a positive-sense single-stranded RNA genome and are divided into four genera, *Vesivirus, Lagovirus, Norovirus* (NV) and SV [1–3]. Human SV is a causative agent of gastroenteritis in humans as are the NV GI and GII strains [4, 5]. However, in spite of their similar clinical symptoms, a number of morphological as well as genetic differences are observed between SV and NV [6].

The prototype strain of SV, Hu/SV/GI/Sapporo/77/JP (Sapporo virus), was first detected in 1977 by electron microscopy of stool specimens from gastroenteritis patients [7]. Since the complete nucleotide sequence of the Manchester virus was reported in 1995 [8], the complete and partial nucleotide sequences of several SV strains have also been reported [9–15]. Recently, SV was divided into five genogroups (GI–GV) including human (GI, GII, GIV and GV) and animal (GIII) strains based on the sequence of the capsid protein [15]. Schuffenecker et al. [16] conducted comparative analysis of the genetic clustering of SV based on three different genome regions and demonstrated that the best clustering was obtained when the whole capsid protein gene was used for phylogenetic analysis. Recently, Chen et al. [17] reported comparable results with the viral particle of caliciviruses based on structural and genetic studies of the capsid protein. In addition, the hypervariable region of the calicivirus capsid protein is known to be involved in host specificity, cell interaction and strain diversity [17–20]. However, to date, only a

few nucleotide sequences including the whole capsid protein gene of SV have been reported.

The objective of the present study was to analyze the capsid protein of SV strains in Japan and genetically characterize the variability in the capsid protein among SVs. To this end, nucleotide sequences including the whole capsid protein gene of 15 SV strains detected in Japan were genetically analyzed and compared with previously published SV sequences.

Materials and methods

Samples, RT-PCR and molecular cloning

Of 125 SV strains [13, and unpublished data] detected in Japan between 1999 and 2004, 15 strains that branched from the other strains with a difference of more than 8% at the 5′ end of the capsid region (approximately 400 bp) were selected. Approximately 2.2 kb of the 3′ terminus of the genome of each strain, including partial open reading frame (ORF) 1, ORF2 and the 3′ non-coding region, was amplified with a forward primer (SV-F11 or SV-F21; [13]) and reverse primer (dT25VN; T25V(A/C/G)N(A/G/C/T)). The PCR products were purified and cloned into a plasmid vector using the pGEM-T Vector System (Promega, Madison, USA) or TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA), and then used to transform competent DH5α cells. The nucleotide sequences within the plasmid were sequenced using the PRIZM BigDye Terminator Cycle Sequencing Kit and Genetic Analyzer 3100-Avant (Applied Biosystems, California, USA).

Sequence analysis

Nucleotide sequences were processed using GENETYX-MAC software, and multiple alignment and phylogenetic analysis using the neighbor-joining (NJ) method were carried out using the Clustal X multiple alignment program (Ver.1.83) [21]. The phylogenetic tree was drawn with Tree View software [22] and the PHYLIP package (Ver. 3.6) [23] was used to calculate molecular distances. Nucleotide sequences determined in this study were submitted to the EMBL database and assigned accession numbers AJ606689–AJ606699 and AJ786349–AJ786352.

Results

Genetic characteristics of the 15 SV strains examined in this study along with 12 reference strains are summarized in Table 1. A phylogenetic tree obtained using the NJ method based on the whole capsid gene sequences is shown in Fig. 1. SV is clearly separated from other caliciviruses and can be divided into six distinct genetic clusters. Of these, four clusters (GI, GII, GIV and GV) contain only human strains while the other two are composed of the animal strains PEC (GIII) and MEC. As shown in Fig. 1, five of the strains analyzed in this study were grouped in GI with the Manchester virus and Parkville virus, and 6 were grouped with the London virus in GII. The remaining four strains were classified two strains each as GIV and GV, which are represented by Hou7-1181 and Arg39, respectively. The nucleotide (nt) identities of the 2.2 kb sequences of the 3′ terminus of the15 SV strains examined in this study ranged between 56.7 and 99% (data not shown). The human SV strains had two ORFs (ORF 1 and 2) and a 3′ non-coding region (NCR), and moreover, those classified into GI, GIV and GV had an additional ORF overlapping the capsid region (Column OL-ORF in Table 1).

The capsid protein of human SV was predicted as consisting of 551–569 amino acids (aa) (1656–1710 nt). The aa identities between strains within the same genogroup were 75.7–99.8%, whereas those between different genogroups were 45.4–53.0%. PEC and MEC had 40.3–44% identity with the human SV strains. Distribution analysis of pair-wise distances revealed four distinct clusters corresponding to the distances between intra-genotype, inter-genotype, inter-genogroup and inter-species strains (data not shown). The independencies of these four clusters were significant ($P < 0.01$) in both the nt- and aa-based analyses. The pair-wise distances corresponding to the genogroup clusters were 0.227 ± 0.129 (mean value ± 3SD; aa) and 0.337 ± 0.091 (nt), respectively, and those of the genotype clusters were 0.023 ± 0.073 (aa) and 0.057 ± 0.090 (nt), respectively. The cutoff values of the genotype clusters based on distance analysis were < 0.098 with the aa sequences and < 0.246 with the nt sequences. Farkas et al. [15] reported 6 genetic clusters among the GI and GII strains (GI/1-GI/3, GII/1-GII/3). In this study, four additional new clusters were identified, and subsequently, GI and GII were reclassified into 6 genotypes, GI/1–GI/6, and 4 genotypes, GII/1–GII/4, respectively (Table 1 and Fig. 1).

Amino acid alignment of the capsid protein region indicated that it is separated into four regions by sequence variability: an N-terminal variable region (NVR) corresponding to aa 1–43 of the Manchester virus (observed in all the SV strains), well conserved sections following the second half of the N-terminal region (N) (aa 44–285) and one quarter of the

Table 1 Summary of sapoviruses examined in this study

Genus	Species	Strain	Origin[b]	Accession Number	Tentative Genotype[c]	Length of nucleotide residues				
						Total[d]	ORF1 (Capsid)	ORF2	3′ NCR	ORF3[e]
		Manchester		X86560	GI/1	2262	1686	498	82	486
		Chiba/010250[a]	M, 2Y, Feb 2000	AJ606695	GI/1	2260	1686	498	80	486
		Parkville		U73124	GI/2	2293	1716	498	89	492
		Chiba/010658[a]	M, 2Y, Mar 2001	AJ606696	GI/3	2273	1701	498	78	489
		Mex14917		AF435813	GI/3	2272	1701	498	77	489
		Chiba/000496[a]	F, 5Y, Mar 2000	AJ606693	GI/4	2267	1686	498	87	486
		Chiba/000764[a]	M, 1Y, May 2000	AJ606694	GI/5	2277	1698	498	85	489
		Ehime/2K-814[a]	M, 42Y, Apr 2000	AJ606698	GI/6	2278	1704	498	80	486
		Bristol/98		AJ249939	GII/1	2317	1683	495	140	No
		Chiba/020003[a]	M, 8M, Oct 2001	AJ606692	GII/1	2316	1686	501	133	No
		Ehime/2K-1948[a]	M, 3Y, Dec 2000	AJ786351	GII/1	2316	1683	501	133	No
Sapovirus	*Sapporo virus*	Mex340		AF435812	GII/2	2285	1677	501	108	No
		Chiba/990727[a]	M, 2Y, May 1999	AJ606689	GII/2	2285	1677	501	108	No
		CS2000		AY289804	GII/3	2275	1680	501	95	No
		Chiba/990763[a]	F, 1Y, Jun 1999	AJ606690	GII/4	2303	1686	501	117	No
		Chiba/991172[a]	F, 1Y, Dec 1999	AJ606691	GII/4	2305	1686	501	119	No
		Chiba/040507[a]	F, 1Y, Apr 2004	AJ786350	GII/4	2305	1686	501	119	No
		Hou7-1181		AF435814	GIV	2255	1662	504	93	486
		Ehime/99-1596[a]	M, 1Y, Jul 1999	AJ606697	GIV	2254	1656	504	98	486
		Chiba/000671[a]	M, 12Y, Dec 1999	AJ786349	GIV	2247	1656	504	91	486
		Arg39		AY289803	GV	2290	1710	501	83	468
		Ehime/01-1669[a]	F, 10Y, Dec 2001	AJ606699	GV	2290	1710	501	83	468
		Ishikawa/03-158[a]	M, 12Y, Nov 2003	AJ786352	GV	2290	1710	501	83	468
	PEC	Cowden		AF182760	GIII	2181	1635	495	55	No
Lagovirus	*RHDV*			M67473		2133	1740	354	59	No

[a] SV analyzed in this study

[b] Sex, Age (Y, years; M, months), Date of sample collection

[c] GI; genogroup I, GII; genogroup II, GIII; genogroup III, GIV; genogroup IV, GV; genogroup V

[d] Nucleotide residues from the putative starting codon of the capsid protein to 3′ end of the genome

[e] Region overlapping with the capsid region

C-terminal region (C) (aa 442–561), and a central variable region (CVR) (aa 286–441). The NVR is not observed in other caliciviruses. Only 6.1% and 5.9% of the aa residues were conserved in the NVR and CVR, respectively, which is strikingly low compared with the N and C regions (40.5% and 27.3%, respectively). The aa identities of the CVR section between intra-genotype, inter-genotype and inter-genogroup strains were 88.2–100%, 59.1–80.8% and 25.2–35.2%, respectively. Chen et al. [17] previously reported that the CVR was homologous to the P2 domain of the Norwalk virus, which is located on the viral surface and is concerned with strain specificity; for example, antigenicity.

ORF2 encoded 165–167 aa but did not contain any cysteine residues. The aa identities of ORF2 between strains belonging to the same genogroup were 68.5–99.4% and those belonging to different genogroups were 32.5–54.5%. The 3′ NCR was 80–130 nt long and contained a C/T rich region and two conserved sequences. The nucleotide sequences CWCACGCG-TYCGGGWG and TAAGCGA were highly conserved within all SV strains.

Discussion

Recently, Farkas et al. [15] classified SV into 9 genetic clusters within 5 genogroups, including one genogroup represented by the animal sapovirus PEC. In this study, 2.2 kb sequences from the 3′ end of the genomes of 15 human SV strains detected in Japan were analyzed and compared with other calicivirus sequences. They were classified into four distinct genogroups (GI, GII, GIV, and GV). This is the first report to identify four SV genogroups in a single country.

NV genogroups were determined mainly based on the capsid gene sequence, and the aa identities among the complete capsid gene of strains belonging to the same genogroup were previously shown to be more than 65% [24–27]. In this study, the aa identities of SV strains belonging to the same genogroup were more than 75%. In contrast, those of SV strains belonging to different genogroups were less than 53%. We also used distribution analysis to determine the pair-wise distances of the complete capsid region of 32 SV strains, revealing six and four genotype clusters in the GI and

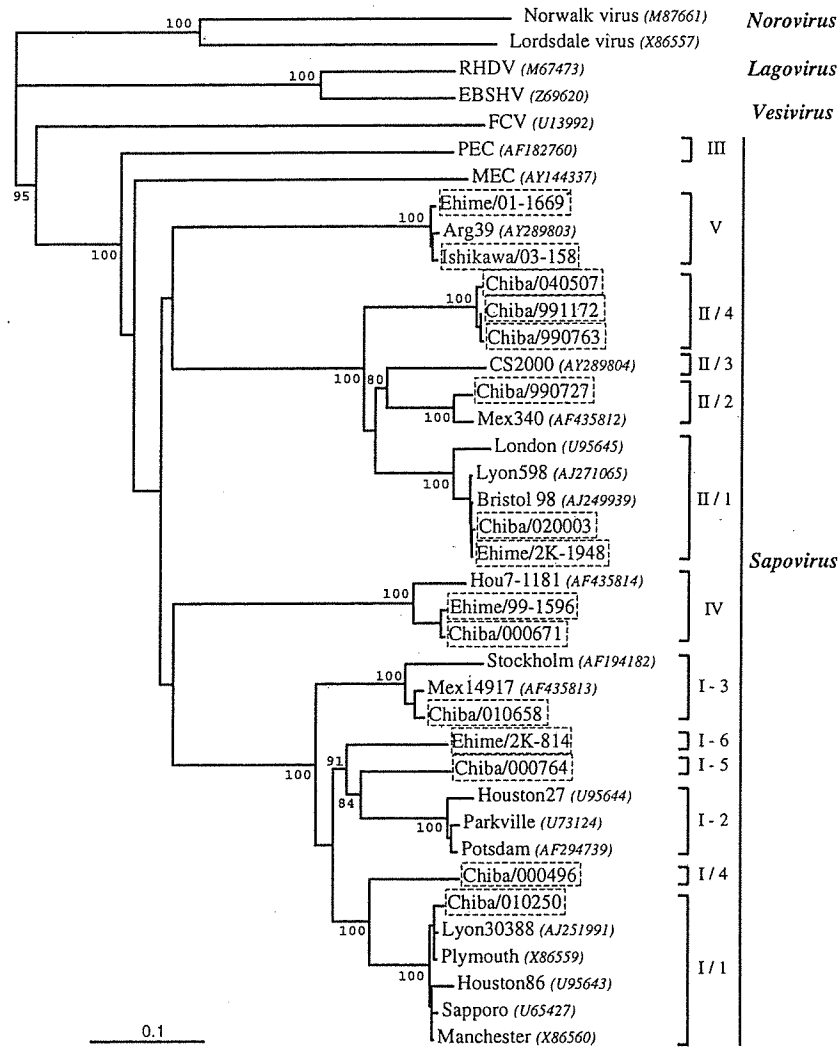

Fig. 1 Phylogenetic tree obtained based on the amino acid sequences of the capsid protein region using the neighbor-joining method. Bootstrap values of major nodes are indicated. Strains analyzed in this study are shown in boxes. Putative genogroups and genotypes are shown

GII genogroups, respectively. These results seem to agree with the findings on NV.

Structural analysis of the Norwalk virus capsid protein revealed that the central variable region (the P2 domain) is located on the viral surface and concerns strain specificity; for example, antigenicity [18]. Chen et al. [17] reported comparable results with regard to the structure of caliciviruses, revealing common genetic and structural characteristics such as the composition of the shell and protruding region of the capsid protein. They also reported that the CVR of SV is homologous to the Norwalk virus P2 domain. In other RNA viruses, the relationship between the genetic identities within genotypes and serotypes has been evaluated. For example, putative border values for the genetic divergence allowing genotyping of the P1 and VP1 region of enteroviruses and the VP1/2A region of poliovirus were estimated as 15–20% [28–30]. In our study, aa identities of the SV capsid region between inter-genotype strains ranged from 75.7 to 84.3%. In addition, lower aa identities were observed in the CVR region, ranging from 59.1 to 80.8% between inter-genotype strains. The CVR of the SV capsid protein showed more than a 20% difference in aa identities with the gapped sequence within different genotype strains and only a few aa residues were conserved. It is therefore suggested that the genotypes identified in this study might show different antigenic profiles.

Recently, Hansman et al. [31, 32] reported antigenic divergence between SV genogroups using baculovirus-expressed virus-like particles (VLP) and hyperimmune serum against VLP. Hyperimmune serum against VLP did not react against VLP of other genogroups. Similarly, Kamata et al. [33] reported that hyperimmune serum against recombinant VLP of NV showed low reactivity against VLP of other geneotypes. It is

therefore suggested that capsid based-genotyping is strongly correlated with antigenic properties, indicating the usefulness of the present findings in antigenic studies of SV.

At present, the relationship between SV genotypes and serotypes cannot be discussed because the antigenic diversity among SV genotypes has yet to be evaluated. However, the genetic classification described here seems to correlate well with the antigenicity of SV, reflecting the genetic diversity of the P2-like CVR. We suggest that the CVR of SV might be concerned with the antigenic specificity of SV strains; however, further studies of the antigenicity of SV are required.

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Outbreak of Sapovirus Infection Among Infants and Children With Acute Gastroenteritis in Osaka City, Japan During 2004–2005

Tung Gia Phan,[1] Quang Duy Trinh,[1] Fumihiro Yagyu,[1] Kumiko Sugita,[1] Shoko Okitsu,[1] Werner E.G. Müller,[2] and Hiroshi Ushijima[1]*

[1]*Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[2]*Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Mainz, Germany*

One hundred and twenty five fecal specimens were collected from sporadic cases of acute gastroenteritis in a pediatric clinic in Osaka City, Japan from July 2004 to June 2005 and tested for the presence of rotavirus, norovirus, sapovirus, astrovirus, and adenovirus by RT-multiplex PCR. Among diarrheal viruses detected, norovirus was the most prevalent (19.2%, 24 of 125), followed by group A rotavirus (18.4%, 23 of 125), astrovirus (1.6%, 2 of 125), and adenovirus (0.8%, 1 of 125), respectively. Interestingly, sapovirus infection was identified with high incidence of 17.6% (22 of 125). Sapovirus was subjected to molecular genetic analysis by sequencing. It was found that sapovirus detected in this study was classified into four genotypes (GI/1, GI/4, GI/6, and GI/8), and sapovirus GI/6 was predominant, followed by GI/8 and accounted for 81.8% (18 of 22) and 9.1% (2 of 22), respectively. It was noteworthy that sapovirus GI/6 infection was apparently confined within the period of 5 months (August 2004 through December 2004). This pattern of infection implied the outbreak of sapovirus GI/6 in these patients, which was the first outbreak of acute gastroenteritis attributed to sapovirus in Osaka City. Another interesting feature of the study was the appearance of two novel, naturally occurring recombinant sapoviruses circulating in the Japanese population studied. This report confirmed the presence as well as the importance of sapovirus causing acute gastroenteritis among Japanese infants and children. ***J. Med. Virol. 78:839–846, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: sapovirus; Osaka; outbreak

INTRODUCTION

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths [Murray and Lopez, 1997; Parashar et al., 2003a]. Among different types of diarrheal viruses, rotavirus is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [Parashar et al., 2003b]. Sapovirus, however, is also considered to be a significant global enteropathogen in association with sporadic cases as well as outbreaks of gastroenteritis in such settings as kindergartens, schools, and nursing home for the elderly [Chiba et al., 1979; Matson et al., 1989; Chiba et al., 2000; Akihara et al., 2005; Zintz et al., 2005]. Etiologic studies of acute gastroenteritis in infants conducted in Japan with RT-PCR showed that sapovirus was one of the most common causes of outbreaks of viral gastroenteritis among infants in Sapporo [Chiba et al., 1979, 2000]. Immunological and seroepidemiological studies have indicated a worldwide distribution of sapovirus [Sakuma et al., 1981; Nakata et al., 1985; Lopman et al., 2002]. The age-related prevalence of antibody against this virus also has shown that infections commonly occur in children less than 5 years old. It was also found that serum antibody level to sapovirus was lowest in the first year of

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*Correspondence to: 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.
E-mail: ushijima@m.u-tokyo.ac.jp

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life, increasing after 2 years of age [Matsui and Greenberg, 2000].

Norovirus (formerly known as "Norwalk-like virus") and sapovirus (formerly known as "Sapporo-like virus") are distinct genera within the family *Caliciviridae*. These viruses contain a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The norovirus genome contains three ORFs (ORF1, 2, and 3). The ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase (RdRp), ORF 2 encodes the capsid proteis and ORF3 enodes a small capsid protein. In the two sapovirus ORFs (ORF 1 and 2), however, it is ORF 1 that encodes the non-structural as well as the capsid proteins while ORF2 encodes a small protein. The prototype strain of sapovirus is the Sapporo virus (Hu/SaV/Sapporo virus/1977/JP), which was discovered originally during an outbreak a home for infants in Sapporo, Japan in 1977 [Chiba et al., 1979]. Sapovirus has a typical "Star of David" configuration by electron microscopy (EM), and sapovirus strains are antigenically identical to each other by immune EM [Chiba et al., 2000]. Sapovirus is divided into five genogroups, among which only genogroups I, II, IV, and V are known to infect humans. Recently, the diversity of sapoviruses was described in which genogroup I and II sapoviruses could be classified into eight and five genotypes, respectively [Farkas et al., 2004; Akihara et al., 2005; Phan et al., 2005b].

The objectives of this study were: to determine the incidence of diarrheal virus infection in infants and young children with acute gastroenteritis in Osaka City, Japan; to characterize the detected sapovirus according to genogroup and genotype; and to analyze the genetic diversity among these viruses. Additionally, the age-related distribution and seasonal pattern of sapovirus infection were also studied.

MATERIALS AND METHODS

Fecal Specimens

One hundred twenty-five fecal specimens were collected from sporadic cases of acute gastroenteritis in a pediatric clinic in Osaka City, Japan during the period of July 2004 to June 2005. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000*g* for 10 min. The supernatants were collected and stored at −30°C until use for the detection of 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), except for adenovirus, 4 μl of extracted viral genome was added with a reagent mixture consisting of 5× first strand buffer (Invitrogen, Carlsbad, CA), 10 mM dNTPs (Roche, Mannheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/μl) (Invitrogen), random primer (1μg/μl) (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and MilliQ water. The total of reaction mixture is 8 μl. The RT step was carried out at 50°C for 1 hr, followed by 99°C for 5 min and then held at 4°C [Phan et al., 2005a].

Polymerase Chain Reaction (PCR)

The first group of viruses including astrovirus, norovirus (GI, GII), and sapovirus and the second group including group A, B, and C rotaviruses and adenovirus were detected by mutiplex PCR with specific primers as reported previously by Phan et al. [2005a]. These mutiplex PCR assays are the sensitive and specific methods that have been used with the success to accurately define the true burden of diarrheal disease due to viruses. The identification of the first group of viruses was performed with 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, 814, 352, and 482 bp, respectively in one PCR tube. For the detection of the second group of viruses, specific primers PreCAP1 and 82b; G1SKF and G1SKR; COG2F and G2SKR, SLV5317 and SLV5749 specifically generated four different sizes of amplicons of 719, 330, 387, and 434 bp for astrovirus, norovirus (GI, GII), and sapovirus, respectively (Table I). Sapovirus polymerase region was also amplified to identify recombinant sapovirus using primers P290 and P289 [Jiang et al., 1999]. To eliminate the possibility of co-infection of two different genotypes in case of sapovirus recombination, flanking polymerase and capsid regions with their junction was amplified with primers P290 and SLV5749 to generate 1,163-bp product [Jiang et al., 1999; Phan et al., 2005a]. PCR was carried out with 1 μl of cDNA in 10 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (2.5 mM/μl), primers (33 μM/μl), Taq DNA polymerase (5 U/μl) (Promega) and MilliQ water. PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C. In order to monitor the quality of extraction and RT-PCR, Hu/SVGII/Karachi/928/1994, and MilliQ water were used as positive and negative controls, respectively. In experiment, to avoid false positive results instructions (autoclaved solutions, reagents into aliquots, disposable gloves, avoidance of splashes, and positive displacement pipettes) to prevent cross contamination were followed strictly [Kwok and Higuchi, 1989].

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide

TABLE I. A List of Specific Primers Used in the Study to Detect Multiple Target Viruses Among Infants and Children With Diarrhea in Japan by RT-PCR

Virus	Target region	Primer name	Polarity[a]	Sequence (5′–3′)[b]	Amplicon size (bp)	Position (nt number)	Representative strain's accession number
Group A rotavirus	VP7	Beg9	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	395	1–395	D16343
		VP7-1′	−	ACTGATCCTGTTGGCCATCCTTT			
Group B rotavirus	VP7	B5-2	+	GGCAATAAAATGGCTTCATTGC	814	1–814	AY539856
		B3-3	−	GGGTTTTTACAGCTTCGGCT			
Group C rotavirus	VP7	NG8S1	+	ATTATGCTCAGACTATCGCCAC	352	353–704	AB086966
		NG8S2	−	GTTTCTGTACTAGCTGGTGAAC			
Adenovirus	Hexon	Ad1	+	TTCCCCATGGCICAYAACAC	482	1834–2296	M12411
		Ad2	−	CCCTGGTAKCCRATRTTGTA			
Astrovirus	Capsid	PreCAP1	+	GGACTGCAAAGCAGCTTCGTG	719	4235–4953	L23513
		82b	−	GTGAGCCACCAGCCATCCCT			
Norovirus GI	Capsid	G1-SKF	+	CTGCCCGAATTYGTAAATGA	330	5342–5671	M87661
		G1-SKR	−	CCAACCCARCCATTRTACA			
Norovirus GII	Capsid	COG2F	+	CARGARBCNATGTTYAGRTGGATGAG	387	5003–5389	X86557
		G2-SKR	−	CCRCCNGCATRHCCRTTRTACAT			
Sapovirus	Capsid	SLV5317	+	CTCGCCACCTACRAWGCBTGGTT	434	5083–5516	X86560
		SLV5749	−	CGGRCYTCAAAVSTACCBCCCCA			
Sapovirus	Polymerase	P290	+	GATTACTCCAAGTGGGACTCCAC	331	4354–4684	X86560
		P289	−	TGACAATGTAATCATCACCATA			

[a]+, Forward primer; −, Reverse primer.
[b]Within nucleotide sequence of primers, B = C, G or T; H = A, C or T; N: Any base; R = A or G; S = G or C; V = A, C or G; W = A or T; Y = C or T.

for 20 min, then visualized under ultraviolet (UV) light, and the results were recorded by photography.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for sapovirus were determined directly with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). Phylogenetic tree with 100 bootstrap resamples of the nucleotide alignment data sets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3) was used to compare recombinant sapovirus sequences. The capsid and polymerase sequences of sapovirus strains 5786/Osaka/JP, 5800/Osaka/JP, 5836/Osaka/JP, 5862/Osaka/JP, 5862/Osaka/JP, and 5821/Osaka/JP, had been submitted to the DDBJ DNA/GenBank database and had been assigned accession numbers AB242322 and DQ307499, AB242323 and DQ 307500, AB242324 and DQ307501, DQ401095 and DQ 401096, AB242325, and AB242326, respectively. Reference sapovirus strains and accession numbers used in this study were as follows: PEC (AF182760), Lyon/598/97/F (AJ271056), London/92 (U95645), Mex340/90 (AF435812), Cruiseship/00 (AY289804), Hou7-1181/90 (AF435814), Arg39 (AY289803), Parkville/94 (U73124), Sapporo/82 (U65427), Manchester/93 (X86560), Karachi/730/1992 (AB126249), Karachi/874/1992 (AB181129), Karachi/928/1994 (AB181128), Karachi/1017/1990 (AB 181227), Karachi/876/1993 (AB181132), Houston/90 (U95644), Stockholm/97 (AF194182), 12/DCC/Tokyo/Japan/44 (AB236380), Karachi/872/1991 (AB181231), 4408/Maizuru/Japan (AB180209), and Mex14917/2000 (AF435813).

RESULTS

Detection of Diarrheal Viruses

A total of 125 fecal specimens were collected from infants and children with acute gastroenteritis in Osaka City, Japan, during the period of July 2004 to June 2005. For the pediatric population, the lowest age was 5 months, the highest was 8 years, and the average age was 1.2 years (14 months). Among all children with acute gastroenteritis, 91.2% were aged less than 36 months. Males accounted for 57%. All fecal specimens were tested for the presence of rotavirus, norovirus, sapovirus, astrovirus, and adenovirus by RT-multiplex PCR. Diarrheal viruses were detected in 72 out of 125 (57.6%) specimens tested. Among diarrheal viruses detected, norovirus was the most prevalent (19.2%), followed by 18.4% of group A rotavirus, 1.6% of astrovirus, and 0.8% of adenovirus, respectively. It was interesting that sapovirus infection was identified with the high incidence and accounted for 17.6%. No group B and C rotaviruses were found in these patients.

Nucleotide Sequencing and Phylogenetic Analysis of Sapovirus Genogroup and Genotype

The PCR products of sapovirus were sequenced in order to characterize further the genetic relationship among the sapovirus isolates detected in infants and children with acute gastroenteritis in Osaka City, Japan. Their partial nucleotide sequences were compared to each other as well as to those of reference sapovirus strains available in the DDBJ DNA/GenBank database by BLAST. The nucleotide sequence of the 5′ ends of the sapovirus capsid gene was determined by direct sequencing with the amplified fragments. This region has been shown to be suitable for genotyping [Farkas et al., 2004; Phan et al., 2005b]. A total of 22 sapovirus sequences were analyzed by phylogenetic analysis and grouped using the recent sapovirus capsid region classification scheme [Farkas et al., 2004; Akihara et al., 2005; Phan et al., 2005b]. In the present study, all of the sapovirus sequences were classified into only one distinct genogroup I (Fig. 1). The results indicated that sapovirus group I was a dominant genogroup. The sapovirus GI sequences clustered into four genotypes (GI/1, GI/4, GI/6, and GI/8) and these presented 4.5% (1 of 22), 4.5% (1 of 22), 81.8% (18 of 22), and 9.1% (2 of 22), respectively. Using CLUSTAL X, it was also noted that these sapoviruses had a high identity on the amino acid level and the nucleotide level with other reference sapovirus strains previously registered in the DDBJ DNA/GenBank database in the same genotype ranged from 90% to 100%.

Nucleotide Sequencing and Genetic Analysis of Sapovirus Polymerase

The polymerase regions of all sapovirus strains were amplified and sequenced to examine genetically the virus found during this study. In 20 sapoviruses belonging to GI/1, GI/4, and GI/6, the genotypes were maintained in both polymerase and capsid regions. They also shared a high identity at the nucleotide level and the amino acid level of the polymerase with other reference sapovirus strains registered previously in the DDBJ DNA/GenBank database in the same genotype ranged from 95% to 100%. It was found that two sapoviruses with GI/8 capsid, the 5862/Osaka/JP and the 5821/Osaka/JP, were classified into the Sapporo/82 cluster (known as GI/1), not into the 12/DCC/Tokyo/Japan/44 cluster (known as GI/8) when polymerase-based grouping was performed (Fig. 2).

Novel Recombinant Sapoviruses

It was found that the polymerase and capsid regions of the 5862/Osaka/JP and the 5821/Osaka/JP shared 96% and 85% of the amino acid similarity with the Sapporo/82, respectively. However, they had a high homology (99%) at the amino acid level of the capsid region with the 12/DCC/Tokyo/Japan/44. Therefore, these two strains were all recombinant viruses with a GI/8 capsid and GI/1 polymerase. When the sequences of the 5862/Osaka/JP and the 5821/Osaka/JP were compared with that of the Sapporo/82 using the SimPlot, an apparent region of genetic recombination was found at the polymerase- and capsid-junction. Before this junction, the 5862/Osaka/JP, the 5821/Osaka/JP, and the Sapporo/82 were homologous. After the junction, however, the homology was notably different. The SimPlot analysis showed a sudden drop in the identity for the 5862/Osaka/JP and the 5821/Osaka/JP. The 100% homology at the nucleotide as well as the amino acid levels of polymerase and capsid among the 5862/Osaka/JP and the 5821/Osaka/JP was also noted.

Outbreak of Sapovirus

The results shown in Figure 3 indicated that although the fecal specimens were collected over the period of 12 months (July 2004 to June 2005), the sapovirus GI/6 infection was apparently confined within a period of 5 months (August 2004 through December 2004). This pattern of infection indicated an outbreak of sapovirus GI/6 in these subjects and this would be the first outbreak of acute gastroenteritis attributed to sapovirus in Osaka City. It was found that the nucleotide as well as the amino acid sequences of the capsid and polymerase regions among 18 sapovirus isolates were identical (100%). A phylogenetic tree of the amino acid sequences of these isolates and the reference strains was constructed and all of 18 sapovirus GI/6 isolates formed a distinct cluster. These results indicated that sapovirus GI/6 isolates detected in infants and children in Osaka City were closely homologous to each other, suggesting that they were probably derived from the same source of infection. In addition, majority (94.4%, 17 of 18) of sapovirus GI/6 infected cases were confined to infants and young children with an age of less than 3 years. This observation demonstrated that sapovirus infection in this outbreak occurred mainly in infants and young children.

DISCUSSION

Viral gastroenteritis is still a health burden and one of the most frequently encountered problems in developed and developing countries [Parashar et al., 2003a; Mulholland, 2004]. In this study, diarrheal viruses were detected in 57.6% of fecal specimens tested. These findings suggested that acute gastroenteritis in infants and children in Osaka City about 57.6% might be due to the diarrheal viruses and 42.4% caused by other etiologic agents. Among the diarrheal viruses detected, norovirus was found to be the most prevalent and became a leading cause of viral gastroenteritis in infants and children in Osaka City, followed by group A rotavirus, sapovirus, astrovirus, and adenovirus. These viruses were also considered to be significant global enteropathogens and were associated with sporadic cases as well as outbreaks of gastroenteritis [Noel and Cubitt, 1994; Carter and Willcocks, 1996; Bon et al., 1999; Deneen et al., 2000; Marks et al., 2000].

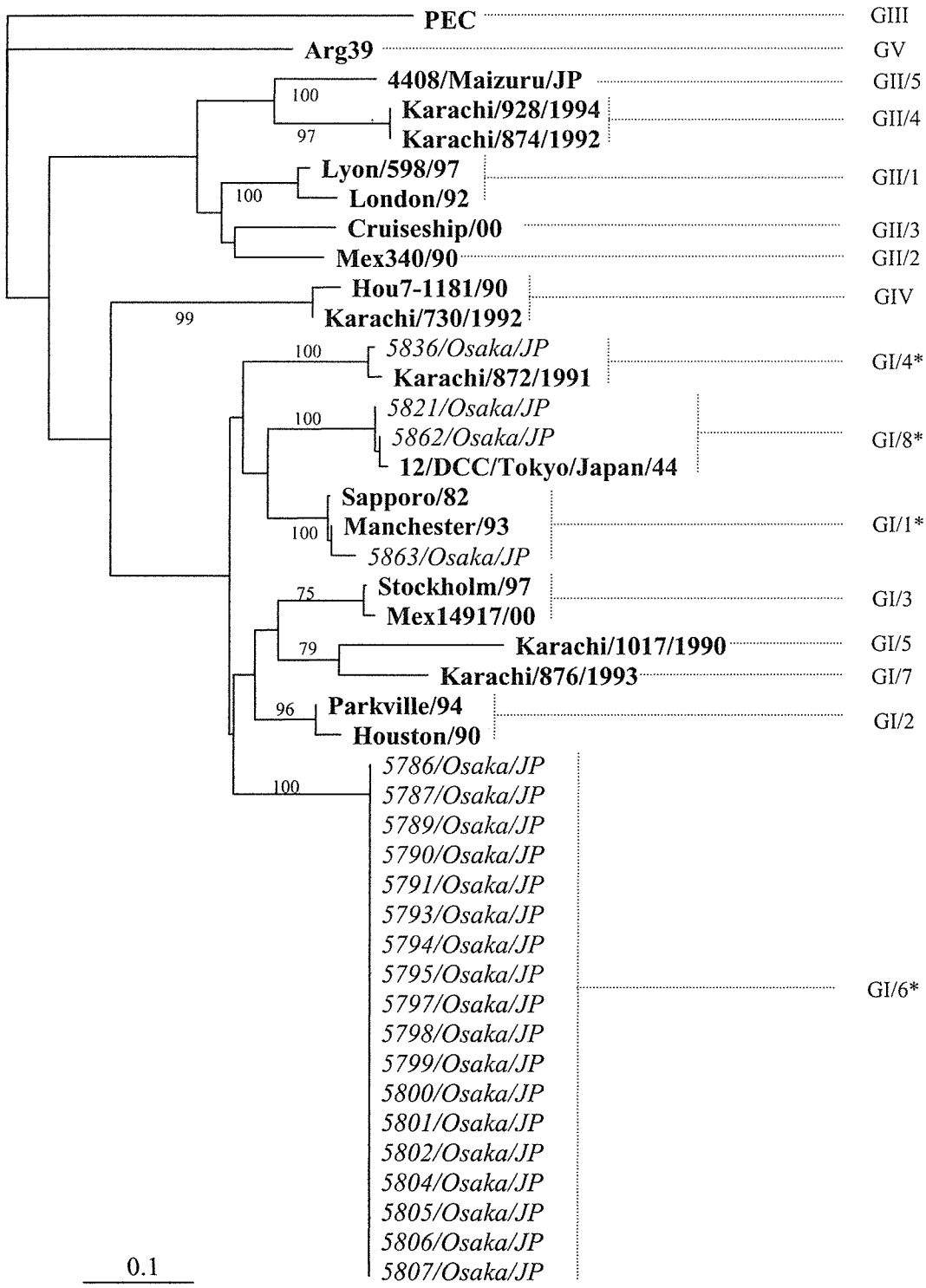

Fig. 1. Phylogenetic tree of nucleotide sequences of Japanese sapovirus. All of the sapovirus sequences were classified into only one distinct genogroup I and the sapovirus genogroup I sequences clustered into four genotypes (GI/1, GI/4, GI/6, and GI/8). The tree was constructed from partial amino acid sequences of capsid region of sapovirus isolates detected in Osaka City, Japan. Reference strains of sapovirus were selected from DDBJ/GenBank under the accession number indicated in the text. Japanese sapovirus is highlighted in italic. PEC strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. *, Genotype contains Japanese sapovirus detected in the study.

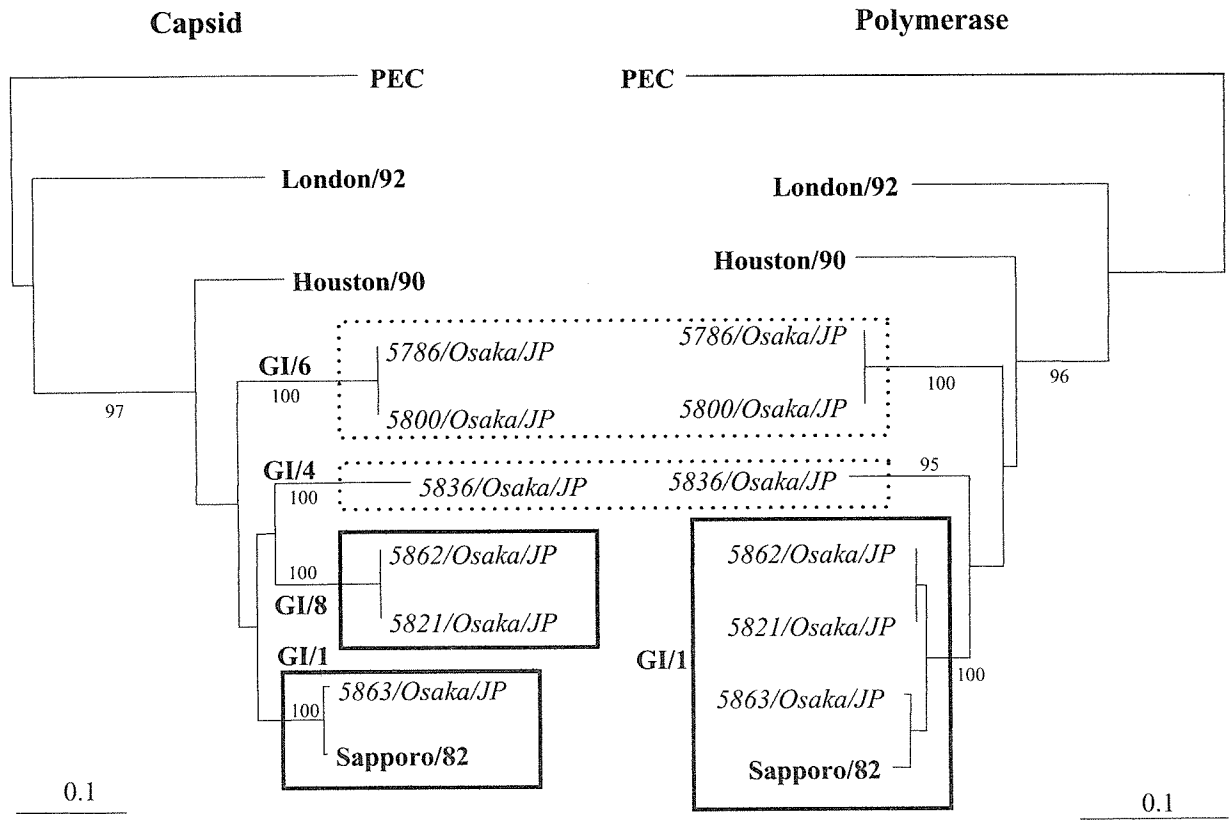

Fig. 2. Observation of changes of sapovirus genotypes on the basis of phylogentic trees of amino acid sequences. The novel recombinant sapoviruses, the 5862/Osaka/JP and the 5821/Osaka/JP, were classified into two distinct genotypes GI/1 and GI/8 when the polymerase- and capsid-based groupings were performed. In other sapoviruses detected, the genotypes were maintained in both polymerase and capsid regions. The trees were constructed from partial amino acid sequences of capsid and polymerase regions of Japanese sapovirus isolates. Reference strains of sapovirus were selected from DDBJ/GenBank under the accession number indicated in the text. Japanese sapovirus was highlighted in italic. PEC strain was used as an outgroup strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values.

Sapovirus is recognized as one of the causes of the diarrheal illness in humans worldwide and its prevalence was shown to range from 0.3% to 9.3% and usually much lower than norovirus [Oh et al., 2003; Yan et al., 2003; Phan et al., 2004]. It was interesting that sapovirus infection was identified with high prevalence, accounting for 17.6%. The results in this study showed that all Japanese sapovirus sequences belonged to only one sapovirus genogroup I with four distinct genotypes (GI/1, GI/4, GI/6, and GI/8). Of note, the sapovirus GI/6 isolates detected were predominant (81.8%) and closely homologous to each other, suggesting that they came from the same source of infection. It was noted that the sapovirus GI/6 infection was confined only within a short period of 5 months (August 2004 through December 2004). Moreover, only two sapovirus GI/6 strains were found in 2002–2003 during a 7-year (1996–2004) survey of sapovirus infection in diarrheal fecal specimens of infants and children collected from Osaka City, Japan and no sapovirus GI/6 was detected in 2003–2004 [Phan et al., 2005b]. Taken together, this is the first report of an outbreak attributed to the sapovirus GI/6 among infants and children in Japan and this would be the first outbreak of acute gastroenteritis attributed to sapovirus in Osaka City.

In this outbreak, the majority of infants and children with sapovirus infection (94.4%) were aged less than 36 months. This result was in line with published reports on sapovirus epidemiology worldwide in which sapovirus infection causes acute gastroenteritis in all age group, through it occurs predominantly in infants and young children [Chiba et al., 2000; Okada et al., 2002; Akihara et al., 2005]. Our findings also confirmed sapovirus as one of the enteropathogens responsible for viral gastroenteritis among infants and children in Japan. According to most studies, sapovirus infection has been found mainly during the cold season [Chiba et al., 1979; Phan et al., 2004, Yan et al., 2005]. In contrast, the present study has demonstrated that the outbreak associated with sapovirus lasted continuously from August to December as the middle of summer to the end of autumn in Japan. This observation clearly indicated that sapovirus infection could occur not only in the cold season but also in the hot season.

Recombination is among the major driving forces of viral evolution. Recombination of viral RNA is known to

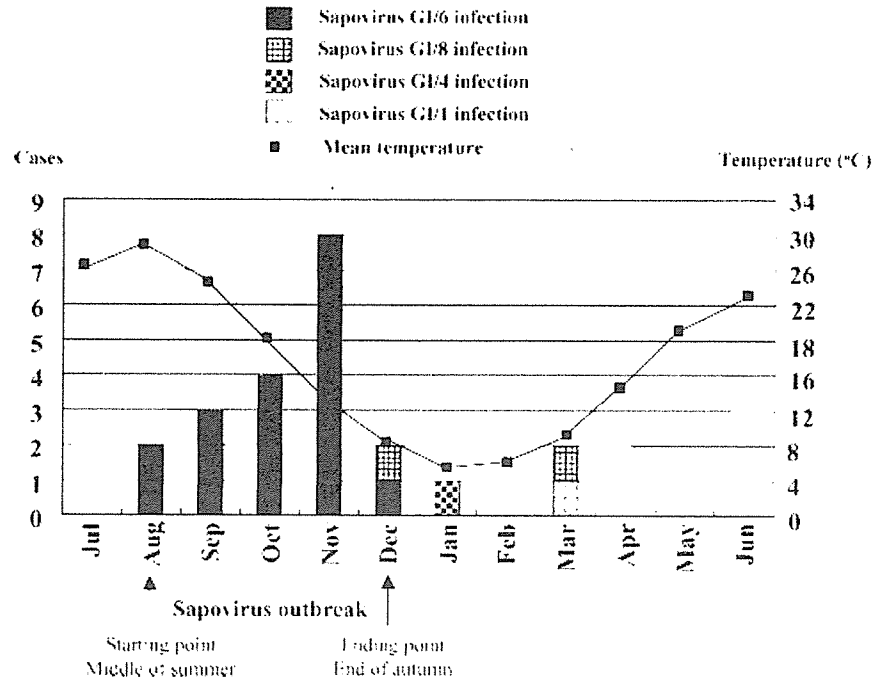

Fig. 3. Monthly distribution of sapovirus infection in infants and children with acute gastroenteritis in Osaka City, Japan, during the period July 2004 to June 2005. The sapovirus GI/6 infection was apparently confined within a period of 5 months (August 2004 through December 2004). The duration of outbreak of sapovirus GI/6 infection is shown.

depend upon various immunological and intracellular constraints that may allow the emergence of viable recombinant [Worobey and Holmes, 1999]. Recombination in viruses can greatly affect phylogenetic groupings, confuse molecular epidemiologic studies, limit the virus control programs, and have major implications in viral vaccine design [Matson, 2003; Bull et al., 2005]. Although several recombinant noroviruses causing sporadic cases and outbreaks of acute gastroenteritis have been reported, the data of recombinant sapovirus are limited. Another interesting feature of this study was the presence of two novel, naturally occurring recombinant sapoviruses circulating in the Japanese population studied. These isolates were obtained from a male patient and a female patient both aged 1 year and with clinical manifestations of acute gastroenteritis in Osaka City, Japan. These isolates had total homology (100%) at the nucleotide as well as amino acid levels of the capsid and polymerase. The findings demonstrated that they likely represented the same strain. Based on genetic analysis, these isolates shared a high level of sequence identity (96%) in the polymerase region and a low level of amino acid identity in the capsid region (85%) with the Sapporo/82 cluster (known as GI/1). However, they had a high level of amino acid identity (99%) in capsid region with 12/DCC/Tokyo/Japan/44 cluster (known as GI/8). Altogether, these sapovirus strains demonstrated a novel recombination with GI/1 polymerase and GI/8 capsid.

In conclusion, this is the first report of outbreak associated with the sapovirus GI/6 infection among infants and children with acute gastroenteritis in Osaka City, Japan and warns of the threat they pose. This is also the first study, to our best knowledge, demonstrating the existence of the novel recombinant sapovirus causing diarrheal illness in Osaka City, Japan.

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