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ARTICLE

Emergence of rare sapovirus genotype among infants and children with acute gastroenteritis in Japan

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Abstract A total of 1,154 fecal specimens from infants and children with acute gastroenteritis in five cities in Japan (Maizuru, Tokyo, Sapporo, Saga, and Osaka), collected from July 2003 to June 2005, were tested for the presence of diarrheal viruses by reverse transcriptase multiplex PCR. Overall, 469 of 1,154 (40.6%) were positive for diarrheal viruses, of which 49 (10.4%) were positive for sapovirus. The peak of sapovirus infection shifted from April–June in 2003–2004 to October–December in 2004–2005. The observations show that maximum sapovirus prevalence can occur during warmer seasons. Sapovirus was subjected to molecular genetic analysis by sequencing. The results indicated that sapovirus genogroup I was a dominant group (100%). Sapovirus strains detected in this study were further classified into four genotypes (GI/1, GI/4, GI/6, and GI/8). Of these, sapovirus GI/1 was the most predominant, followed by sapovirus GI/6; these accounted for 93% (13 of 14) and 7% (1 of 14), respectively, in 2003–2004. However, it was noteworthy that sapovirus GI/6 suddenly emerged to become the leading genotype, accounting for 77% (27 of 35) of isolates in 2004–2005. This is believed to be the first report of the changing distribution of sapovirus genotypes and of the emergence of the rare sapovirus GI/6.

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Introduction

Acute gastroenteritis is a common illness in humans worldwide and has a great impact on public health [1]. It is recognized as one of the leading causes of death by infectious disease [1, 2]. The mortality of infants and children due to acute gastroenteritis is greater in developing than in developed countries [3]. While rotavirus is recognized as the most important cause of severe gastroenteritis in infants and young children worldwide, sapovirus (SaV) is also considered a significant global enteropathogen of acute gastroenteritis [4–7].

SaV, previously referred to as Sapporo-like virus, is a distinct genus within the family *Caliciviridae*. The prototype strain of SaV is the Sapporo virus (Hu/SaV/Sapporo virus/1977/JP), which was derived from an outbreak in a home for infants in Sapporo, Japan, in 1977 [8]. SaV has a positive-sense single-strand RNA genome surrounded by an icosahedral capsid. The SaV genome is 7.3–7.5 kb long and contains two main open reading frames. The open reading frame 1 encodes a polyprotein that undergoes protease processing to produce several nonstructural proteins, including an RNA-dependent RNA polymerase and a capsid protein. The open reading frame 2 encodes a small basic protein with unknown function [8]. On the basis of the sequence analysis of the capsid gene, SaV is divided into five genogroups, among which only genogroups I, II, IV, and V are known to infect humans [9–12]. Recently, the diversity of SaV was described by Akihara et al. [13], who found that SaV genogroup I and genogroup II could be classified into eight and five genotypes, respectively. Immunological and seroepidemiologic studies have indicated a worldwide distribution of SaV. The age-related prevalence of antibody against this virus also has shown that infections commonly occur in children under 5 years of age. Furthermore, it was

found that serum antibody level to SaV was lowest in the first year of life but increased after 2 years of age [14–16].

The objectives of this study were to determine the incidence of diarrheal virus infections in infants and children with acute gastroenteritis in five different cities in Japan during 2003–2005; to characterize the SaV detected according to genogroup and genotype; and to describe the genetic diversity among them. Additionally, the age-related distribution and the seasonal pattern of SaV infection were determined.

Materials and methods

Fecal specimens

A total of 1,154 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics from five different cities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) in Japan during the period of July 2003 to June 2005. The ages of the subjects ranged from 2 months to 15 years, the median age being 28 months. This age distribution in the periods 2003–2004 and 2004–2005 was similar. The fecal specimens were suspended in distilled water to prepare 10% suspensions, which were clarified by centrifugation at 10,000×*g* for 10 min; the supernatants were stored at −30°C.

Extraction of viral genome

The genomes of both RNA and DNA viruses were extracted from 140 μl of 10% fecal suspensions using a single QIAamp spin-column kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Reverse transcription

In reverse transcription (RT), 4 μl of extracted viral genome was added to a reagent mixture consisting of 5× first strand buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/μl) (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (1 μg/μl) (Takara, Shiga, Japan), RNase inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and MilliQ water. The total volume of reaction mixture was 8 μl. The RT step was carried out at 50°C for 1 h, then 99°C for 5 min, after which it was stored at 4°C [17].

Viral detection and polymerase chain reaction

Two multiplex polymerase chain reaction (PCR) procedures using mixtures of primers previously reported were used to identify two groups of diarrheal viruses. The first group includes astrovirus, norovirus (GI, GII), and SaV, and the second group includes group A, B, and C rotaviruses and adenovirus [17]. In the first multiplex PCR, the primer set included equimolar mixes of PreCAP1 and 82 b for astrovirus, G1SKF and G1SKR for norovirus genogroup I, COG2F and G2SKR for norovirus genogroup II, and SLV5317 and SLV5749 for SaV. The respective products of these reactions consisted of amplicons of 719, 330, 387, and 434 bp for astrovirus, norovirus (GI, GII), and SaV, respectively. In the second multiplex PCR, the primer set included equimolar mixes of Beg9 and VP7-1, B5-2 and B3-3, and G8NS1 and G8NA2 for group A, B, and C rotaviruses, respectively, and Ad1 and Ad2 for adenovirus. The respective products of these reactions consisted of amplicons of 395, 814, 352, and 482 bp for group A, B, and C rotaviruses and adenovirus, respectively. The PCR was performed at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 7 min, after which the products were stored at 4°C.

Genotype of group A rotavirus

Genotyping of group A rotavirus was conducted using the G-type specific primers from the method described previously by Das et al. in 1994 [18]. The full-length of *VP7* gene was reversely transcribed and then further amplified with Beg9 and End9 primers. The expected size of the PCR product generated from the full-length *VP7* gene was 1,062 bp in length. The second amplification was performed using the first PCR product as the template with G-type specific mixed primers (9T1-1, 9T1-2, 9T-3P, 9T-4, and 9T-B) for downstream priming and 9con1 for upstream priming in an amplification of *VP7* genes of G1–G4 and G9, respectively. These primers specifically generated five different sizes of amplicons of 158, 224, 466, 403, and 110 bp for G1, G2, G3, G4, and G9, respectively.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, after which they were stained with ethidium bromide for 20 min and then examined under ultraviolet light. The results were photographed.

Latex agglutination test

The Diarlex test (Orion Diagnostica, Espo, Finland), a commercial latex agglutination test, was used for the detection of group A rotavirus infection as a confirmatory test in the fecal specimens that were found by RT-PCR to harbor coinfection with SaV and group A rotavirus. A drop of the fecal supernatant was mixed with a drop of test latex on a slide, and the reaction was observed after 2 min. Development

of distinct agglutination in the Diarlex reagent was considered a positive result. If agglutination was seen in the negative control latex, the result was considered uninterpretable.

Nucleotide sequencing and phylogenetic analysis

The nucleotide sequences of RT-PCR products (DNA) positive for SaV and norovirus 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 resamples of the nucleotide alignment datasets was generated using the neighbor-joining method with Clustal X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). The sequences of SaV strains detected in the present study—5786/Osaka/JP, 5800/Osaka/JP, 5836/Osaka/JP, 5862/Osaka/JP, 5862/Osaka/JP, and 5821/Osaka/JP—had been submitted to GenBank and had been assigned accession numbers AB242322, AB242323, AB242324, DQ401095, AB242325, and AB242326, respectively. Reference SaV 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 (AB181227), Karachi/876/1993 (AB181132), Houston/90 (U95644), Stockholm/97 (AF194182), 12/DCC/Tokyo/Japan/44 (AB236380), Karachi/872/1991 (AB181231), 4408/Maizuru/JP (AB180209), 4724/Osaka/JP (AB180212), and Mex14917/2000 (AF435813).

Results

Molecular epidemiology of viral infections

RT-PCR analysis revealed the presence of viruses in 469 fecal specimens (Table 1). The ages of the infants and children with viral gastroenteritis ranged from 2 months to 11 years. The highest rate of viral infection was in the age group 12–23 months (42%) and the lowest rate was in the age group <6 months (2%). There was no difference in age distribution between the periods 2003–2004 and 2004–2005. In 2003–2004, group A rotavirus was the most prevalent (43.3%, 78 of 180), followed by norovirus (GI and GII) (29.5%, 53 of 180). Interestingly, norovirus (GI and GII) dominated over group A rotavirus and became the leading cause of acute gastroenteritis in 2004–2005, accounting for 45.3% (131 of 289) of cases. Moreover, a

Table 1 Distribution of viral infection in infants and children with acute gastroenteritis in five cities in Japan

Year	No. of specimens tested	No. (%[a]) positive for viruses	Monoinfection (%[b])								Mixed infection (%[b])						
			RV			NoV		SaV	Ade	AsV	Ade+ AsV	SaV + RA	Ade+ NoV GII	RA + NoV GII	Ade + RA	SaV + NoV GII	AsV + RA
			A	B	C	I	II										
2003–2004	402	180 (44.8)	78 (43.3)	0 (0)	1 (0.6)	1 (0.6)	52 (28.9)	12 (6.7)	22 (12)	5 (2.8)	1 (0.6)	2 (1.1)	3 (1.7)	2 (1.1)	1 (0.6)	0 (0)	0 (0)
2004–2005	752	289 (38.4)	77 (26.6)	0 (0)	0 (0)	2 (0.7)	129 (44.6)	30 (10.4)	32 (11.2)	8 (2.8)	0 (0)	0 (0)	1 (0.3)	2 (0.7)	1 (0.3)	5 (1.7)	2 (0.7)
Total	1,154	469 (40.6)	155 (33)	0 (0)	1 (0.2)	3 (0.6)	181 (38.6)	42 (9)	54 (11.5)	13 (2.8)	1 (0.2)	2 (0.4)	4 (0.9)	4 (0.9)	2 (0.4)	5 (1.1)	2 (0.4)

RV rotavirus, *NoV* norovirus, *SaV* sapovirus, *Ade* adenovirus, *AsV* astrovirus, *NoV GI* norovirus genogroup I, *NoV GII* norovirus genogroup II, *RA* group A rotavirus

[a] Refers to total number of specimens tested

[b] Refers to number of viral-positive specimens tested

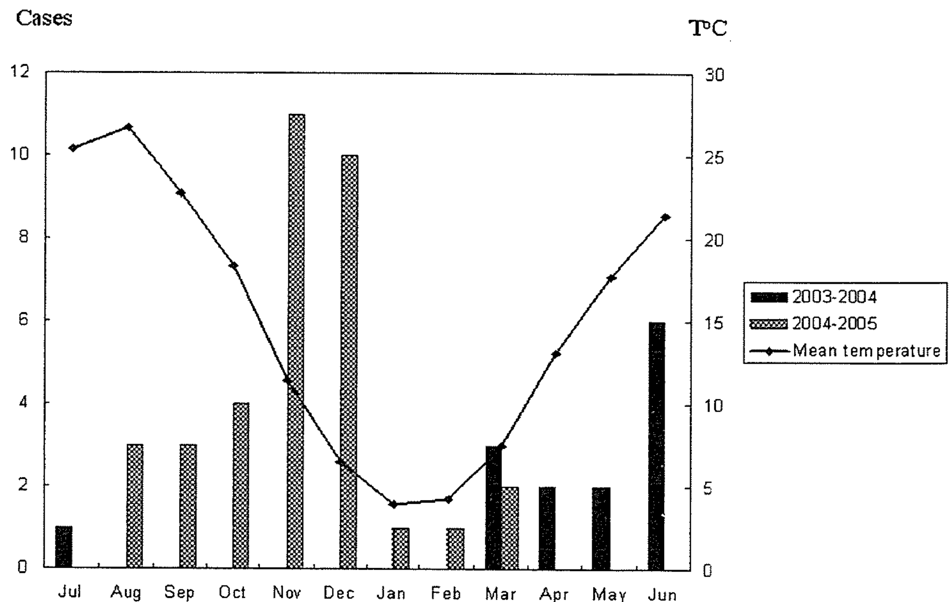

Fig. 1 Monthly distribution of SaV infection in infants and children with acute gastroenteritis in five different cities of Japan, July 2003 to June 2005. The mean temperature in Japan during the molecular epidemiology study of SaV infection is shown

rather high rate (4.3%, 20 of 469) of viral coinfections was shown in this study during 2003–2005. It was found that the number of SaV infections increased sharply from 14 cases in 2003–2004 to 35 cases in 2004–2005. The incidence of SaV was highest in the 12–23-month-old group (45%, 22 of 49) and lowest in infants aged <6 months (2%, 1 of 49). Of note, most of the SaV infections (82%, 40 of 49) occurred in infants and children <3 years of age.

Seasonal variation of sapovirus infection

A comparison of rate of detection of SaV between the periods 2003–2004 and 2004–2005 is shown in Fig. 1. The monthly mean temperature in the five cities in Japan is also shown. The summer lasts from June to September, and the hottest temperature was recorded in August. The coldest month is January, when the temperature might dip as low as 4°C. The incidence of SaV was highest in April–June (ten cases) and was second highest in January–March (three cases). The lowest rate of detection was recorded in October–December (zero cases) in 2003–2004 ($p<0.001$). In contrast, SaV infection was identified continuously from August to February in 2004–2005, and the highest number of cases was recorded in October–December (25 cases), followed by July–September (six cases) ($p<0.001$). The peak of SaV infection shifted from April–June in 2003–2004 to October–December in 2004–2005.

Coinfection with sapovirus and other viral enteropathogens

In total, 49 fecal specimens were determined to be positive for SaV by RT-PCR. Seven (14%) of these specimens harbored coinfection with other viral enteropathogens: norovirus in five cases and group A rotavirus in two. The

Table 2 Characteristics of seven cases of mixed infection with SaV and other enteropathogens among the Japanese pediatric population

Case no.	Patient no.	Sex	Age (months)	City	Month	Year	Sapovirus		Other diarrheal viruses detected			
							Genogroup	Genotype	RT-PCR	Diarlex[a]	Sequence[b]	Genotype[c]
1	5,060	F	14	Maizuru	Apr	2004	I	1	Group A rotavirus	Positive	ND	G3
2	5,299	F	17	Maizuru	Apr	2004	I	1	Group A rotavirus	Positive	ND	G3
3	5,720	F	46	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
4	5,721	F	31	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
5	5,735	M	49	Osaka	Dec	2004	I	6	Norovirus	ND	GII/4	ND
6	5,797	F	10	Osaka	Oct	2004	I	6	Norovirus	ND	GII/3	ND
7	5,806	M	11	Osaka	Nov	2004	I	6	Norovirus	ND	GII/4	ND

ND not done
[a] Used only to detect group A rotavirus
[b] Used only to determine genotype of norovirus
[c] Used only to genotype group A rotavirus

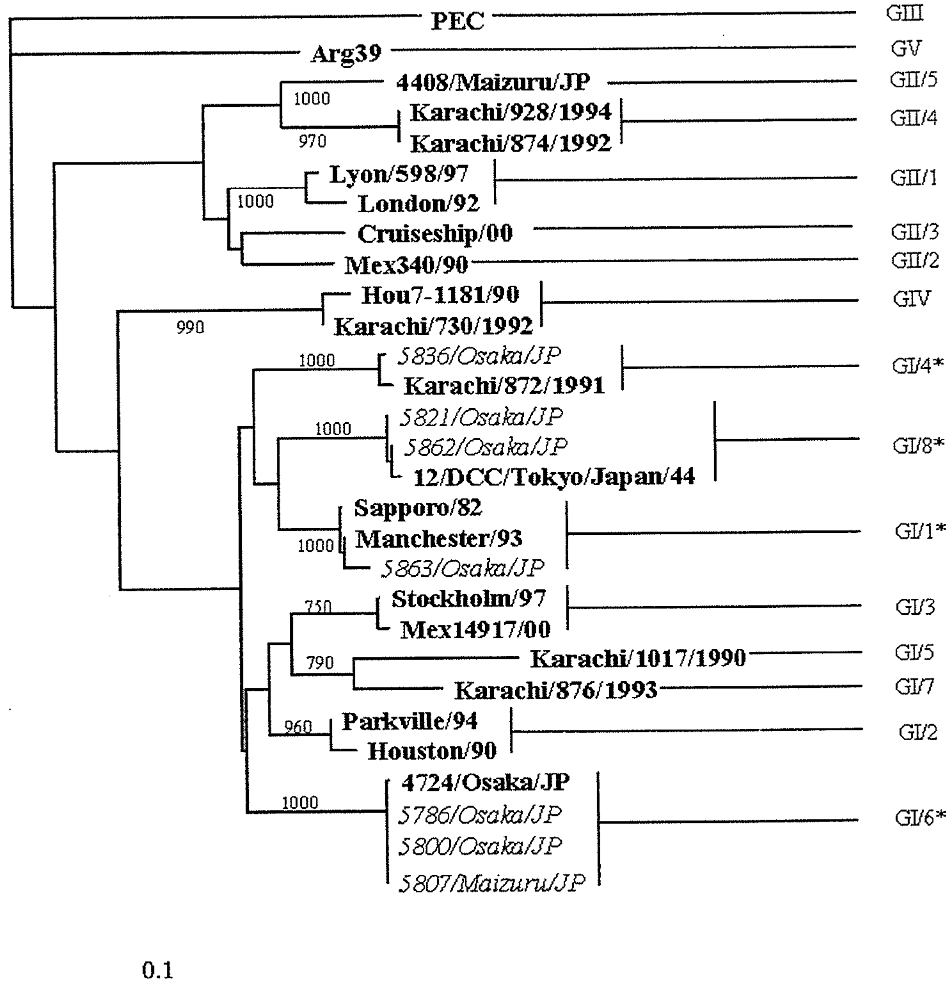

Fig. 2 Phylogenetic tree of nucleotide sequences of SaV strains detected in infants and children with acute gastroenteritis in five different cities in Japan. All of the SaV sequences were classified into only one distinct genogroup (I), and the SaV genogroup I sequences were clustered into four genotypes (GI/1, GI/4, GI/6, and GI/8). The tree was constructed from partial amino acid sequences of the capsid region of SaV strains. Reference SaV strains were selected from GenBank under the accession numbers indicated in the text. Japanese SaV is highlighted in *italic*. The PEC strain was used as an outgroup strain for phylogenetic analysis. The scale indicates amino acid substitutions per position. The numbers in the branches indicate the bootstrap values. *Genotype contains Japanese SaV detected in the study

five coinfections with norovirus were detected in Osaka and the two with group A rotavirus in Maizuru. All diarrheal viruses detected as coinfections with SaV (norovirus and group A rotovirus) were confirmed and further characterized by G typing, latex agglutination test, or sequencing analysis. The five noroviruses belonged to genogroup I, genotype 4 in four cases and genogroup I, genotype 3 in one case, while both group A rotaviruses were identified as G3 genotype 3 (Table 2).

Nucleotide sequencing and phylogenetic analysis of sapovirus

The PCR products of SaV were sequenced in order to further characterize the genetic relationship among the SaV strains detected in infants and children with acute gastroenteritis in Japan. The sequence of the 5′ end of the *SaV* capsid gene was targeted for genotyping [10, 13]. Their partial amino acid sequences were compared to each other as well as to those of reference SaV strains available in GenBank by BLAST. A total of 49 SaV sequences were analyzed by phylogenetic analysis and were grouped using the recent SaV capsid region classification scheme of Akihara et al. [13]. All of SaV sequences clustered into a single genogroup, genogroup I (GI). Most of the SaV GI sequences (93%, 13 of 14) in 2003–2004 belonged to genotype 1 (SaV GI/1) (typified by the Manchester virus cluster). Only one SaV GI sequence (7%, 1 of 14) clustered

Table 3 Distribution of SaV genotypes based on the sequencing genetic analysis among infants and children with acute gastroenteritis in five different cities in Japan

Date of fecal specimen collection	No. of specimens positive for sapovirus	No. (%) identified as sapovirus genogroup I			
		Genotype 1	Genotype 4	Genotype 6	Genotype 8
July 2003–June 2004	14	13 (93)	0 (0)	1 (7)	0 (0)
July 2004–June 2005	35	4 (11)	2 (6)	27 (77)	2 (6)

into the 4724/Osaka/JP virus cluster (known as SaV GI/6) (Fig. 2). In 2004–2005, SaV strains were classified into four distinct genotypes (GI/1, GI/4, GI/6, and GI/8). Interestingly, SaV GI/6 emerged as the predominant genotype in 2004–2005, representing 77% (27 of 35) of the strains, followed by SaV GI/1 (11%, 4 of 35) (Table 3).

Discussion

SaV infection causes acute gastroenteritis in all age groups, through it occurs predominantly in infants and young children [5, 6, 19, 20]. Overall, 49 of 1,154 fecal specimens tested were positive for SaV, and positive specimens were found in all age groups of the subjects included in the study. However, most (82%) of the SaV infections occurred in infants and children <3 years of age. These results were in line with previously published reports on SaV epidemiology worldwide, in which SaV prevalence was shown to range from 0.3 to 9.3%, far below the prevalence of either rotavirus or norovirus [19–22]. Our findings also confirmed SaV as one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. According to surveillance on pediatric cases of viral gastroenteritis in Japan, the main peak of SaV infection was in winter [6, 9]. Interestingly, the highest number of SaV infections in our study was identified in the April–June period in 2003–3004 and in the October–December period in 2004–2005. The observations show that maximum SaV prevalence can occur during warmer seasons.

To date, coinfections with various enteric viruses have been widely reported [12, 20]. Interestingly, we found a rather high rate (14%, 7 of 49) of coinfections with SaV and other viral pathogens. Coinfection of SaV with rotavirus and with norovirus was confirmed by the Diarlex test and G typing. These results underscore that coinfection with SaV and other enteropathogens is not rare.

All of the Japanese SaV sequences belonged to only one SaV genogroup I with four distinct genotypes (GI/1, GI/4, GI/6, and GI/8). The findings clearly indicated that SaV GI was the dominant group to cause acute gastroenteritis among the pediatric population in Japan. In strong agreement with previous reports [7, 10, 13], SaV strains most frequently detected in sporadic gastroenteritis in infants and children in 2003–2004 belonged to the Manchester cluster (GI/1). Of note, SaV GI/6 strains were the most predominant (77%) in 2004–2005 and closely homologous to each other, suggesting that they came from the same source of infection. Moreover, only two SaV GI/6 strains were found in 2002–2003 during a 7-year (1996–2004) survey of SaV infection in diarrheal fecal specimens from Japanese infants and children [20]. In the present study, only one SaV GI/6 was detected in 2003–2004. Taken together, there was an emergence of rare SaV GI/6, and this was the first evidence of the changing distribution of the SaV genotype in association with acute gastroenteritis in Japan. This sudden emergence of SaV GI/6 indicates that the pediatric population in Japan might lack antibody protection to these strains and that these rare strains could be more virulent than those usually associated with pediatric gastroenteritis. Continuous surveillance of SaV infection in Japan is recommended in order to determine whether these rare strains remain dominant in the coming years.

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

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Abstract

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Fecal specimens

A total of 752 fecal specimens were collected from sporadic cases of acute gastroenteritis in pediatric clinics in four localities (Maizuru, Tokyo, Sapporo, and Osaka) in Japan from July 2004 to June 2005. Of these, 231 specimens were from Osaka, 51 from Sapporo, 24 from Tokyo, and 446 from Maizuru. The case definition of diarrhea used in this study was as at least three passings of unformed (loose and watery) stool a day. Acute gastroenteritis was defined as the occurrence of diarrhea and other symptoms such as vomiting, fever, and abdominal pain. The ages of the subjects ranged from 2 months to 15 years, with a median of 27 months. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at $10,000 \times g$ for 10 min. The supernatants were collected and stored at −30 °C until use for the detection of group A rotavirus.

2.2. Extraction of viral genome

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

2.3. Reverse transcription

For reverse transcription (RT), 4 μl of extracted viral genome was added to 4 μl of a reagent mixture consisting of 5× first strand buffer (Invitrogen, Carlsbad, CA, USA), dNTPs (10 mM/μl) (Roche, Mannheim, Germany), DTT (Invitrogen), superscript reverse transcriptase III (Invitrogen), random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (Toyobo, Osaka, Japan), and MilliQ water. The total reaction mixture was 8 μl. The RT step was carried out at 50 °C for 1 h, followed by 99 °C for 5 min and then held at 4 °C (Phan et al., 2005).

2.4. Polymerase chain reaction (PCR)

Using PCR with specific primers as previously reported resulted in the identification of rotavirus. Primers Beg9 (5′-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3′) and VP7-1′ (5′-ACTGATCCTGTTGGCCATCCTTT-3′) was used to amplify VP7 of rotavirus and specifically generated a size of amplicon of 395 bp (Phan et al., 2005). PCR was carried out with 1 μl of cDNA in 10 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/μl), primers (33 μM), Taq DNA polymerase (5 U/μl) (Promega) and MilliQ water. PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

2.5. Group A rotavirus G-typing

G-typing of group A rotavirus was preformed using the protocol from the method previously presented by Das et al. (1994). The full-length of the VP7 gene was reversely transcribed and then further amplified with Beg9 and End9 primers. The expected size of the PCR product generated from the full-length VP7 gene was 1062 bp in length. The second amplification was performed using the first PCR product as the template with G-type specific mixed primers (9T1-1, 9T1-2, 9T-3P, 9T-4, and 9T-B) for downstream priming and 9con1 for upstream priming in an amplification of G1–G4 and G9 VP7 genes, respectively. These primers specifically generated five different sizes of amplicons: 158, 224, 466, 403, and 110 bp for G1, G2, G3, G4, and G9, respectively. The samples whose G-type could not be identified by the first set of primers, described by Das et al. (1994) were then identified using another alternative set of type-specific primers previously reported by Gouvea et al. (1990).

2.6. Group A rotavirus P-typing

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

2.7. Electrophoresis

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

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

2.8. Nucleotide sequencing and phylogenetic analysis

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

3. Results

3.1. Molecular epidemiology of group A rotavirus infection

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

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

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

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

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

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

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

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

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

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

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

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

3.5. Alignment of the partial amino acid sequences of VP4

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

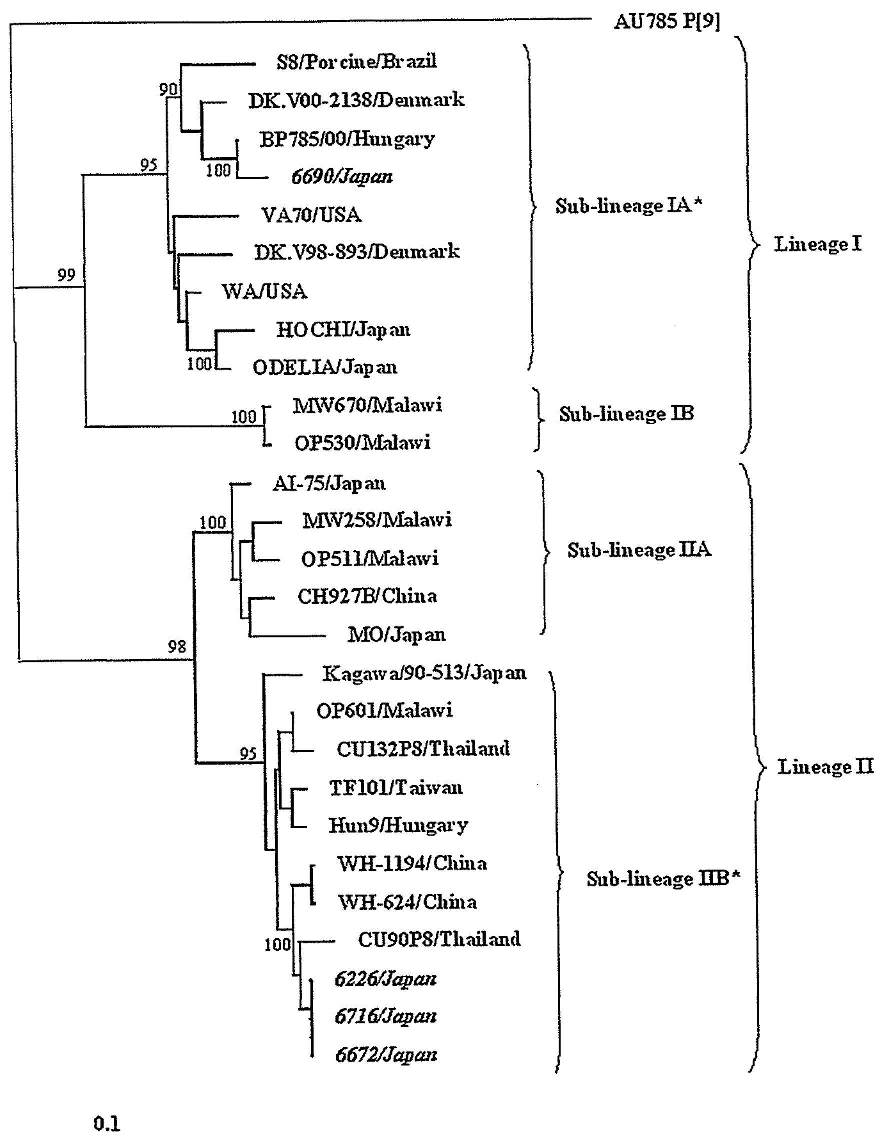

Fig. 2. Phylogenetic tree obtained from nucleotide sequences of the group A rotavirus P[8] VP8* gene. Reference group A rotavirus strains were selected from GenBank under the accession number indicated in the text. Japanese A rotavirus isolates detected are highlighted in italics. The AU785 P[9] strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. (*) Indicates the sub-lineage containing the rotavirus detected in this study.

Table 2
The amino acid substitutions from sequence data of the P[8] VP8* gene specific for lineages and sub-lineages. The amino acid substitutions highlighted in italics and in bold are specific for differentiation of lineages and sub-lineages, respectively. Shaded boxes indicate variable regions located at rotavirus VP8*. The phylogenetic lineage- and sub-lineage-defining sequences are indicated by a horizontal bar

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

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

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

4. Discussion

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

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

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

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

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An outbreak of *adenovirus serotype 41* infection in infants and children with acute gastroenteritis in Maizuru City, Japan

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Abstract

A total of 337 fecal specimens were collected from infants and children with acute gastroenteritis in Maizuru 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 (13.6%, 46 of 337), followed by *adenovirus* (8%, 27 of 337), *group A rotavirus* (5%, 17 of 337), *astrovirus* (1.8%, 6 of 337), and *sapovirus* (1.8%, 6 of 337), respectively. *Adenovirus* was subjected to molecular genetic analysis by sequencing. *Adenovirus* detected in this study was classified into five serotypes, namely Ad1, Ad2, Ad3, Ad5, and Ad41. Of these, Ad41 was the most predominant serotype that accounted for 85.2% (23 of 27). It was noteworthy to point out that Ad41 infection was apparently confined only to the period of 4 months (October 2004 through January 2005). This pattern of infection implied the outbreak of Ad41 in these subjects, which was the first outbreak of acute gastroenteritis attributed to *adenovirus* in Maizuru City, Japan. Another interesting feature of the study was the existence of two Ad41 subtypes co-circulating in this outbreak. This report confirmed the presence of *adenovirus* as one of an important cause of acute gastroenteritis among Japanese infants and children.
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Keywords: *Adenovirus*; Outbreak; Maizuru

1. 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). Among different kinds of diarrheal viruses, *rotavirus* is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide (Mulholland, 2004). *Adenovirus*, however, is also considered to be a significant enteropathogen in association with sporadic cases as well as outbreaks of gastroenteritis in such settings as kindergartens, schools, and hospitals (Chiba et al., 1983; Van et al., 1992; Akihara et al., 2005).

Human *adenovirus* belongs to the *Mastadenovirus* of the family *Adenoviridae*. *Adenovirus* causes a variety of diseases such as acute respiratory, gastrointestinal, and urinary tract infections. To date, 51 *adenovirus* serotypes have been recognized and classified into six subgenera from A to F. This classification scheme is generally consistent with subgroupings of *adenoviruses* on the basis of their physicochemical, biological and genetic properties (Hierholzer et al., 1988; Schnurr and Dondero, 1993; De Jong et al., 1999). Among six subgenera, subgenus F, represented by two *adenovirus* serotypes, *adenovirus* serotype 40 (Ad40) and Ad41, was the most important in association with acute gastroenteritis and accounting for 1–20% of cases. They had a global distribution and were of comparable prevalence both in outpatients and hospitalized children in both the developed and developing countries (Brandt et al., 1985; Shinozaki et al., 1991; Li et al., 2004).

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Over the past decades, neutralization test, ELISA, or virus isolation had been used for the detection and identification of *adenovirus* serotypes. However, these methods are relatively complicated, labor intensive, time consuming, of low sensitivity, and sometimes require the cell culture techniques. In addition, isolation of *adenovirus* is sometimes unsuccessful because of the low viral titer in clinical specimens (Van der Avoort et al., 1989; Takeuchi et al., 1999; Li et al., 2004). Those disadvantages lead to a limitation of their use. Amplification of the viral genome by RT-PCR has been introduced as a convenient and powerful alternative for molecular diagnosis. Highly sensitive and specific RT-PCR assay is currently available for the detection of *adenovirus*. Additionally, genome amplification allows further characterization of the *adenovirus* serotype by sequence analysis (Takeuchi et al., 1999; Phan et al., 2005b).

The objectives of this study were to determine the prevalence of diarrheal virus infection in infants and young children with acute gastroenteritis in Maizuru City, Japan, to identify the serotype and to characterize the genetic diversity among *adenoviruses* detected in this study. Additionally, the age-related distribution and seasonal pattern of *adenovirus* infection were also described.

2. Materials and methods

2.1. Fecal specimens

A total of 337 fecal specimens were collected from infants and children with acute gastroenteritis in a clinic in Maizuru 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 for the detection of diarrheal viruses.

2.2. Extraction of viral genome

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

2.3. Reverse transcription (RT)

For reverse transcription (RT), 4 μl of extracted viral genome was added with a reagent mixture consisting of 5× First strand buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/μl) (Invitrogen, Carlsbad, CA, USA), random primer (1 μg/μl) (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase Inhibitor (33 U/μl) (Toyobo, Osaka, Japan), and MilliQ water. The total volume of the reaction mixture was 8 μl. The RT step was carried out at 50 °C for 1 h, followed by 99 °C for 5 min and held at 4 °C (Phan et al., 2005b).

2.4. Detection of diarrheal viruses by 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 RT-PCR with primers as previously reported by Phan et al. (2005b). 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 bp, 814 bp, 352 bp and 482 bp, respectively. For the detection of the second group of viruses, specific primers PreCAP1 and 82b, G1SKF and G1SKR, COG2F and G2SKR, SLV5317 and SLV5749 were utilized to specifically generate four different sizes of amplicons of 719 bp, 330 bp, 387 bp and 434 bp for *astrovirus*, *norovirus* (*GI*, *GII*), and *sapovirus*, respectively. PCR was carried out with 1 μl of cDNA in 10 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/μl), primers (33 μM/μl), Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA) and MilliQ water. PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

2.5. Electrophoresis

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

2.6. Serotyping of adenovirus by PCR and sequence analysis

2.6.1. Amplification of hexon hypervariable regions (HVRs) by PCR

Seven hypervariable regions of the hexon gene of *adenovirus* were amplified by specific primers S29 (for sense 5′-GCCAGCACRTWCTTTGACAT-3′) and S53 (for antisense 5′-CCCATGTTGCCAGTGCTGTTGTARTACA-3′) to generate the amplicon size of 1286 bp (Takeuchi et al., 1999). PCR was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °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.

2.6.2. Nucleotide sequencing and phylogenetic analysis of HVRs

The nucleotide sequences of PCR products (DNA) positive for *adenovirus* were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.). Sequence analysis was performed using CLUSTAL X software (Version 1.6). A phylogenetic tree with 1000 bootstrap resamples of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTER X. The genetic distance was calculated using

Kimura's two-parameter method (PHYLIP). The nucleotide sequence data of *adenovirus* serotype 41 strains 5918/JP and 5950/JP had been submitted to GenBank and had been assigned accession number DQ336390 and DQ336391, respectively. Reference *adenovirus* strains and accession numbers used in this study were as follows: *adenovirus* serotype 31 (X74661), *adenovirus* serotype 1 (X67709), *adenovirus* serotype 2 (XJ01917), *adenovirus* serotype 5 (M73260), *adenovirus* serotype 6 (X67710), *adenovirus* serotype 3 (X76549), *adenovirus* serotype 4 (X84646), *adenovirus* serotype 8 (X74663), *adenovirus* serotype 19 (X98359), *adenovirus* serotype 37 (X98360), *adenovirus* serotype 41-subtype 1 (AB103349), *adenovirus* serotype 41-subtype 2 (AB103344), and *adenovirus* serotype 40 (X51782).

3. Results

3.1. Molecular epidemiology of diarrheal viruses

A total of 337 fecal specimens were collected from infants and children with acute gastroenteritis in Maizuru City, Japan, during the period of July 2004 to June 2005. For the pediatric population, the lowest age was 3 months, the highest was 14 years, and the average age was 1.3 years (15 months). Among all children with acute gastroenteritis, 92% were aged less than 36 months. Moreover, the number of males accounted for 53.4%. RT-multiplex PCR was performed to test all fecal specimens for the presence of *rotavirus*, *norovirus*, *sapovirus*, *astrovirus*, and *adenovirus*. The results shown in Table 1 revealed that diarrheal viruses were detected in 102 out of 337 (30.3%) specimens tested. Among the diarrheal viruses detected, *norovirus* was the most prevalent (13.6%), followed by *adenovirus* (8%), *group A rotavirus* (5%), *astrovirus*, and *sapovirus* (1.8% each), respectively. No *group B and C rotaviruses* were found in these subjects. Since *adenovirus* was detected with a high prevalence, it was interesting to further characterize its serotypes and genetic relationships.

3.2. Nucleotide sequencing and phylogenetic analysis of adenovirus

The PCR products of *adenovirus* were sequenced in order to further characterize the genetic relationship among the *adenovirus* isolates detected in infants and children with acute gastroenteritis in Maizuru City, Japan. Their nucleotide sequences containing seven hypervariable regions of the hexon gene were compared to each other as well as to those of reference *adenovirus* strains available in GenBank by BLAST. A total of 27 *adenovirus* sequences were analyzed by phylogenetic analysis using the recent seven-hypervariable regions of the hexon gene-based classification scheme of Li et al. (2004). *Adenoviruses* detected in the present study were classified into five serotypes, Ad1, Ad2, Ad3, Ad5 and Ad41. Of these, Ad41 predominated over other serotypes and represented 85.2% (23 of 27) while one of each was Ad1, Ad2, Ad3 and Ad5, respectively (Fig. 1). Using CLUSTAL X, it was also noticed that these *adenoviruses* had a high identity on

Table 1
Distribution of diarrheal viruses detected in infants and children with acute gastroenteritis in Maizuru City, Japan during 2004 and 2005 (number of fecal specimens tested: 337)

Target virus				
Norovirus	*Adenovirus*	*Group A rotavirus*	*Sapovirus*	*Astrovirus*
46	27	17	6	6
13.6%	8%	5%	1.8%	1.8%

the nucleotide level as well as on the amino acid level with corresponding *adenovirus* reference strains previously registered in GenBank ranging from 94% to 100%.

3.3. Outbreak of adenovirus serotype 41

The results shown in Fig. 2 revealed that although the fecal specimens were collected over a period of 12 months (July 2004

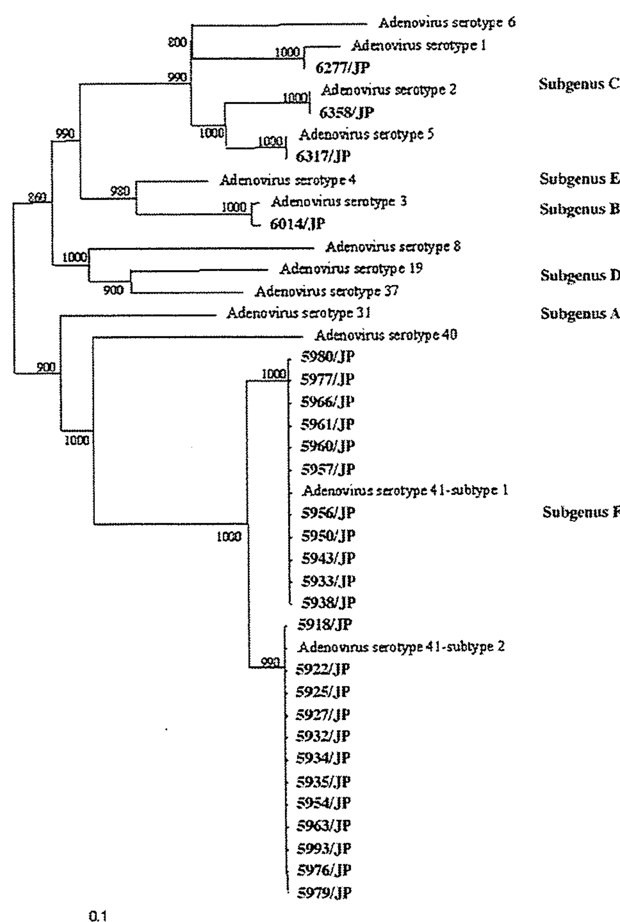

Fig. 1. Phylogenetic tree of nucleotide sequences of *adenoviruses* detected in acute gastroenteritis infants and children in Maizuru City, Japan, in 2004–2005. The tree was constructed from nucleotide sequences of seven hypervariable regions of the hexon gene of *adenovirus* isolates detected in Maizuru City, Japan. Reference strains of human *adenovirus* were selected from GenBank under the accession number indicated in the text. *Adenoviruses* detected in this study were highlighted in bold. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values.

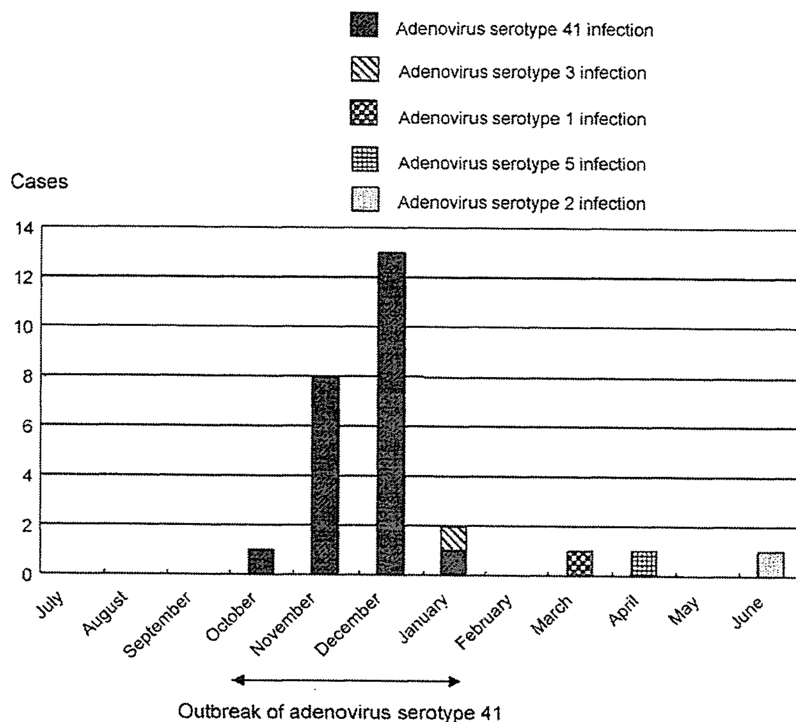

Fig. 2. Monthly distribution of *adenovirus* infection in infants and children with acute gastroenteritis in Maizuru City, Japan during the period of July 2004 to June 2005. The duration of outbreak of Ad41 infection was shown.

to June 2005), the Ad41 infection was apparently confined to a period of 4 months (October 2004 through January 2005). This pattern of infection indicated an outbreak of Ad41 in these subjects, and this would be the first outbreak of acute gastroenteritis attributed to *adenovirus* in Maizuru City, Japan. A phylogenetic tree of the nucleotide sequences of these Ad41 isolates and the reference strains was constructed and all of 23 Ad41 isolates formed two distinct subtypes 1 and 2. It was also found that the nucleotide as well the as amino acid sequences of HVRs among Ad41 isolates in each subtype were of significantly high identity (99–100%). Altogether, the results clearly indicated that two subtypes of Ad41 had been co-circulating in this outbreak. In addition, the majority (91.3%, 21 of 23) of Ad41 infected cases were confined to infants and young children aged less than 3 years (Table 2). This observation demonstrated that Ad41 infection in this outbreak occurred mainly in infants and young children.

4. Discussion

Viral gastroenteritis is still a health burden and one of the most frequently encountered problems in developed and developing countries (Murray and Lopez, 1997). In this study, diarrheal viruses were detected in 30.3% of fecal specimens tested. These findings suggested that about 30% acute gastroenteritis in infants and children in Maizuru City might be due to the diarrheal viruses and 69.7% caused by other etiologic agents. Interestingly, *norovirus* dominated over *group A rotavirus* and became a leading cause of viral gastroenteritis among infants and children in the present study. According to the epidemiological survey (1996–2003) of diarrheal viruses conducted in Maizuru City, the incidence of *group A rotavirus* was always higher than that of *norovirus*, ranging from 10.2% to 23.5% (Zhou et al., 2003; Phan et al., 2005a; Yoshinaga et al., 2006). Taken together, there was a changing predominance of viruses causing diarrheal illness among infants and children in Maizuru City. It was possible that infants and children in Japan might have enough antibody protection against *rotavirus*, which was triggered by the previous group A rotavirus infection. However, it might be due to the co-existence of multiple factors such as changes of climate, water, and others. Further research should be conducted in order to investigate this phenomenon.

In Maizuru City, the detection rate of *adenovirus* infection ranged from 3.8% to 4.8% (Zhou et al., 2003; Li et al., 2004). In this study, it was interesting that *adenovirus* infection was identified with a high incidence (8%) and was recognized as the second common agent of acute gastroenteritis in Maizuru City during 2004–2005. Sequence analysis showed that *adenovirus* detected in this study belonged to three distinct subgenera (B, C and F) with five serotypes (Ad1, Ad2, Ad3, Ad5 and Ad41). Of note, high prevalence (85.2%) of Ad41 with a sudden appearance and disappearance pattern was confined to a short period of 4 months (October 2004 through January 2005) suggesting an outbreak of Ad41 in Maizuru City. By contrast, only one *adenovirus* was found from October to January of previous years (2002–2004) in diarrheal fecal specimens of children collected in Maizuru City, Japan (data not shown).

Table 2
Characteristics of *adenovirus* type 41 outbreak in infants and children with acute gastroenteritis in Maizuru City, Japan

No.	Patient	Age	Sex	Date of stool collection	Laboratory findings			
					Adenovirus	*Adenovirus* type	Subtype	Other virus
1	5918	2 y	M	25 October 2004	+	41	2	–
2	5922	1 y 11 m	M	2 November 2004	+	41	2	–
3	5925	7 m	M	8 November 2004	+	41	2	–
4	5927	1 y 11 m	F	16 November 2004	+	41	2	–
5	5932	10 m	F	24 November 2004	+	41	2	–
6	5933	1 y 5 m	M	24 November 2004	+	41	1	–
7	5934	1 y 1 m	F	24 November 2004	+	41	2	–
8	5935	2 y 9 m	F	24 November 2004	+	41	2	–
9	5938	2 y 2 m	M	25 November 2004	+	41	1	–
10	5943	1 y 7 m	F	2 December 2004	+	41	1	–
11	5950	2 y 4 m	M	11 December 2004	+	41	1	–
12	5954	10 m	F	13 December 2004	+	41	2	–
13	5956	2 y 5 m	F	13 December 2004	+	41	1	–
14	5957	2 y 3 m	M	13 December 2004	+	41	1	–
15	5960	6 y 8 m	M	15 December 2004	+	41	1	–
16	5961	1 y 2 m	F	15 December 2004	+	41	1	–
17	5963	1 y 3 m	F	16 December 2004	+	41	2	*Norovirus*
18	5966	9 y 3 m	M	18 December 2004	+	41	1	–
19	5976	9 m	F	22 December 2004	+	41	2	–
20	5977	3 y 1 m	F	22 December 2004	+	41	1	–
21	5979	3 m	M	24 December 2004	+	41	2	–
22	5980	1 y 2 m	F	24 December 2004	+	41	1	–
23	5993	3 y 6 m	F	6 January 2005	+	41	1	–

Note. M, male; F, female; y, year; m, month; +, positive; –, negative.

This is the first report of an outbreak attributed to Ad41 infection among infants and young children in Maizuru City, Japan. Another interesting feature of the study clearly demonstrated that two distinct Ad41 subtypes, subtypes 1 and 2, were co-circulating in this outbreak.

Although it has been reported that the prevalences of Ad40 and Ad41 were approximately equal (Shinozaki et al., 1991; Phan et al., 2004), none of Ad40 was detected in the present study. However, this finding was in line with recent studies that reported a decrease in the detection rate of Ad40 and a concomitant increase of Ad41 to become the predominant serotype. This phenomenon might reveal the occurrence of an antigenic drift of Ad41. Such changes of antigenicity might have allowed the Ad41 to escape from acquired immunity and cause an increase of Ad41 infection for the susceptible individuals within the community (Van der Avoort et al., 1989; Li et al., 2004).

In this outbreak, the majority of infants and children with Ad41 infection (91.3%) were aged less than 36 months. This observation was consistent with the studies on *adenovirus* epidemiology worldwide in which *adenovirus* infection associated with acute gastroenteritis occurs predominantly in infants and young children (Chiba et al., 1983; Jarecki-Khan et al., 1993; Akihara et al., 2005). Our findings also confirmed *adenovirus* as one of the enteropathogens responsible for viral gastroenteritis among infants and children in Japan. According to some studies conducted in Japan, *adenovirus* infection has been found mainly in summer (Li et al., 2004; Akihara et al., 2005). In contrast, the present study demonstrated the outbreak of *adenovirus* in the cold season, spanning from October 2004 to January 2005. This observation clearly indicates that *adenovirus* infection can occur not only in the hot season but also in the cold season.

In conclusion, this report provided further evidence of the existence of the multiple co-circulating viruses in causing diarrheal illness in Maizuru City, Japan. It is also the first, to our best knowledge, demonstrating an outbreak associated with the *adenovirus* 41 infection in infants and children with acute gastroenteritis in Maizuru City and warns of the threat it poses.

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