Acknowledgements

This study was supported by Grants-in-aid from the Ministry of Education and the Ministry of Health and Welfare, Japan. This study was also supported by the Heiwa Nakajima Foundation, the Sumitomo Foundation and the Kaiun Mishima Memorial Foundation in Japan. We would like to thank Dr. Osamu Nishio, Dr. Kenji Abe from National Institute of Infectious Diseases (NIID), Tokyo and Dr. Hideaki Shimizu from Kawasaki City Institute of Public Health, Kawasaki City, Japan for providing us the positive viral controls in this study.

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Microbiol. Immunol., 49(2), 121–128, 2005

Molecular Epidemiology of Adenovirus Infection among Pediatric Population with Diarrhea in Asia

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Received October 4, 2004; in revised form, November 22, 2004. Accepted December 6, 2004

Abstract: **A total of 3,577 fecal specimens from infants and children with acute gastroenteritis in Japan, Korea and Vietnam during 1998 and 2001 were tested for adenovirus by the ELISA method. Of these, adenovirus was detected in 158 (4.4%). The detection rate of adenovirus was highest in Korea (8.7%, 20/231) followed by 5.0% (100/1,991) in Japan and 2.8% (38/1,355) in Vietnam. All adenoviruses were further serotyped by PCR-RFLP. The diversity of adenovirus serotypes including Ad2, 3, 5, 8, 31, 40 and 41 was demonstrated. Worth of note was a decrease in the rate of isolation of Ad40 (7.6%, 12/158) and a concomitant increase of Ad41 (63.9%, 101/158) to become the predominant serotype. Another interesting feature of the study was the presence of Ad3 (4.0% and 30%) and Ad8 (8.0% and 20%) in Japan and Korea, respectively, which is mainly associated with keratoconjunctivitis worldwide. Our result underscored the importance of adenovirus in association with acute gastroenteritis in Asian countries.**

Key words: **PCR-RFLP, ELISA, Serotype, Enteropathogen**

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to acute gastroenteritis is greater in developing than in the developed countries. Acute gastroenteritis consistently ranks as one of the top six causes of all deaths. Apart from group A rotavirus as the most common cause of acute gastroenteritis, adenovirus is considered to be significant global enteropathogen. This virus is also associated with sporadic outbreaks of acute gastroenteritis worldwide (7, 31).

Human adenovirus belongs to the *Mastadenovirus* of family *Adenoviridae*. Comprehensive controlled studies revealed that the so-called "noncultivable" or "enteric" adenovirus (EAd) is, in contrast to the conventional cultivable "nonenteric" adenovirus (NEAd), which is also commonly detected in fecal specimens, a frequent primary cause of pediatric gastroenteritis (4). Fifty-one human adenovirus serotypes have been distinguished on the basis of their resistance to neutralization by antisera to other known adenovirus serotypes. The various serotypes are classified into six subgenera from A to F based on their hemagglutination reaction with human and animal erythrocytes (10, 12, 15, 27). Epidemiological studies performed in various industrialized countries demonstrated that EAd ranked only second in the etiology of viral gastroenteritis in infants and young children and its positive rate of infection in sporadic cases and outbreaks of acute gastroenteritis ranged from 1.1% to 12.0% (2, 6, 29). Among six subgenera, subgenus F adenoviruses were the most important etiologic agents of severe acute gastroenteritis, accounting for 1 to 20% of cases. They had a global distribution and were of comparable prevalence both in outpatients and hospitalized children in developed and developing countries (14, 18, 21, 25).

The objectives of this study were to determine the incidence of adenovirus infection in infants and children with acute gastroenteritis in Japan, Korea and Vietnam and to characterize the detected viruses into serotype. Additionally, the age-related, and geographi-

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Abbreviations: CPE, cytopathic effect; EAd, "enteric" adenovirus; ELISA, enzyme linked immunosorbent assay; NEAd, "nonenteric" adenovirus; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

cal distributions, as well as seasonal patterns of enteric adenovirus infections, were also described.

Materials and Methods

Fecal specimens. A total of 3,577 fecal specimens were collected from infants and children with acute gastroenteritis from Japan, Korea and Vietnam. These specimens included 1,991 from five places (Tokyo, Osaka, Maizuru, Saga and Sapporo) in Japan from July 1998 to June 2001; 231 from Seoul in Korea from January 1998 to July 1999; and 1,355 from Ho Chi Minh City in Vietnam from December 1999 to November 2000. Japanese and Korean specimens were collected from outpatients. Vietnamese specimens were obtained from hospitalized children. Fecal specimens were prepared as approximately 10% suspension in distilled water, vortexed 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.

Screening of adenovirus by ELISA. Ten percent supernatants of specimens were used for screening of adenovirus by enzyme linked immunosorbent assay (ELISA) with polyclonal antibodies (23). The polyclonal antibodies could detect all adenoviruses regardless of serotype. An 96-well polyvinyl microplate was coated with 100 µl anti-adenovirus rabbit serum diluted 1:8,000 in carbonate buffer (pH 9.6) and kept overnight at 4 C. The plate was washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), and 50 µl PBS and 50 µl supernatant of specimens was added. After shaking for 1 hr at room temperature (RT), the plate was kept overnight at 4 C. The plate was washed three times with PBS-T, and 100 µl guinea pig antiserum against adenovirus diluted 1:8,000 with 1% bovine serum albumin (BSA) in PBS-T, was added to the wells, and then shaken for 1 hr at RT. After washing three times, 100 µl horseradish peroxidase (HRP) conjugated rabbit anti-guinea pig IgG diluted 1:3,000 with 1% BSA in PBS-T was added and the plate was shaken for 1 hr at RT. The plate was washed four times and 100 µl of *O*-phenylenediamine (OPD)-H_2O_2 was added. The plate was shaded for 30 min and the reaction was stopped by 50 µl of 20% sulfuric acid. If the OD value was more than 0.2 as well as 2-fold greater than the negative control well, the specimen was considered positive. Positive specimens were stored at −30 C until use for serotyping with PCR and restriction fragment length polymorphism (RFLP).

Serotyping adenoviruses by PCR-RFLP of hexon gene. Neutralization testing is a typical and golden method to identify adenovirus serotype (10). However, virus isolation culture and formation of cytopathic effect (CPE) usually take at least 2 weeks. It is inefficient for managing a large quantity of clinical specimens. Therefore, in this study, in order to determine the serotypes of adenoviruses detected by screening ELISA, PCR-RFLP analysis was conducted.

Extraction of viral DNA from fecal specimens. Adenovirus DNA for PCR-RFLP was extracted from 10% fecal suspensions by using the glass powder method (30). In a 1.5 ml centrifuge tube, 250 µl of 10% fecal supernatant was added to the same volume of 6 mol/liter guanidine thiocyanate and 6 µl of glass powder (Asahiglass, Tokyo). The mixture was shaken gently at 4 C for 20 min. Then centrifugation was performed at 790×*g* for 1 min and the supernatant was discarded. After washing the sediment with 1 ml of washing buffer for three times, 500 µl of 99.9% ethanol was added to dissolve the sediment, and then centrifuged at 12,637×*g* for 5 min at 4 C. The supernatant was removed and pellet was dried in a vacuum centrifuge at 55 C for 10 min. Then, 30 µl of distilled water was added and the tube was incubated at 65 C for 10 min. After centrifugation at 12,637×*g* for 5 min at room temperature, the DNA-containing supernatant was collected and stored at −30 C until use.

Polymerase chain reaction. Polymerase chain reactions (PCRs) were performed in a 0.5 ml reaction tube in a total volume of 25 µl. They were conducted in two steps: first and second amplifications. Two pairs of primers were used for amplification targeting the downstream conserved region of the hexon gene (26). The primer pair Ad TU 7 and Ad TU 4′ for the first PCR amplified a 1,004 bp product, and the second pair Adn U-S′ and Adn U-A, used for the nested PCR, amplified a 956 bp product.

Three microliters of extracted DNA was heated at 98 C for 15 min, then immediately placed on ice for at least 5 min. Then 22 µl of reaction mixture was added to the tube. The mixture solution consisted of 17.4 µl of distilled water, 2.5 µl of 10-fold *Taq* DNA polymerase reaction buffer, 1 µl of 2.5 mM deoxynucleotide triphosphate mixture (dNTP), 0.1 µl *Taq* polymerase (5 U/µl, Promega Corp., Madison), and 0.5 µl of 33 µmol/liter of each primer. The amplification reaction was performed in a PCR Thermal Cycler TP2000 (TaKaRa, Biotechnology Co., Ltd., Japan) with preliminary denaturation for 3 min at 94 C, followed by 35 cycles of denaturing at 94 C for 1 min, annealing at 45 C for 2 min, and primer extension at 72 C for 3 min, and a final product extension at 72 C for 7 min.

The nested PCR amplification was carried out with 3 µl of the first PCR product, undiluted or diluted 100-fold in distilled water. This PCR amplification was

conducted by using the same conditions used for the first PCR except the primers. After PCR, 4 μl of the amplified product was electrophoresed in 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and photographed under ultraviolet (UV) light.

Restriction fragment length polymorphism (RFLP) analysis. The 956 bp product of the second PCR was digested by the three restriction endonucleases *Eco*T14I, *Hae*III and *Hinf*I (TaKaRa Biotechnology Co., Ltd.) for 3 hr at 37 C, and then electrophoresed (26). In order to determine the serotype, the restriction patterns of the clinical specimens were compared with the standard patterns of adenovirus prototypes.

Nucleotide sequencing and phylogenetic analysis. In order to understand the genetic relationship between Ad41 strains detected from three countries in this study and Ad41-TAK prototype, eight Ad41 representative strains were randomly selected. The nucleotide sequences of PCR products (DNA) positive for Ad41 were determined with a Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc.). Sequence analysis was performed using E-CLUSTAL W (Version 1.6). The nucleotide sequence data for hypervariable regions of the hexon gene from Ad41 strains had been submitted to the DDBJ DNA database and had been assigned accession numbers as follows: Ad41-D1-VN47 (AB103341), Ad41-D4-VN28 (AB103342), Ad41-D12-JP3171 (AB103343), Ad41-D22-Km079 (AB103344), Ad41-D26-JP3106 (AB103345), Ad41-D27-JP2149 (AB103346), Ad41-D25-Ks35 (AB103347) and Ad41-D28-VN1020 (AB103348). Reference strains and accession numbers used in this study were Ade41-TAK (X51783) and Ad40-Dugan (X51782).

Results

Detection of Adenovirus among Three Countries

A total of 3,577 fecal specimens were collected from children with acute gastroenteritis in Japan, Korea and Vietnam during the period of 1998 to 2001. All fecal specimens were tested for the presence of adenovirus by ELISA, based on polyclonal antibodies. The results shown in Table 1 revealed that adenovirus was detected in 158 out of 3,577 (4.4%) specimens tested. Among the three countries in this study, the detection rate of adenovirus was highest in Korea (8.7%, 20/231) followed by 5.0% (100/1,991) in Japan and 2.8% (38/1,355) in Vietnam.

Serotype Distribution of Adenovirus

All of the 158 adenoviruses detected by screening ELISA were further serotyped by PCR-RFLP analysis

Table 1. Serotype distribution of enteric adenovirus by ELISA and PCR-RFLP in Japan, Korea and Vietnam

Country	City	Date of fecal collection	No. (%) of specimens								No. (%) of Ad positive	No. of specimens
			Ad41	Ad40	Ad2	Ad3	Ad8	Ad5	Ad31	ND		
Japan	Five places[a]	07 1998–06 2001	66 (66.0)	2 (2.0)	9 (9.0)	4 (4.0)	8 (8.0)	7 (7.0)	1 (1.0)	3 (3.0)	100 (5.0)	1,991
Vietnam	Ho Chi Minh	12 1999–11 2000	25 (65.8)	10 (26.3)	1 (2.6)	0	0	1 (2.6)	0	1 (2.6)	38 (2.8)	1,355
Korea	Seoul	01 1998–07 1999	10 (50.0)	0	0	6 (30.0)	4 (20.0)	0	0	0	20 (8.7)	231

Ad, adenovirus; ND, serotype of specimens could not be determined.
[a] Tokyo, Osaka, Maizuru, Saga and Sapporo.

(Table 1). In the three countries, the serotype distribution of adenovirus was different. In Japan, 66 out of 100 (66.0%) were typed as Ad41, the most common serotype, followed by Ad2 (9/100, 9.0%) (Fig. 1). Other serotypes including two Ad40 s were also identified as being co-circulating. In Vietnam, Ad41 was the most frequently detected adenovirus serotype (25/38, 65.8%). The incidence of Ad40 as the second common serotype of adenovirus associated with acute gastroenteritis was 26.3% (10/38). In Korea, among positive adenoviruses, Ad41 was also predominant (10/20, 50.0%) followed by Ad3 (6/20, 30.0%) and Ad8 (4/20, 20.0%). No Ad40 was found in these subjects. Meanwhile, four specimens positive for adenovirus remained untypeable because the amount of Ad DNA was too small for PCR-RFLP.

Age Distribution of Adenovirus in Japan and Vietnam

Detailed data for Korean patients could not be obtained. Therefore, only age and monthly distributions of adenovirus infection in Japan and Vietnam were compared. In Japan, adenovirus was detected throughout the year at a frequency of 2.6–15.4% (Fig. 2). The detection rate was highest in July and the lowest

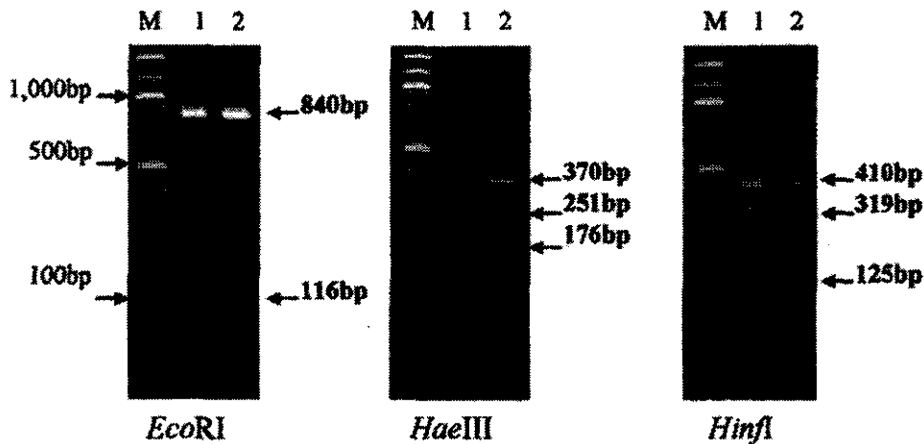

Fig. 1. PCR-PFLP of an adenovirus positive sample. M, 100 bp DNA molecular marker; Lane 1, Ad 41 positive control; lane 2, a Vietnamese isolate from a clinical fecal specimen analyzed with *EcoT14I*, *HaeIII* and *HinfI* (TaKaRa Biotechnology Co., Ltd.).

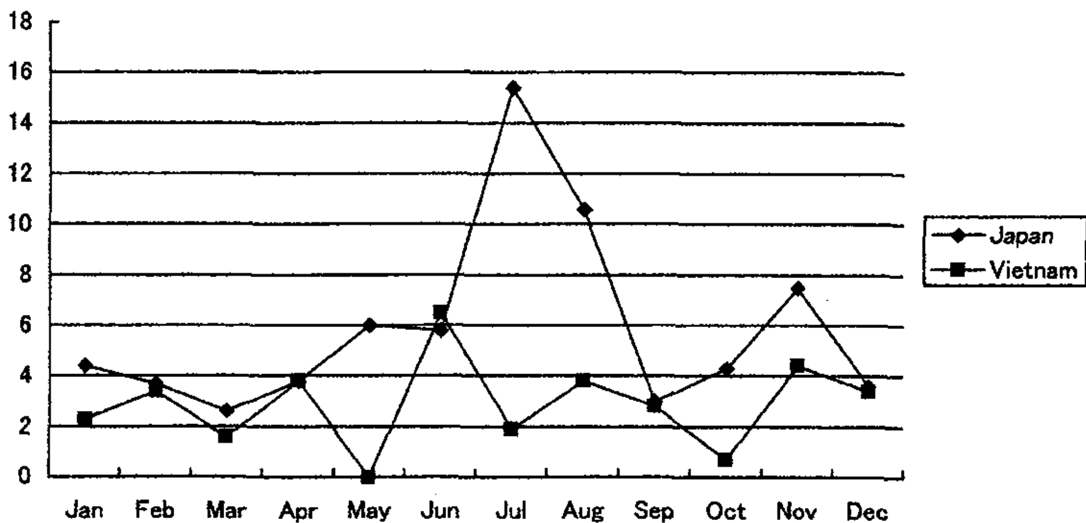

Fig. 2. Monthly distributions of adenovirus identified in fecal specimens collected from infants and children in five places (Tokyo, Osaka, Maizuru, Saga and Sapporo) in Japan and in Ho Chi Minh City, Vietnam, during 1998 and 2001.

rate was in March. In Vietnam, adenovirus was also identified every month except in May and was found highest in June (Fig. 2). The patients in the present study were divided into 5 age groups: <6 months, 6–11 months, 12–23 months, 24–36 months and >36 months of age. The data for the adenovirus rate in each age group in Japan and Vietnam are shown in Table 2. In Japan, the highest incidence group was the 24–36 month old (7.9%), and the second highest fell into infants less than 6 months old (5.4%). Similarly, the adenovirus infectious rate was also highest in the 24–36 month old group (6.7%) in Vietnam but followed by the

Table 2. Age distribution of infants and children infected with adenovirus in Japan and Vietnam

		Age group						
		0–5 months	6–11 months	12–23 months	24–36 months	>36 months	ND[a]	Total
Japan	No. of infants and children	149	354	655	392	328	113	1,991
	No. (%) of adenovirus positive	8 (5.4)	18 (5.1)	32 (4.9)	25 (6.4)	9 (2.7)	8 (7.1)	100 (5.0)
Vietnam	No. of infants and children	235	509	490	89	32	0	1,355
	No. (%) of adenovirus positive	5 (2.1)	8 (1.6)	17 (3.5)	7 (7.9)	1 (3.1)	0	38 (2.8)

[a] ND: age of patients was not recorded.

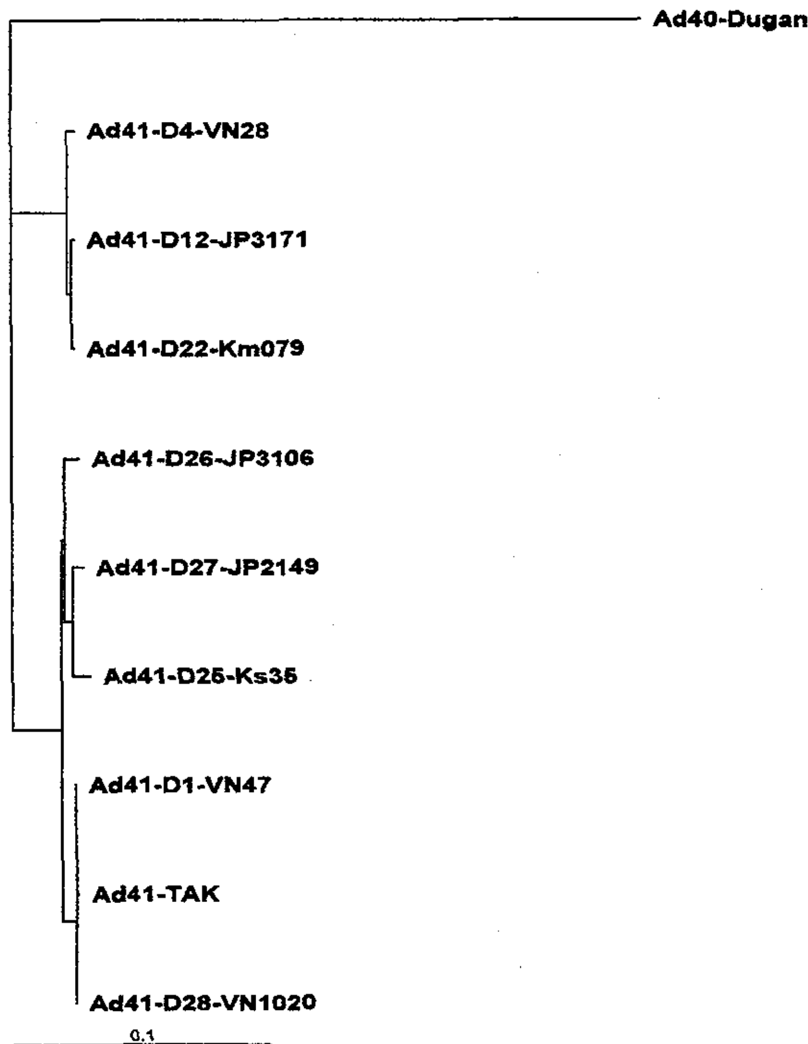

Fig. 3. Phylogenetic tree of nucleotide sequences of 8 isolates of Ad41. The tree was constructed from nucleotide sequences of 8 isolates of Ad41 detected in Japan, Vietnam and Korea. Reference strain of Ad41-TAK was selected from DDBJ/GenBank under the accession number indicated in the text. Ad41-Dugan strain was used as an out-group strain for phylogenetic analysis.

12–23 month age range. Taken together, the adenovirus infectious rate was highest in the 24–36 month old group (6.7%) and lowest in children over 36 months of age (2.8%). Surprisingly, infants under 6 months of age in both countries had a higher rate of viral infections than the group of 6–11 months old.

Nucleotide Sequence and Phylogenetic Analyses of Ad41 Isolates

The PCR products of their hexon gene were sequenced in order to further characterize the genetic relationship among the 8 isolates of Ad41 randomly selected in infants and children in Japan, Vietnam and Korea. A phylogenetic tree of the nucleotide sequences of these isolates and the reference strain was constructed (Fig. 3). Their nucleotide sequences were compared to each other as well as to the Ad41-TAK prototype available in the GenBank database by BLAST. It was found that the nucleotide sequence identity among the 8 Ad41 isolates ranged from 94% to 100%. Moreover, identities of 94%–99% at the nucleotide sequence level among these Ad41 strains and the Ad41-TAK prototype were also noted.

Discussion

Adenovirus is one of the enteropathogens responsible for viral gastroenteritis among infants and children in developing and developed countries. Even though numerous reports of adenovirus infection in many countries have been published, the specific epidemiological data for illness caused by this virus in Korea and Vietnam were very limited. In this study, adenovirus was detected in 4.4% of fecal specimens tested. This finding suggested that about 4.4% of acute gastroenteritis in infants and children in Japan, Korea and Vietnam was due to adenovirus and 95.6% caused by other etiologic agents. This result was consistent with previously published reports worldwide and confirmed adenovirus as one of the important etiological agents for acute gastroenteritis among infants and children (13, 14). Among all children with acute gastroenteritis, 88.9% were aged less than 36 months. Adenovirus was detected mostly in the under 36-month age range. The highest incidence of cases fell into the 24–36 month old group, and the rate of incidence decreased with an increase at age over 36 months. It was worthy of note that infants under 6 months of age had a rather higher rate of viral infections than young children in the 6–11 month old range, especially in Vietnam. Perhaps because of limited breastfeeding or for other reasons such as rapid socio-economic change, marketing by formula manufacturers, and some traditional perceptions about childbirth (22), these infants might not have enough maternally acquired passive antibody against viral enteropathogens. Studies conducted in Vietnam showed a decline in breastfeeding because of the workload of mothers (9, 16, 20). At hospitals and clinics in Vietnam, some infant formula companies continued to give out free samples and to distribute posters and calendars promoting their formulas, so many women in Ho Chi Minh City, Vietnam, preferred to give their babies formula instead of breastfeeding. As a result, the prevalence of breastfeeding was relatively low in Ho Chi Minh City, Vietnam (20).

The results of this study were in line with the previous findings that adenovirus infection occurred throughout the 12-month period (11, 24). However, Shinozaki et al. reported the early autumn as a peak season for adenovirus infection in Japan in 1991. In the present study, it was interesting to note that the adenovirus incidence rates were highest in July and August, known as summer. The climate in Ho Chi Minh City, Vietnam, is distinctively seasonal. The dry season lasts from November to April, the hottest months are from February to May and the temperature may rise over 37 C. The rainy season characterized by sudden heavy rain begins in May and ends in October. The wettest months are from June to September. In this study, no specific seasonal pattern was presented even though adenovirus was observed to be the most prevalent in June. To date, only one study on adenovirus in Vietnam was reported by Landaeta et al. (19) in which just two strains (Ad40/41) were identified in feces from Vietnamese diarrheal children during September and December 2000. This finding also was very different from the previous studies in other tropical countries in Asia. Jarecki-Khan et al. (17) presented a seasonal increase in adenovirus infection during the dry cool months in Bangladesh. The study conducted in India, where the climate is similar, found adenovirus to be more common in the wet warm months of August to September (3).

Early surveys showed that the prevalence of Ad40 and Ad41 remained approximately equal (11, 17). However, the findings in the present study were in line with recent studies that reported a decrease in the rate of isolation of Ad40 and a concomitant increase of Ad41 to become the predominant serotype associated with acute gastroenteritis. It was found that the homology between nucleotide sequences of Ad41 isolates in the present study and the Ad41-TAK prototype was down to 94%. This phenomenon might reveal the occurrence of antigenic drift for Ad41 (32). Such changes of antigenicity might allow the Ad41 to escape from acquired immunity and cause an increase of Ad41 infection for

susceptible individuals within the community (5, 32).

The results indicated that Ad40/41 were dominant serotypes causing acute gastroenteritis among children in three countries. It was also found that a wide range of other adenovirus serotypes in this study such as Ad2, 3, 5, 8, 31 were also identified as being co-circulating. Up to now, there are only few reports on enteric Ad40/41 conducted in Korea and Vietnam. Therefore this was the first research on molecular epidemiology of enteric adenovirus infection other than Ad40/41 in three countries in Asia, especially in Korea and Vietnam.

Human adenovirus causes a variety of diseases such as acute respiratory, ocular, and gastroenteritis infections. Until now, 51 serotypes have been identified. Among them adenovirus type 3, 4, 7, 8, 19 and 37 have been found to be responsible for keratoconjunctivitis worldwide (1, 8). Interestingly, in the present study Ad3 was detected with the rather high incidences 4.0% and 30% and Ad8 with 8.0% and 20.0% in Japan and Korea, respectively. This finding clearly indicated that "nonenteric" adenovirus also had played an important role in causing acute gastroenteritis in infants and children. In conclusion, these data have described wide diversity among adenovirus serotypes co-circulating in Asian countries and increased the evidence for the worldwide distribution of this virus.

This study was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare, Japan. This study was also supported by the Heiwa Nakajima Foundation and the Sumitomo Foundation in Japan.

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Arch Virol (2005) 150: 371–377
DOI 10.1007/s00705-004-0401-0

Archives of Virology
Printed in Austria

Genetic diversity of sapovirus in fecal specimens from infants and children with acute gastroenteritis in Pakistan

Brief Report

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Received June 16, 2004; accepted July 14, 2004
Published online October 11, 2004 © Springer-Verlag 2004

Summary. A total of 517 fecal specimens collected from infants and children with acute gastroenteritis in Karachi city, Pakistan during 1990–1994 were examined for the presence of sapovirus by RT-PCR and sequence analysis methods. Sapovirus was identified in 17 of 517 (3.2%) specimens. Sapovirus was further clustered into three distinct genogroups (I, II and IV) and these presented 70.6%, 23.5% and 5.9%, respectively. Our results clearly indicated that sapovirus could be classified into 7 GI and 4 GII genotypes. It was noteworthy to point out that sapovirus detected among Pakistani infants and children with acute gastroenteritis demonstrated the great genetic diversity and presented novel sapovirus genotypes.

*

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to acute gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis consistently ranks as one of the top six causes of all deaths. Apart from rotavirus as the major etiologic agent of gastroenteritis in children and young animals, sapovirus is considered to be a significant global enteropathogen [6, 7].

Sapovirus was first named after its discovery in an outbreak of acute gastroenteritis in a home for infants in Sapporo, Japan in October 1977 [1]. They have a typical "Star of David" configuration by EM and are antigenically identical to each other by immune EM [2]. Sapovirus contains a positive sense single-strand RNA genome surrounded by an icosahedral capsid. Sapovirus genome contains

two ORFs (ORF1 and 2); ORF 1 encodes the non-structural as well as the capsid proteins. Based on the sequence analysis of the capsid gene, sapovirus is divided into three genogroups (I, II and IV) and currently genogroup V as known to infect human [3, 9, 15]. Moreover, porcine enteric calicivirus (PEC) is reported to be a member of genogroup III [4].

Etiologic studies of acute gastroenteritis in infants conducted in Japan with sensitive reverse transcription–PCR methods showed that sapovirus was one of the most causes of outbreaks of viral gastroenteritis among infants in Sapporo [1, 2]. This virus also is associated with sporadic outbreaks of gastroenteritis worldwide and is recognized just as important as rotavirus infecting children less than 2 years of age in Finland [10, 11]. Recently, seroepidemiologic studies have indicated a worldwide distribution of sapovirus [6, 7]. The aged-related prevalence of antibody against this virus also has shown that infections commonly occur in children less than 5 years old. The pattern of acquisition of the antibody is similar to that of other common virus infection [14].

The objectives of this study were: to analyze the epidemiology of sapovirus among Pakistani pediatric population with acute gastroenteritis, to characterize the detected sapovirus according to genogroup and genotype, and to describe the genetic diversity of sapovirus.

Five hundreds and seventeen fecal specimens were collected from infants and children with acute gastroenteritis in the Civil Karachi Hospital, Dow Medical College, Karachi city, Pakistan during the period of January 1990 to December 1994. All fecal specimens were determined previously to be negative for rotavirus and adenovirus [8]. These specimens were diluted with Eagle's minimum essential medium to 10% suspensions, and clarified by centrifugation at 5000 g for 20 min. The supernatants were collected and stored at −30 °C until use for the detection of sapovirus. The viral genome was extracted from 140 μl of a 10% fecal suspension using a spin column technique according to the instructions in the QIAamp® Viral RNA Mini Kit Handbook, Germany. Sapovirus was detected by RT-PCR analysis of extracted viral RNA with specific primers previously published [19]. Briefly, a pair of published primers (SLV5317 and SLV5749) for amplifying capsid region of sapovirus was used. The PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C [19]. PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr, 5 mg/ml) for 20 min then visualized under ultraviolet (UV) light, and the results were recorded by photography. The nucleotide sequences of PCR products (DNA) positive for sapovirus 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 E-CLUSTAL W (Version 1.6). Reference strains and accession numbers used in this study are as follows: Southampton (L07418), PEC (AF182760), Bristol/89 (AJ249939), Lyon/598/97/F (AJ271056), London/92 (U95645), Mex340/90 (AF435812), Cruise ship/00 (AY289804), Hou7-1181/90 (AF435814), Arg39 (AY289803), Stockholm/97 (AF194182), Mex14917/00 (AF435813), Houston/90 (U95644), Parkville/94 (U73124),

Houston/86 (U95643), Sapporo/82 (U65427), Manchester/93 (X86560), Plymouth/92 (X86559), Lyon/30388/98 (AJ251991), Southampton (L07418).

Out of 517 fecal specimens collected from infants and children with acute gastroenteritis in Karachi city, Pakistan, 168 were collected in 1990; 86 in 1991; 76 in 1992; 99 in 1993; and 88 in 1994. For the pediatric population, the lowest age was under 1 month, the highest was 5 years, and the average age was 1 year (12 months). Moreover, the number of male (60.9%) was higher than that of female (39.1%). All fecal specimens were tested for the presence of sapovirus by RT-PCR. The results shown in Table 1 revealed that sapovirus was detected in 17 out of 517 (3.2%) specimens tested. Sapovirus was identified in each of the five years. The highest incidence fell into 1991 (29.4%, 5 of 17) and the lowest in 1993 (5.9%, 1 of 17). The age at sapovirus infection ranged from 5 months to 3 years. The viral infectious rate was highest in the 6–11 months old group (41.2%) and lowest in children over 35 months or less than 6 months of age (5.9%). Moreover, the number of male infected sapovirus with acute gastroenteritis (58.8%) was higher than that of female.

A total of 17 sapovirus amino acid sequences were analyzed by phylogenetics and grouped using the recent sapovirus capsid region classification scheme of Farkas et al. (2004). Majority of the sapovirus sequences clustered into two distinct genogroupes I and II (GI, GII). Interestingly, one of the sequenced specimens positive for sapovirus, Karachi/730/1992, turned out to belong to a cluster called

Table 1. Characteristics of seventeen sapovirus infections among infants and children with acute gastroenteritis in Karachi city, Pakistan during 1990 and 1994

No.	Patient	Year	Month	Sex	Age	Isolate	Genogroup	Genotype	Accession No.
1	877	1990	August	M	3 y	Karachi/877/1990	GI	5*	AB181133
2	878	1990	August	F	5 m	Karachi/878/1990	GI	6*	AB181228
3	953	1990	September	M	1 y	Karachi/953/1990	GII	1	AB181131
4	1017	1990	October	F	2 y	Karachi/1017/1990	GI	5*	AB181227
5	824	1991	January	M	1 y	Karachi/824/1991	GII	1	AB181130
6	842	1991	May	F	8 m	Karachi/842/1991	GI	1	AB181232
7	872	1991	July	F	6 m	Karachi/872/1991	GI	4*	AB181231
8	934	1991	October	M	9 m	Karachi/934/1991	GI	1	AB181247
9	937	1991	October	M	2 y	Karachi/937/1991	GI	6*	AB181229
10	874	1992	March	M	2 y	Karachi/874/1992	GII	4*	AB181129
11	730	1992	August	M	10 m	Karachi/730/1992	GIV#	N/A	AB126249
12	1021	1992	August	F	9 m	Karachi/1021/1992	GI	4*	AB181230
13	1026	1992	September	F	9 m	Karachi/1026/1992	GI	5*	AB181134
14	876	1993	September	F	1 y	Karachi/876/1993	GI	7*	AB181132
15	928	1994	March	M	1 y	Karachi/928/1994	GII	4*	AB181128
16	938	1994	April	M	9 m	Karachi/938/1994	GI	1	AB181248
17	997	1994	July	M	1 y	Karachi/997/1994	GI	1	AB181233

Note. *No.*, Number; *M*, Male; *F*, Female; *y*, Year; *m*, Month; #Rare genogroup
*New genotype; *N/A*, Not available

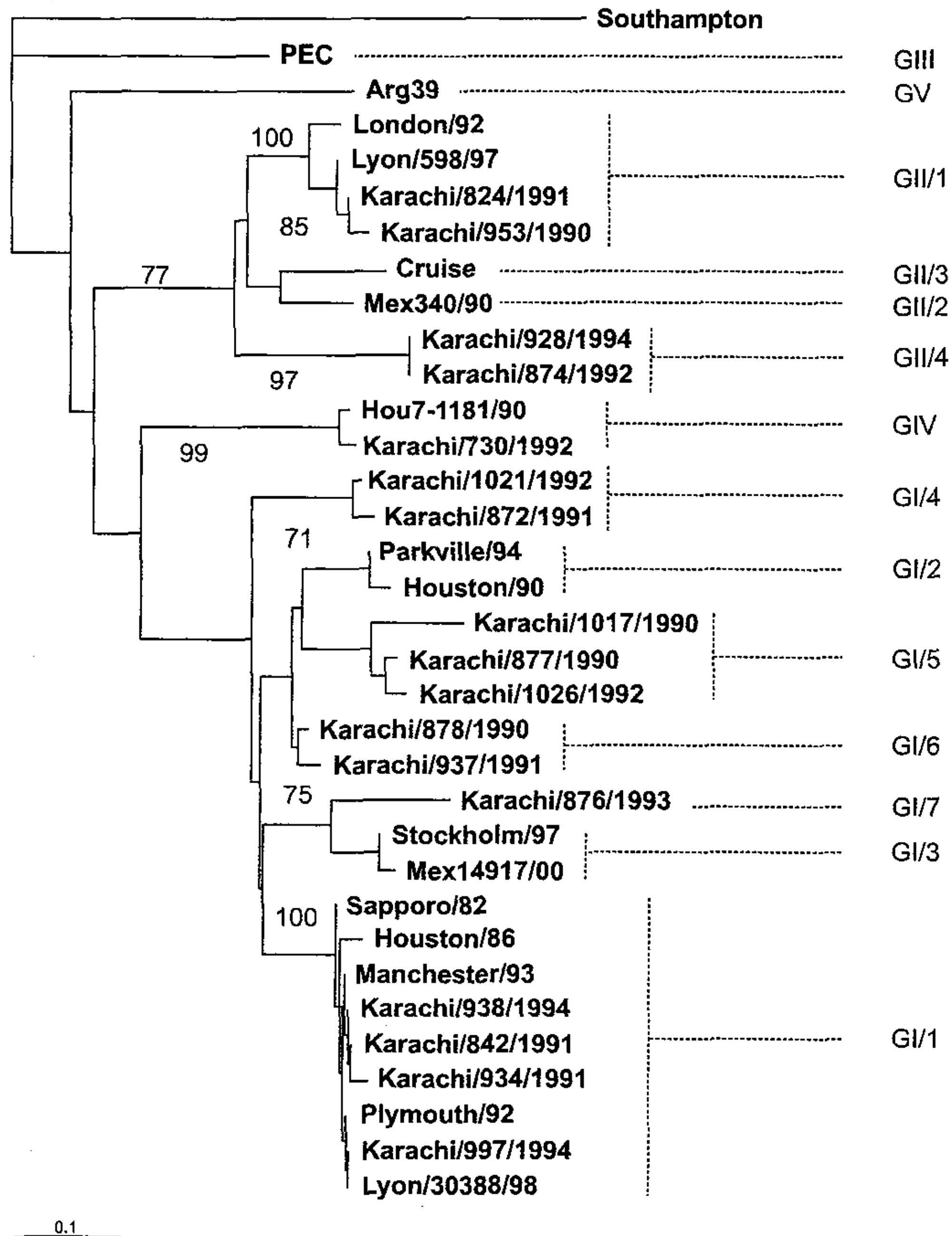

Fig. 1. Phylogentic tree of amino acid sequences of 17 isolates of sapovirus based on the recent sapovirus capsid region classification scheme of Farkas et al. [3]. The tree was constructed from partial amino acid sequences of the capsid region of the seventeen sapoviruses detected in Karachi city, Pakistan during the period of 5 years (1990 through 1994). The numbers in the branches indicate the bootstrap values. Reference strains of sapovirus were selected from DDBJ/GenBank under the accession number indicated in the text. Southampton was used as an out-group strain for phylogenetic analysis

a genogroup IV (known as the Hou7-1181/90 virus). The nucleotide identity between Karachi/730/1992 and Hou7-1181/90 was 96%, and the amino acid identity was 97%.

Most of the sapovirus sequences (70.6%, 12 of 17) belonged to sapovirus GI (known as the Manchester virus cluster). Our results indicated that sapovirus group I was a dominant genogroup. The sapovirus GI sequences clustered into one distinct GI genotype, GI/1 (typified by the Sapporo/82 virus cluster) (33.3%, 4 of 12). In addition we also identified eight isolates (3 in 1990, 2 in 1991, 2 in 1992 and 1 in 1993), forming four novel sapovirus GI genotypes tentatively called GI/4, GI/5, GI/6 and GI/7, respectively (Fig. 1). These sapoviruses had a low identity on the amino acid with other reference strains in the same genogroup ranged from 65% to 86%.

Our findings showed that two (50%) of the sapovirus GII sequences were classified into GII/1 (known as the Lyon/598/97 virus). Interestingly, two isolates named Karachi/928/1994 and Karachi/874/1992 in the present study did not belong to any the genetic clusters and presented a novel sapovirus GII genotype tentatively called GII/4. The sequences with the closest matches to these isolates were from strain isolated in France (Lyon/598/97), showing only 73% amino acid identity.

Sapovirus is one of the leading causes of infantile viral gastroenteritis and also is associated with sporadic outbreaks of gastroenteritis worldwide. In this study, sapovirus was detected in 3.2% fecal specimens tested. These findings suggested that acute gastroenteritis in infants and children in Karachi, Pakistan about 3.2% might be due to sapovirus and 96.8% caused by other etiologic agents. Among all children with acute gastroenteritis due to sapovirus, 94.1% were aged less than 36 months. This result was consistent with previously published reports on epidemiology of sapovirus worldwide in which the prevalence was shown to be 0.3–9.3% [5, 9, 16–18]. It also confirmed sapovirus as one of the enteropathogens responsible for viral gastroenteritis among infants and children worldwide. Furthermore, the highest incidence was the 6–11 month old group, the lowest fell into the infants aged less than 6 months, and the rate of incidence decreased with increasing age over 1 year. Quite possibly, children aged from 6 to 11 months might lack antibody protection to sapovirus, whereas by the time children have reached 1 year old they have begun to acquire viral immunity.

The climate in Karachi city, Pakistan is distinctively seasonal. The summer lasts from April to July, and the hot temperature may reach over 37 °C. The cold season characterized by less rain begins in August and ends in March. The coldest month is January when the temperature may dip as low as 5 °C. In the present study, almost sapoviruses were found in August, September and October. Our results were in line with previous reports of other investigators that sapovirus was observed to prevail in the cold winter months [9, 12, 13, 18].

Up to date, numerous molecular epidemiological studies have revealed a global distribution of sapovirus. However, the genetic analysis on sapovirus in Pakistani children with acute gastroenteritis is not available. The results in this study showed

the majority of Pakistani sequences belonged to two distinct sapovirus genogroups I and II. Of these, the positives were further classified into one sapovirus GI genotype and one sapovirus GII genotype according to the recent sapovirus capsid region classification scheme of Farkas et al. (2004). Moreover, other eight sapoviruses did not cluster with any the published references and presented four novel sapovirus genotypes tentatively called GI/4, GI/5, GI/6, GI/7 and GII/4, respectively. These sapoviruses had a low identity on the nucleotide as well as the amino acid with other reference strains in the same genogroup previously registered in the DDBJ DNA database. It was noteworthy that sapovirus could be classified into 7 GI and 4 GII genotypes.

Another interesting feature was a high identity at the nucleotide and the amino acid between Karachi/730/1992 and only one representative of a genogroup IV, Hou7-1181/90. Both of them, which revealed unique sequences distinct from known sapoviruses in the DDBJ, were recovered from fecal specimens over 10 years ago. Possibly, these special strains might be emerging in the past. Taken together, our findings clearly indicated that sapovirus strains co-circulating among infants and children with acute gastroenteritis in Karachi city, Pakistan demonstrated the great genetic diversity. Additionally, these data have described the molecular epidemiology as well as the importance of sapovirus causing acute gastroenteritis in Pakistan and increased the evidence for the worldwide distribution of this virus. This is the first indication on molecular epidemiology of sapovirus infection conducted in Karachi city, Pakistan, showing the genetic diversity among them.

Acknowledgements

This study was supported by Grants-in-aid from the Ministry of Education and the Ministry of Health, Labor and Welfare, Japan. This study was also supported by the Heiwa Nakajima Foundation and the Sumitomo Foundation in Japan.

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Journal of Medical Virology 75:475–481 (2005)

Outbreak of Acute Gastroenteritis Associated With Group A Rotavirus and Genogroup I Sapovirus Among Adults in a Mental Health Care Facility in Japan

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An outbreak of acute gastroenteritis consisting of 57 cases occurred in a mental health care facility in Takasaki city, Japan during 6th February and 27th March 2002. A total of 18 fecal specimens collected from 17 residents and one member of the medical staff during this outbreak were tested for the presence of viral enteropathogens by RT-PCR and latex agglutination. Group A rotavirus and sapovirus were detected in 5 out of 18 fecal specimens (55.6%). To our knowledge, this is the first finding of an outbreak of gastroenteritis associated with co-circulation of different kinds of viruses such as group A rotavirus and sapovirus. All of group A rotaviruses were typed further as P[4]G2 strains. Both rotavirus and sapovirus were subjected to molecular analysis by sequencing. It was noteworthy that all rotaviruses and sapoviruses had high homologies, respectively, to each other and sapoviruses presented a potential novel sapovirus genogroup I (GI) genotype, which was obviously different from any GI genotypes (GI-a, b, c, and d). The outbreak associated with these viruses spread gradually from dormitory to dormitory, suggesting a spread by person-to-person contact, although investigation on the route of transmission of the outbreak is lacking. The findings confirm the presence of group A rotavirus and sapovirus are important in acute gastroenteritis among adults in Japan. ***J. Med. Virol. 75:475–481, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: multiplex PCR; genogroup; enteropathogen

INTRODUCTION

Acute gastroenteritis is a major cause of morbidity and mortality among infants and young children in both developed and developing countries. Among other enteropathogenic viruses, rotaviruses are recognized as the major etiologic agents of gastroenteritis in children and young animals. Rotaviruses are classified into seven groups (A–G) on the basis of distinct antigenic and genetic properties [Bridger, 1994; Saif and Jiang, 1994]. Human infection has been reported with group A, B, and C rotaviruses. Of these, group A rotaviruses is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide [Kapikian et al., 2001]. The two outer capsid proteins (the glycoprotein VP7; the protease-sensitive protein VP4) of rotavirus particle identify G and P serotypes, respectively [Estes, 2001]. To date, at least 15 G-types and 21 P-types have been found in humans and animals [Bridger, 1994; Pang et al., 2000]. Apart from rotavirus as the major etiologic agent of gastroenteritis in children and young animals, sapovirus is considered to be a significant global enteropathogen. Etiologic studies of acute gastroenteritis in infants conducted in Japan with sensitive reverse transcription-PCR methods showed that sapovirus was one of the most common causes of outbreaks of viral gastroenteritis among infants in Sapporo [Chiba et al., 1979, 2000]. This virus is also associated with sporadic outbreaks of gastroenteritis worldwide and is recognized just as important as

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology, Japan; Grant sponsor: Ministry of Health, Labor and Welfare, Japan; Grant sponsor: Sumitomo Foundation, Japan; Grant sponsor: Japanese Food Hygiene Association.

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Accepted 23 November 2004
DOI 10.1002/jmv.20292
Published online in Wiley InterScience (www.interscience.wiley.com)

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rotavirus infecting children under 2 years of age in Finland [Pang et al., 1999, 2000]. Numerous molecular epidemiological studies have shown a global distribution of these viruses in infants and children worldwide. However, specific epidemiological data for illness caused by group A rotavirus, especially sapovirus among adults in Japan are rare. The objectives of this study were to detect enteropathogenic viruses causing an outbreak of acute gastroenteritis in a mental health care facility in Takasaki city, Japan and to characterize the viruses.

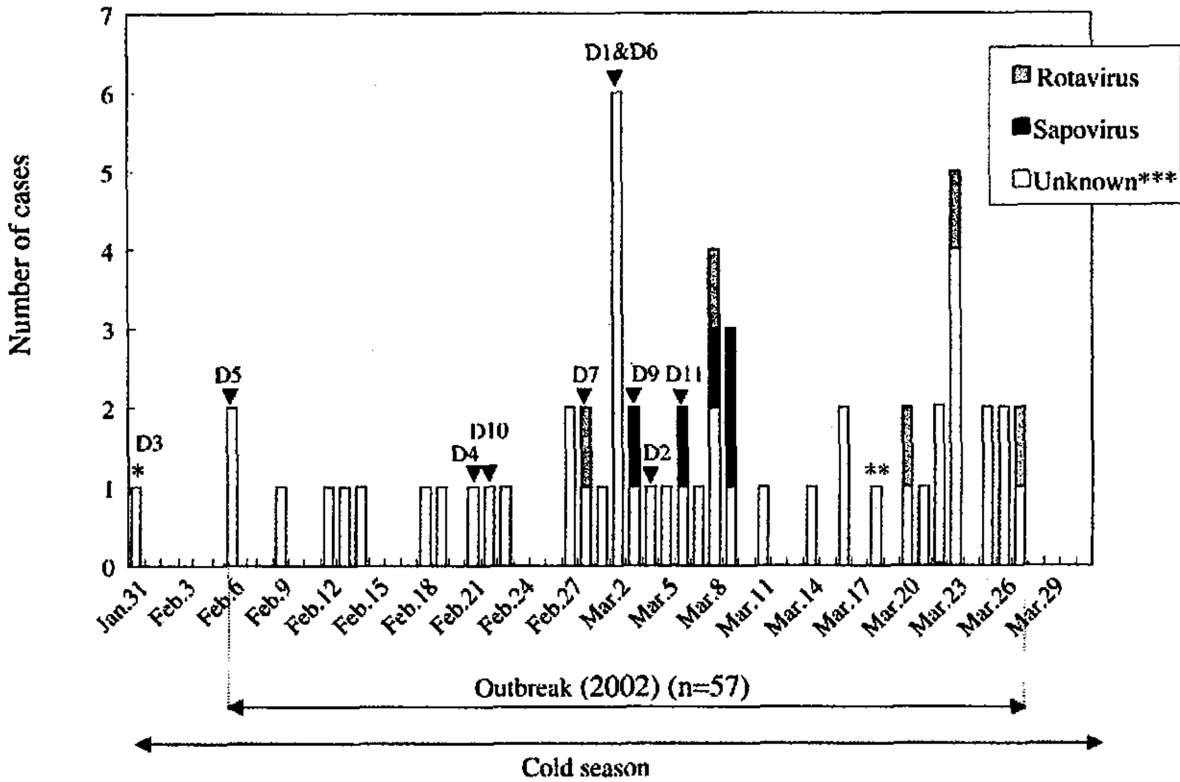
Description of the Outbreak

During 6th February to 27th March 2002 an outbreak of acute gastroenteritis consisting of 57 cases occurred in a mental health care facility in Takasaki city, Japan. The facility includes 11 separate dormitories (termed as D1–D11 in this study) and each has about 22–25 adult persons with mental disorder. Out of 258 residents aged from 36 to 81 years in the facility, 56 (21.7%) persons experienced one episode of acute gastroenteritis with a total of 56 episodes. All dormitories except D8 had residents who reported the symptoms of acute gastroenteritis. One healthcare worker in this facility had symptoms of gastroenteritis during the outbreak. These 57 cases became ill with symptoms including diarrhea (100%), nausea (21.1%), and fever (33.3%). The highest duration of symptoms was 4 days, the lowest was 1 day and the mean was 1.4 days. The index case, a 52-year-old female in D3, had an onset with diarrhea and nausea on 31st January 2002 (Fig. 1). The first cases of this outbreak, one 51-year-old female in D3 and one 50-year-old male in D5, were reported on 6th February 2002. Three days later, the second case (male in D5, aged 36 years) occurred with diarrhea. Since then other residents developed symptoms of acute gastroenteritis gradually from dormitory to dormitory (Fig. 1). The largest number of reported cases fell into around the fourth week of the outbreak. Overall, the outbreak showed a relatively slow pattern of progression.

MATERIALS AND METHODS

Fecal Specimens

A total of 18 fecal specimens were collected from 17 residents, from whom single diarrhea fecal specimen



Note: *, index case; **, single staff case; ***, cases which include rotavirus-negative, sapovirus-negative, and not tested due to whom single fecal specimen was unavailable.

Fig. 1. Epidemic pattern of the outbreak of acute gastroenteritis among adults associated with group A rotavirus and sapovirus in a mental health care facility in Takasaki city, Japan during 6th February and 27th March 2002. A single staff case was included in the outbreak with a total of 57 episodes. Arrow and number (D1–D7, D9–D11) upper the bar indicate date of the first onset in each dormitory. The epidemic shifted gradually in sequence from dormitory to dormitory. No case of gastroenteritis illness was reported in dormitory 8 (D8). Cold season which including the epidemic period was also indicated.

was available, and one member of the staff. These specimens were tested for the presence of rotaviruses, noroviruses, sapoviruses, astroviruses, and adenoviruses. 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 Genomes

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

Multiplex RT-PCR

Multiplex RT-PCR was conducted for detecting two groups of diarrhea viruses, in which, the first group of viruses including human astrovirus, norovirus (GI, GII), and sapovirus and the second group including group A, B, and C rotavirus and adenovirus. The identification of the first group of viruses was performed by using multiplex RT-PCR with specific primers as reported previously [Yan et al., 2003]. Briefly, G1-SKF/G1-SKR, and COG2F/G2-SKR for norovirus genogroup I and II, respectively, SLV5317/SLV5749 for sapovirus and PreCAP1/82b for astrovirus. All of the four primer pairs amplify the capsid region of target viral genome, produce four size-specific amplicons of 330, 387, 434, 719 bp for norovirus genogroup I and II, sapovirus and astrovirus, respectively. In order to detect the second group of viruses, after viral extract was heated at 94°C for 3 min followed by the RT step described elsewhere [Yan et al., 2003], four pairs of published primers (Beg9 and VP7-1′, B5-2 and B3-3, G8NS1 and G8NA2 for amplifying *VP7* gene of human group A, B, and C rotaviruses, respectively; Ad1 and Ad2 for hexon gene of all species from A to F adenoviruses) were used [Gouvea et al., 1990; Xu et al., 2000; Kobayashi et al., 2001; Kuzuya et al., 2003]. These primers were specifically generated four different sizes 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 of 94°C 30 sec, 55°C 30 sec, 72°C 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C.

G and P Serotyping RT-PCR

Group A rotaviruses were characterized further for G and P serotypes by using the method described previously by Gouvea et al. [1990] and Gentsch et al. [1992], respectively. Briefly, for the protocol of RT-PCR for G serotyping, the RNA of rotavirus was reverse transcribed and then further amplified with Beg9 and End9, generating a full-length of *VP7* gene in 1,062 bp. The nested PCR was carried out using the first PCR product as the template with G-serotype specific mixed primers (BT1, CT2, ET3, DT4, and FT9) for upstream priming and End9 for downstream priming in an amplification of *VP7* genes of G1 to G4, and G9, respectively [Gouvea et al., 1990]; moreover, for the P serotyping RT-PCR, the RNA of rotavirus was reverse transcribed and then amplified further with Con2 and Con3 primers for the amplification of the *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 used for identification of P[8], P[4], P[6], P[9], P[10], and P[11], respectively [Gentsch et al., 1992].

Latex Agglutination Test

A latex agglutination test was used as a confirmation test for the detection of group A rotavirus by a commercial Rotalex kit (Daiich Kagaku Co., Ltd., Tokyo, Japan). Fecal samples were processed according to the manufacturer's instructions. A drop of the fecal supernatant was mixed with a drop of test latex on a slide, and reaction was observed after 2 min. Development of distinct agglutination in the Rotalex reagent was treated as positive. If agglutination was seen in the negative control latex, the test was considered uninterpretable.

Monoplex PCR for Amplification of Sapovirus Polymerase Region

Sapovirus positives by multiplex PCR in fecal specimens were examined further by monoplex PCR with specific primers SR80 and JV33 [Vinje et al., 2000] for generating a 320 bp amplicom in the polymerase region, with the same thermal cycler program as for the multiplex PCR.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products positive for rotavirus (*VP7* gene) and sapovirus (polymerase gene and capsid gene) were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Tokyo, Japan). Sequence analysis was performed using E-CLUSTAL W (Version 1.6). Reference strains and accession numbers used in this study were as follows: Sapovirus GI strains: Sapporo/82/JP (U65427), Lyon/30388/98/F (AJ251991), Plymouth (X86559), Houston/86/US (U95643), Manchester (X86560), Potsdam/2000/DEU (AF294739), Parkville (U73124), Houston/27/90/US (U95644), Stockholm/318/97/SE (AF194182), Chiba/010658F/2001 (AJ412827), Chiba/000782F/2000 (AJ412813), Chiba/010469F/2001 (AJ412820), Chiba/000527H/2000 (AJ412801); Sapovirus GII strains: London/29845/92/UK (U95645), Lyon/598/97/F (AJ271056), Chiba/010557S/2001 (AJ412821), Chiba/010604F/2001 (AJ412826), Chiba/010592F/2001 (AJ412824), Chiba/010469F/2001 (AJ412820), Chiba/990727S/1999 (AJ-41795), Chiba/010004H/2001 (AJ412816), Chiba/010675F/2001 (AJ412828); and Sapovirus GIV strains: Chiba/000671T/1999 (AJ412805).