**Table 1.** Distribution of diarrheal virus infection in infants with and without acute gastroenteritis in a day care center (DCC) in Tokyo, Japan, during June 1999 to July 2000

| Characteristics of fecal specimen | Number of fecal specimens | Viral positive specimens (%) | Diarrheal virus (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mono-infection | | | | | | Mixed infection | | |
| | | | Rota | AstV | NV | | SV | Ade | Ade/NVGII | Ade/AstV | AstV/NVGII |
| | | | | | GI | GII | | | | | |
| Diarrheal | 88 | 45 (51.1) | 0 (0) | 14 (15.9) | 0 (0) | 13 (14.8) | 2 (2.3) | 11 (12.5) | 0 (0) | 0 (0) | 5 (5.7) |
| Asymptomatic | 833 | 230 (27.6) | 0 (0) | 53 (23.0) | 0 (0) | 46 (20.0) | 22 (9.6) | 96 (41.7) | 4 (0.5) | 6 (0.7) | 3 (0.4) |
| Total | 921 | 275 (29.9) | 0 (0) | 67 (7.3) | 0 (0) | 59 (6.4) | 24 (2.6) | 107 (11.6) | 4 (0.4) | 6 (0.6) | 8 (0.9) |

Note: Rota, Rotavirus; AstV, Astrovirus; NV, Norovirus, GI, Genogroup I; GII, Genogroup II; SV, Sapovirus; Ade, Adenovirus

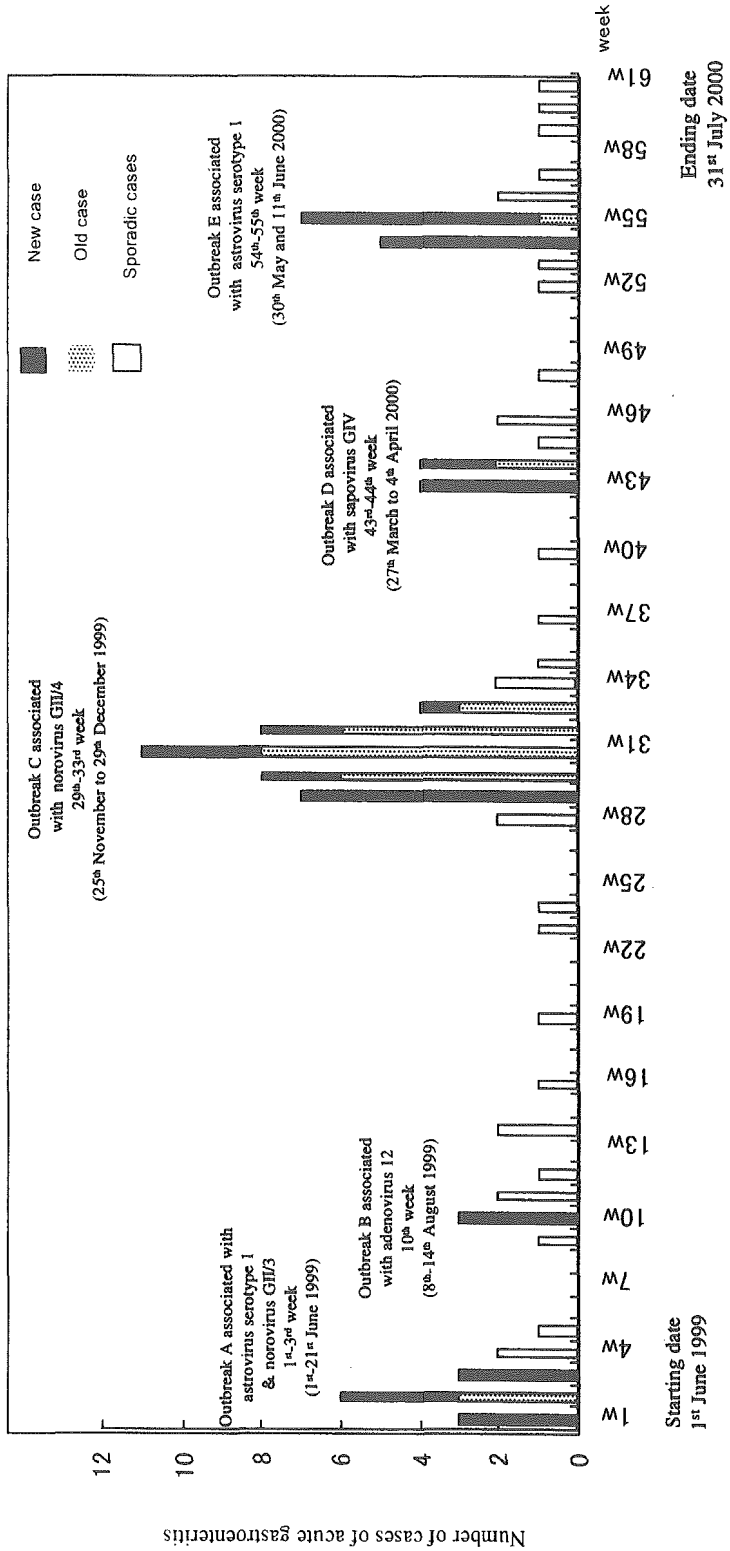



**Fig. 1.** Weekly distribution of acute gastroenteritis in infants in a day care center (DCC) in Tokyo, Japan, during the period June 1999 to July 2000. The duration and viral etiologic agents associated with outbreaks of acute gastroenteritis are shown. Note: The number of infants with acute gastroenteritis each week is the total of new cases that week plus still affected cases from previous weeks showing continuing signs of the illness that week

iii) with diarrhea in at least one negative fecal specimen before the date of fecal collection. Thus 24.8% (57 of 230) of asymptomatic viral infections were due to viral shedding after the latest acute gastroenteritis.

*Multiple outbreak of acute gastroenteritis*

According to the definition above, five separate outbreaks of acute gastroenteritis among infants in a DCC were recognized during 61-week observation (Fig. 1). These outbreaks (1 to 5) were termed from Outbreaks A to E, respectively.

Outbreak A in association with norovirus GII and astrovirus

Outbreak A occurred during the 1st to 3rd weeks (1st–21st June 1999). It was found that 9 infants demonstrated signs of acute gastroenteritis. The lowest age was 2 months and the highest was 10 months. All were positive for diarrheal viruses; 4 infants were infected with astrovirus and 5 infants with mixed infection by astrovirus and norovirus GII.

Outbreak B in association with adenovirus

During the 10th week (8th–14th August 1999), an outbreak of acute gastroenteritis was identified. In this outbreak, 3 infants aged 5, 6 and 10 months, respectively, had the illness. All of them were infected with adenovirus.

Outbreak C in association with norovirus GII

Outbreak C of acute gastroenteritis among 15 infants from 9 to 21 months old was noticed from the 29th to the 33rd week (25th November to 29th December 1999). Norovirus GII was detected in 7 of these infants. Interestingly, it was found that 5 infants (No. 5, 6, 8, 11 and 13) were infected with norovirus GII twice in this outbreak and Outbreak A. No diarrheal virus was found in the other 8 infants.

Outbreak D in association with sapovirus

It was found that Outbreak D of acute gastroenteritis occurred during a 2-week period (43rd and 44th) (27th March to 4th April 2000). A total of 6 infants aged from 8 to 20 months were involved in this outbreak. Only 2 of these infants were infected with sapovirus.

Outbreak E in association with astrovirus

During a two-week period (54th and 55th) (30th May to 11th June 2000), 11 infants were involved in Outbreak E. The lowest age was 4 months and the highest was 25 months. Six of them were infected with astrovirus. No other agent was identified. Interestingly, two infants were infected twice with astrovirus in this outbreak (Outbreak E) and Outbreak A.

*Clinical manifestations*

All clinical symptoms from Japanese infants with acute gastroenteritis in DCC during the research period were reported. All of them had diarrhea. The shortest duration of diarrhea was 1 day and the longest was 17 days. The symptoms were accompanied by vomiting (17.4%) and fever (71.2%). Infants with vomiting vomited from 1 to 8 times per day. The fever rose to 39.8 degrees Celsius. No mucus or blood was found in the feces.

*Viral shedding*

The duration of viral shedding by Japanese infants in the DCC was determined by RT-multiplex PCR of follow-up fecal specimens obtained after the onset of signs of acute gastroenteritis. It was noted that the duration of shedding of adenovirus and astrovirus ranged from 1 day to 10 days and from 1 day to 22 days, respectively. Interestingly, norovirus could be detected for up to 56 days from one nine-month-old girl in Outbreak C. Sapovirus could be seen only on the first and second days of sampling in the fecal specimens. No sapovirus was found from feces of these infants after 7 days (Table 2).

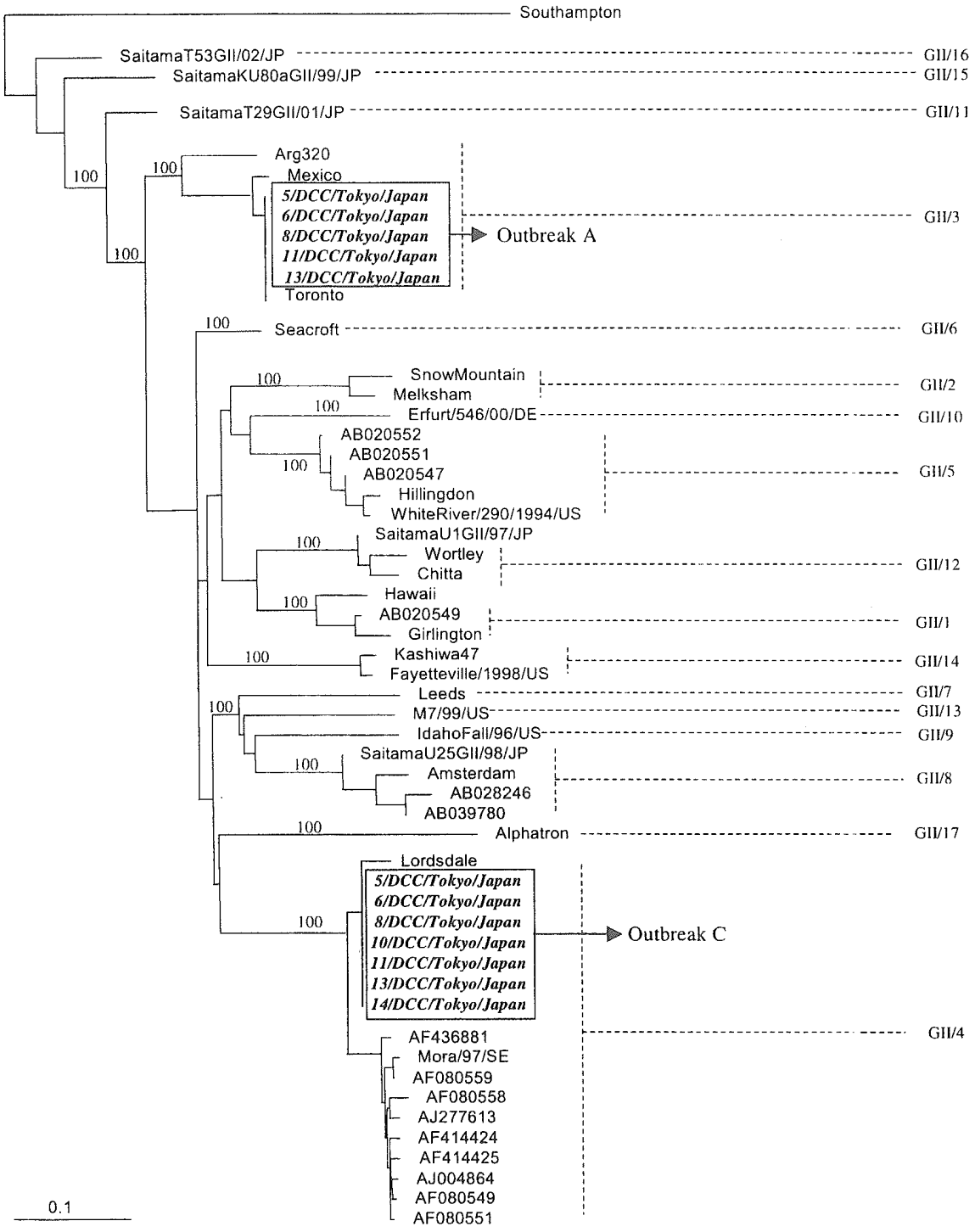
*Astrovirus isolates*

The PCR products of their capsid gene were sequenced in order to further characterize the genetic relationship among the astrovirus isolates detected in the outbreaks of acute gastroenteritis. Their partial nucleotide sequences were compared to each other as well as to those of reference strains available in the GenBank

**Table 2.** The shedding duration of diarrheal viruses in infants with acute gastroenteritis in a day care center (DCC) in Tokyo, Japan, during June 1999 to July 2000

| Diarrheal virus | Shedding duration in the present study | Shedding duration in previously published reports | References |
|---|---|---|---|
| Astrovirus | From 1 day to 22 days | From 1 day to 35 days | Mitchell et al., 1995 |
| Adenovirus | From 1 day to 10 days | From 1 day to 14 days | Van et al., 1992 |
| Norovirus | From 1 day to 56 days | From 1 day to 22 days | Rockx et al., 2002 |
| Sapovirus | 2 days | From 1 day to 15 days | Rockx et al., 2002 |

**Fig. 2.** Phylogenetic tree of nucleotide sequences of norovirus genogroup II (NVGII) isolates. The tree was constructed from partial nucleotide sequences of the capsid region of the NV GII strains, which were identified in Outbreaks A and C of acute gastroenteritis in infants. Reference strains of NVGII were selected from DDBJ/GenBank under the accession number indicated in the text. The NVGII isolates found in Tokyo, Japan, are highlighted in italics and belong to two different clusters. Southampton was used as an out-group strain for phylogenetic analysis


Southampton
SaitamaT53GII/02/JP
SaitamaKU80aGII/99/JP
SaitamaT29GII/01/JP
GII/16
GII/15
GII/11
100
100
100
Arg320
Mexico
5/DCC/Tokyo/Japan
6/DCC/Tokyo/Japan
8/DCC/Tokyo/Japan
11/DCC/Tokyo/Japan
13/DCC/Tokyo/Japan
Toronto
Outbreak A
GII/3
100
Seacroft
GII/6
100
SnowMountain
Melksham
GII/2
100
Erfurt/546/00/DE
GII/10
AB020552
AB020551
AB020547
100
GII/5
Hillingdon
WhiteRiver/290/1994/US
SaitamaU1GII/97/JP
100
Wortley
Chitta
GII/12
Hawaii
100
AB020549
Girlington
GII/1
100
Kashiwa47
Fayetteville/1998/US
GII/14
Leeds
GII/7
100
M7/99/US
GII/13
IdahoFall/96/US
GII/9
SaitamaU25GII/98/JP
100
Amsterdam
AB028246
AB039780
GII/8
100
Alphatron
GII/17
Lordsdale
5/DCC/Tokyo/Japan
6/DCC/Tokyo/Japan
8/DCC/Tokyo/Japan
10/DCC/Tokyo/Japan
11/DCC/Tokyo/Japan
13/DCC/Tokyo/Japan
14/DCC/Tokyo/Japan
100
Outbreak C
AF436881
Mora/97/SE
AF080559
AF080558
AJ277613
AF414424
AF414425
AJ004864
AF080549
AF080551
GII/4
0.1

database by BLAST. It was found that all astroviruses detected in the present study were serotypes 1. The nucleotide sequences of astrovirus isolates in each outbreak were 100% identical. The astrovirus homology between Outbreaks A and E was 96%; at the nucleotide level they were 91% and 95% identical to the prototype Oxford 1 strain, respectively.

*Norovirus GII isolates*

All norovirus genogroup II isolates associated with the outbreaks of acute gastroenteritis were further characterized for their genotypes and genetic relationship with the reference strains based on the recent norovirus capsid region classification scheme of Kageyama et al., 2004 [11]. The phylogenetic tree of nucleotide sequences of the capsid region of the norovirus GII isolates was constructed in comparison with the reference strains. The results (shown in Fig. 2) reveal that isolates of norovirus GII in Outbreak A of acute gastroenteritis formed a cluster with Toronto reference strain known as norovirus genotype 3. The other norovirus GII isolates in association with Outbreak C belong to genotype 4 (known as Lordsdale virus cluster). The homology at the nucleotide as well as the amino acid level among norovirus GII isolates in the same outbreak was 100%. Norovirus GII isolates in Outbreak A and C were 100% and 99% identical to Toronto and Lordsdale, respectively.

*Adenovirus isolates*

The PCR products of adenovirus were sequenced in order to further characterize the genetic relationship among the isolates of adenovirus detected in Outbreak B of acute gastroenteritis. Their partial nucleotide sequences were compared to each other as well as to those of reference strain available in the GenBank database. Interestingly, all adenoviruses detected in the present study were serotype 12. The nucleotide sequences among adenovirus 12 isolates in Outbreak B were 100% identical. The homology between these adenoviruses 12 and the adenovirus reference strain VR-863 (Accession number, X73487) was 92%.

*Sapovirus isolates*

Two sapovirus isolates found in Outbreak D were further characterized for their genogroup and genotypes as well as genetic relationship with the reference strains based on the recent sapovirus capsid region classification scheme of Phan et al., 2005 [27]. The phylogenetic tree of nucleotide sequences of the capsid region of the sapovirus isolates was constructed in comparison with the reference strains. Of interest, it was found that two sapoviruses turned out to be similar to the Chiba000671T strain (known to belong to genogroup IV) (Fig. 3). The 100% homology at the nucleotide as well as the amino acid level was recognized not only between two sapovirus isolates but also between them and the Chiba000671T reference strain.

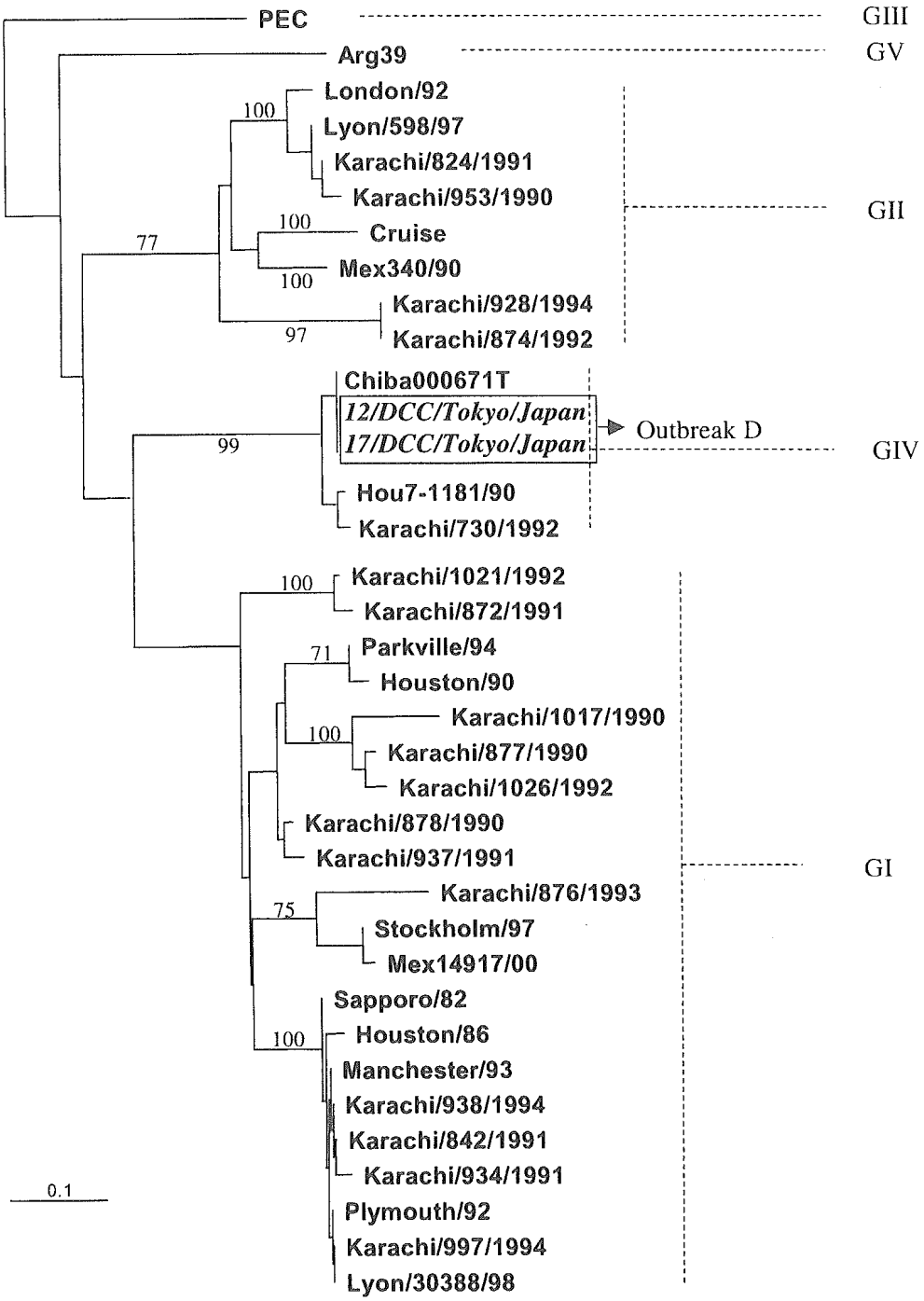



**Fig. 3.** Phylogenetic tree of nucleotide sequences of sapovirus isolates. The tree was constructed from partial amino acid sequences of the capsid region of the sapovirus strains, which were identified in Outbreak D of acute gastroenteritis among infants in the DCC. Reference strains of sapovirus were selected from DDBJ/GenBank under the accession number indicated in the text. The sapovirus isolates found in Japan are highlighted in italics and belong to genogroup IV. PEC was used as an out-group strain for phylogenetic analysis

## Discussion

Viral gastroenteritis is a common disease with a high morbidity reported worldwide and one of the most frequently encountered problems in day care settings [21, 29]. The mortality among children due to gastroenteritis among children is greater in developing than in the developed countries. In this study, enteric pathogenic viruses were detected in 51.1% of diarrheal fecal specimens tested. These findings suggest that about half of cases of acute gastroenteritis in Japanese infants in DCCs are due to diarrheal viruses and half caused by other etiologic agents. Among diarrheal viruses detected, astrovirus was the most prevalent and a leading cause of viral gastroenteritis in Japanese infants in DCCs. Next was norovirus GII, followed by adenovirus and sapovirus. Surprisingly, rotavirus was not found by RT-multiplex PCR in this study. All fecal specimens collected from diarrheal infants were further tested for the presence of rotavirus with monoplex PCR and another common method based on a different principle, ELISA. However, no rotavirus could be detected (data not shown). These findings clearly indicate that diarrheal viruses other than rotavirus cause acute gastroenteritis in Japanese infants in DCCs. It is surprising, because rotavirus is known as the most important and a major cause of severe gastroenteritis in infants and young children in DCCs worldwide.

In order to investigate asymptomatic infection with diarrheal virus, fecal specimens were also collected from infants without signs of acute gastroenteritis. Interestingly, a rather high incidence (27.6%; 230 of 833) of viral infection was identified among Japanese asymptomatic infants in the DCC. Of these, adenovirus was still the most predominant, followed by astrovirus, norovirus GII, and sapovirus. Moreover, 5.7% of normal fecal specimens demonstrated mixed viral infections. However, only 6.8% (57 of 833) of asymptomatic viral infection was due to viral shedding after the latest acute gastroenteritis and up to 20.8% (173 of 833) of healthy infants in DCC had a diarrheal virus infection without clinical manifestations of acute gastroenteritis. Taken together, our findings are the first to clearly indicate that diarrheal viral pathogens cause not only clinical manifestations of acute gastroenteritis but also asymptomatic infection in infants in a DCC in Japan.

To date, several reports by different groups of investigators have described the shedding of diarrheal viruses by infants and children with acute gastroenteritis after the onset of the illness [17, 30, 32]. In line with those findings, viral shedding was also noted in the present study. It was found that the shedding of adenovirus and astrovirus ranged from 1 day to 10 days and from 1 day to 22 days, respectively, and sapovirus could be seen only on the first and second days of sampling in the fecal specimens. These results are in strong agreement with previously published reports that the duration of adenovirus, astrovirus and sapovirus lasted 1–14 days, 1–35 days and 1–15 days, respectively [17, 30, 32]. Norovirus shedding was reported to be detected from 1 to 22 days [17]. Interestingly, the norovirus shedding in our study continued for up to 56 days. This result was consistent with another report that the duration if sapovirus shedding was shorter than that of norovirus [17].

Another interesting feature of the present study was the demonstration of multiple outbreaks of acute gastroenteritis among infants in that day care center during the period June 1999 to July 2000. It is noteworthy to point out that Outbreak D was associated with sapovirus genogroup IV (GIV). To date, only three sapovirus strains belonging to genogroup IV have been found and are regarded as uncommon strains [7, 25, 27]. However, the results of the present study clearly indicate that sapovirus GIV strains are not rare. Furthermore, subgenus F represented by adenoviruses 40 and 41 are known to be the most common adenovirus serotypes of acute gastroenteritis in a DCC (32). Surprisingly, adenovirus 12 belonging to subgenus A, which was mainly associated with meningoencephalitis among children and immunocompromised hosts, was found to be responsible for Outbreak B of acute gastroenteritis in infants in that DCC. This finding underscored that "nonenteric" adenovirus also played an important role in causing acute gastroenteritis in infants in Japan.

It was also found that re-infection with norovirus GII and astrovirus occurred in infants in the DCC in Tokyo during two different outbreaks of acute gastroenteritis. Five infants (No. 5, 6, 8, 11 and 13) in Outbreaks A and C were infected with norovirus GII twice with different genotypes 3 and 4, respectively. Two outbreaks (Outbreaks A and C) in association with astrovirus infections took place at almost the same time (the beginning of June) in the DCC. The outbreak of acute gastroenteritis due to astrovirus appears to have happened in June; further research should be conducted in order to investigate this phenomenon.

In conclusion, this is the first indication to describe the existence of multiple outbreaks of acute gastroenteritis caused by different diarrheal viruses, especially adenovirus 12 and sapovirus genogroup IV in Japanese infants in a DCC. Our data have described the molecular epidemiology as well as the importance of these viruses causing acute gastroenteritis in Japan and increased the evidence for their worldwide distribution.

## Acknowledgements

This study was supported by Grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare, Japan. This study was also supported by the Heiwa Nakajima Foundation, the Mishima Kaiun Foundation and the Sumitomo Foundation in Japan. We thank Mr. Christopher Holmes for help in revising this paper.

## References

1. Bartlett AV, Moore M, Gary GW, Starko KM, Erben JJ, Meredith BA (1985) Diarrheal illness among infants and toddlers in day care centers. II. Comparison with day care homes and households. J Pediatr 107: 503–509
2. Bartlett AV 3rd, Reves RR, Pickering LK (1988) Rotavirus in infant-toddler day care centers: epidemiology relevant to disease control strategies. J Pediatr 113: 435–341
3. Bresee J, Fang ZY, Wang B, Nelson EA, Tam J, Soenarto Y, Wilopo SA, Kilgore P, Kim JS, Kang JO, Lan WS, Gaik CL, Moe K, Chen KT, Jiraphongsa C, Ponguswanna Y,

Nguyen VM, Phan VT, Le TL, Hummelman E, Gentsch JR, Glass R (2004) First report from the Asian Rotavirus Surveillance Network. Emerg Infect Dis 10: 988–995
4. Caceres VM, Kim DK, Bresee JS, Horan J, Noel JS, Ando T, Steed CJ, Weems JJ, Monroe SS, Gibson JJ (1998) A viral gastroenteritis outbreak associated with person-to-person spread among hospital staff. Infect Control Hosp Epidemiol 19: 162–167
5. Deneen VC, Hunt JM, Paule CR, James RI, Johnson RG, Raymond MJ, Hedberg CW (2000) The impact of foodborne calicivirus disease: the Minnesota experience. J Infect Dis 181: 281–283
6. Ferson MJ (1993) Infections in day care. Curr Opin Pediatr 5: 35–40
7. Farkas T, Zhong WM, Jing Y, Huang PW, Espinosa SM, Martinez N, Morrow AL, Ruiz-Palacios GM, Pickering LK, Jiang X (2004) Genetic diversity among sapoviruses. Arch Virol 149: 1309–1323
8. Hansman GS, Doan LT, Kguyen TA, Okitsu S, Katayama K, Ogawa S, Natori K, Takeda N, Kato Y, Nishio O, Noda M, Ushijima H (2004) Detection of norovirus and sapovirus infection among children with gastroenteritis in Ho Chi Minh City, Vietnam. Arch Virol 149: 1673–1688
9. Hansman GS, Katayama K, Maneekarn N, Peerakome S, Khamrin P, Tonusin S, Okitsu S, Nishio O, Takeda N, Ushijima H (2004) Genetic diversity of norovirus and sapovirus in hospitalized infants with sporadic cases of acute gastroenteritis in Chiang Mai, Thailand. J Clin Microbiol 42: 1305–1307
10. Hansman GS, Kuramitsu M, Yoshida H, Katayama K, Takeda N, Ushijima H, Surenkhand G, Gantolga D, Kuroiwa C (2005) Viral gastroenteritis in Mongolian infants. Emerg Infect Dis 11: 180–182
11. Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, Takai R, Oka T, Takeda N, Katayama K (2004) Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. J Clin Microbiol 42: 2988–2995
12. Lie KK (1994) Infection problems connected to child day care centers. Tidsskr Nor Laegeforen 114: 1936–1938
13. Lopman BA, Brown DW, Koopmans M (2002) Human caliciviruses in Europe. J Clin Virol 24: 137–160
14. Lui BL, Clarke IN, Caul EO, Lambden PR (1995) Human enteric caliciviruses have a unique genome structure and are distinct from the Norwalk-like viruses. Arch Virol 140: 1345–1356
15. Matson DO (1994) Viral gastroenteritis in day-care settings: epidemiology and new developments. Pediatrics 94: 999–1001
16. Marks PJ, Vipond IB, Carlisle D, Deakin D, Fey RE, Caul EO (2000) Evidence for airborne transmission of Norwalk-like (NLV) in a hotel restaurant. Epidemiol Infect 120: 481–487
17. Mitchell DK, Monroe SS, Jiang X, Matson DO, Glass RI, Pickering LK (1995) Virologic features of an astrovirus diarrhea outbreak in a day care center revealed by reverse transcriptase-polymerase chain reaction. J Infect Dis 172: 1437–1444
18. Mitchell DK, Matson DO, Jiang X, Berke T, Monroe SS, Carter MJ, Willcocks MM, Pickering LK (1999) Molecular epidemiology of childhood astrovirus infection in child care centers. J Infect Dis 180: 514–517
19. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV (1999) Food-related illness and death in the United States. Emerg Infect Dis 5: 607–625
20. Morrow AL, Townsend IT, Pickering LK (1991) Risk of enteric infection associated with child day care. Pediatr Ann 20: 427–433

21. Mulholland EK (2004) Global control of rotavirus disease. Adv Exp Med Biol 549: 161–168
22. Murray CJ, Lopez AD (1997) Mortality by cause for eight regions of the world: global burden of disease study. Lancet 349: 1269–1276
23. Noel J, Cubitt D (1994) Identification of astrovirus serotypes from children treated at the Hospitals for Sick Children, London 1981–1993. Epidemiol Infect 113: 153–159
24. Oh DY, Gerhard G, Eckart S (2003) Viral agents of acute gastroenteritis in German children: prevalence and molecular diversity. J Med Virol 71: 82–93
25. Okada M, Shinozaki K, Ogawa T, Kaiho I (2002) Molecular epidemiology and phylogenetic analysis of Sapporo-like viruses. Arch Virol 147: 1445–1451
26. O'Ryan ML, Matson DO, Estes MK, Bartlett AV, Pickering LK (1990) Molecular epidemiology of rotavirus in children attending day care centers in Houston. J Infect Dis 162: 810–816
27. Phan TG, Okame M, Tuan AN, Nishio O, Okitsu S, Ushijima H (2005) Genetic diversity of sapovirus in fecal specimens from infants and children with acute gastroenteritis in Pakistan. Arch Virol 150: 371–377
28. Parashar UD, Bresee JS, Glass RI (2003) The global burden of diarrhoeal disease in children. Bull World Health Organ 81: 236–240
29. Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI (2003) Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis 9: 565–572
30. Rockx B, De Wit M, Vennema H, Vinje J, De Bruin E, Van Duynhoven Y, Koopmans M (2002) Natural history of human calicivirus infection: a prospective cohort study. Clin Infect Dis 35: 246–253
31. Thapar N, Sanderson IR (2004) Diarrhoea in children: an interface between developing and developed countries. Lancet 363: 641–653
32. Van R, Wun CC, O'Ryan ML, Matson DO, Jackson L, Pickering LK (1992) Outbreaks of human enteric adenovirus types 40 and 41 in Houston day care centers. J Pediatr 120: 516–521
33. Yan H, Yagyu F, Okitsu S, Nishio O, Ushijima H (2003) Detection of norovirus (GI, GII), sapovirus and astrovirus in fecal samples using reverse transcription single-round multiplex PCR. J Virol Methods 14: 37–44
34. Yan H, Nguyen TA, Phan TG, Okitsu S, Li Y, Ushijima H (2004) Development of RT-multiplex PCR assay for detection of adenovirus and group A and C rotaviruses in diarrheal fecal specimens from children in China. Kansenshogaku Zasshi 78: 699–709

Author's address: Hiroshi Ushijima, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; e-mail: ushijima@m.u-tokyo.ac.jp

Clin. Lab. 2005;51:183-191


ORIGINAL ARTICLE

# Viral Diarrhea in Japanese Children: Results from a One-Year Epidemiologic Study

TUNG GIA PHAN[1], TUAN ANH NGUYEN[1], TOSHIMASA KUROIWA[1], KUNIO KANESHI[1], YUICHI UEDA[1], SHIGEKAZU NAKAYA[1], SHUICHI NISHIMURA[1], TADASHI NISHIMURA[2], ATSUKO YAMAMOTO[3], SHOKO OKITSU[1], HIROSHI USHIJIMA[1]

[1]*Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[2]*Department of Pediatrics, Hokusetsu Hospital, Osaka, Japan*
[3]*Department of Pediatrics, Mitsui Memorial Hospital, Tokyo, Japan*

## SUMMARY

**A total of 557 fecal specimens from infants and children with acute gastroenteritis in five places (Maizuru, Tokyo, Sapporo, Saga and Osaka) in Japan from July 2002 to June 2003 were tested for the presence of diarrheal viruses by RT-PCR, PRHA, RNA-PAGE and latex agglutination methods. Of these, 56.4% (314) were found positive for diarrheal viruses. Among them, group A rotavirus was the most prevalent (43.6%, 137 of 314) followed by norovirus (29.9%, 94 of 314), adenovirus (7.6%, 24 of 314), group C rotavirus (6.4%, 20 of 314), sapovirus (5.1%, 16 of 314) and astrovirus (1.6%, 5 of 314), respectively. A high rate (7.4%, 19 of 314) of viral mixed infections, including one triple infection (adenovirus, norovirus and astrovirus) was demonstrated. Norovirus infection that usually has a peak during November and January in Japan was detected year-round and highest in September in our study. Norovirus was subjected to molecular genetic analysis by sequencing. The results clearly indicated that norovirus group II was a dominant genogroup (94.3%, 100 of 106). It is noteworthy that noroviruses detected in this study were classified into 8 genotypes (GI/1, GI/4, GII/2, GII/3, GII/4, GII/5, GII/6 and GII/12). Of these, NVGII/4 was the predominant genotype, followed by NVGII/6, and these presented 75.6% (80 of 106) and 11.3% (12 of 106), respectively. Another interesting feature in our study was the sudden appearance and disappearance of SaitamaU16-like strains belonging to NVGII/6 in the short period (January 2003 to June 2003). Our findings confirmed the presence of many diarrheal viruses co-circulating among Japanese infants and children and showed the great genetic diversity among norovirus. (Clin. Lab. 2005;51:183-191)**

## KEY WORDS

PCR, norovirus, astrovirus, sapovirus, rotavirus, adenovirus, genotyping, Japan, children

## INTRODUCTION

Viral gastroenteritis is one of the most common illnesses in humans worldwide and has a great impact on people (1, 2). Among children the mortality due to acute gastroenteritis is greater in developing than in developed countries (3). Annual mortality associated with acute gastroenteritis was estimated to be 2.1 million in 2000. Among different kinds of diarrheal viruses, rotavirus is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide (1, 2). Adenovirus, astrovirus, norovirus and sapovirus, however, are also considered to be significant global enteropathogens (4, 5, 6, 7, 8). Transmission routes of these viruses are classified into food-borne, waterborne, person-to-person spread, and there might be some other unknown modes (8, 9, 10, 11, 12).

Norovirus (NV, formerly known as "Norwalk-like virus) and sapovirus (SV, formerly known as "Sapporo-like virus" are the two distinct genera within the family *Caliciviridae*. Norovirus is one of the leading agents of

Manuscript accepted February 5, 2005

**Table 1: Distribution of viral infection in infants and children with acute gastroenteritis in Maizuru, Tokyo, Sapporo, Saga and Osaka, Japan**

| No of specimen tested | No of viral positive (%) | Monoinfection (%) | | | | | | | | Mixed infection (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | RV | | | NV | | SV | Ad | AstV | Ad NVGII | RC NVGII | RA NVGII | RA RC | SV RA | NVGI RA | Ad, NVGII and AstV |
| | | A | B | C | I | II | | | | | | | | | | |
| 557 | 314 | 137 | 0 | 20 | 4 | 90 | 16 | 24 | 5 | 3 | 2 | 4 | 5 | 2 | 2 | 1 |
| | (56.4) | (43.6) | (0) | (6.4) | (1.2) | (28.7) | (5.1) | (7.6) | (1.6) | (0.9) | (0.6) | (1.2) | (1.6) | (0.6) | (0.6) | (0.3) |

Note: No, Number; RV, Rotavirus; NV, Norovirus; SV, Sapovirus; Ad, Adenovirus; AstV, Astrovirus; NVGI, Norovirus genogroup I; NVGII, Norovirus genogroup II; RC, Group C rotavirus; RA, Group A rotavirus

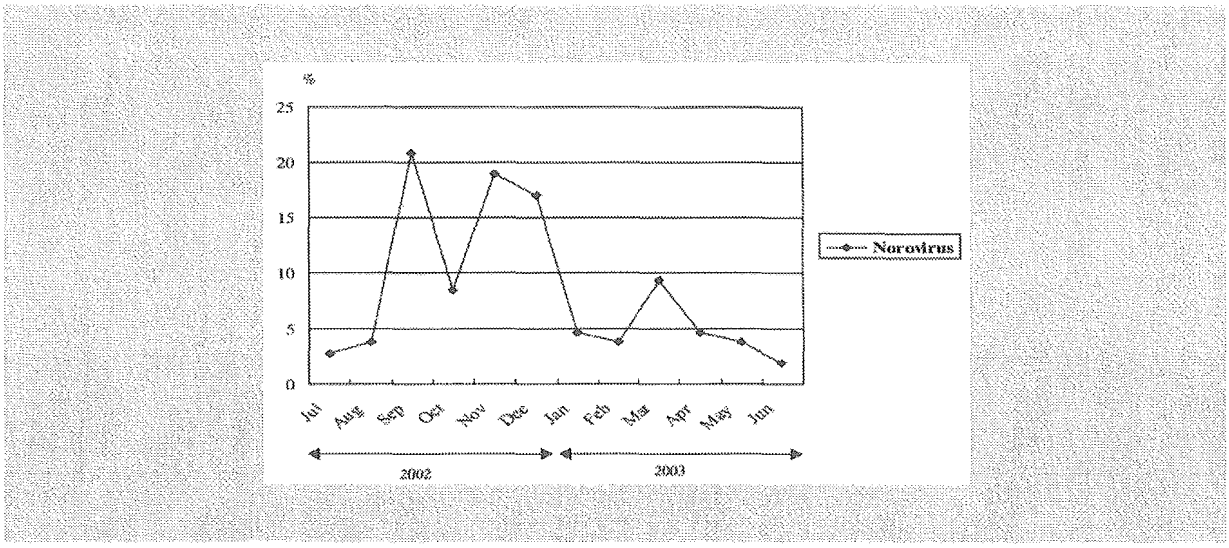



**Figure 1: The seasonal distribution of norovirus is shown. Norovirus was identified throughout the 12-month period from July 2002 to June 2003.**

acute gastroenteritis worldwide and causes outbreaks in various epidemiological settings such as restaurants, schools, day-care centers (DCCs), hospitals, nursing homes and cruise ships (8, 9, 13, 14, 15, 16). Norovirus is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make norovirus a major public health concern (17). The prototype strain of norovirus is the Norwalk virus (Hu/NV/Norwalk virus/1968/US), which was originally discovered in an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA, in 1968. Two types of caliciviruses contain a positive sense single-stranded RNA genome surrounded by an icosahedral capsid. The norovirus genome contains three ORFs (ORF1, 2 and 3). The ORF1 encodes nonstructural proteins, including the RNA-dependent RNA polymerase (RdRp), ORF 2 encodes the capsid protein (VP1) and ORF3 encodes a small capsid protein (VP2) (18). Based on the sequence analysis of the capsid gene, norovirus is divided into genogroups I and II known to infect humans. A recent study indicated that NVGI and NVGII could be classified into 14 and 17 genotypes, respectively (17). To date, norovirus is still uncultivable by standard culture with different cell lines. However, expression of either VP1 alone or both VP1 and VP2 using recombinant baculoviruses formed the virus-like particles (VLPs) that are morphologically and antigenically similar to the native virion (19, 20). Immunological and seroepidemiologic studies indicated a worldwide distribution of norovirus. Moreover, it was found that the serum antibody level to norovirus was lowest in the first year of life, rising after two years of age (8, 21). The objectives of this study were: to determine the incidence of diarrheal virus infections in infants and children with acute gastroenteritis in five different places of Japan during 2002 and 2003; to characterize norovirus detected according to genogroup and genotype and to describe the genetic diversity among them. Additionally, the age-related distribution and seasonal pattern of norovirus infection were also determined.

**Table 2: Characteristics of 12 mixed infection cases with norovirus and other enteropathogens among Japanese pediatric population**

| No | Patient | Sex | Age (Month) | Place | Month | Year | Norovirus | | Other diarrheal virus | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Geno group | Geno type | RT-PCR | PRHA* | Diarlex# | PAGE$ | Sero type& |
| 1 | 4351 | M | 10 | Maizuru | Sep | 2002 | II | 4 | Adenovirus | ND | + | ND | ND |
| 2 | 5397 | M | 15 | Maizuru | Dec | 2002 | II | 4 | Group C rotavirus | + | ND | + | ND |
| 3 | 4400 | F | 15 | Maizuru | Jan | 2003 | II | 4 | Group A rotavirus | ND | + | + | Mix G3G4 |
| 4 | 4403 | F | 21 | Maizuru | Jan | 2003 | II | 6 | Group C rotavirus | + | ND | + | ND |
| 5 | 4471 | F | 11 | Maizuru | April | 2003 | I | 1 | Group A rotavirus | ND | + | + | G3 |
| 6 | 4511 | M | 28 | Maizuru | May | 2003 | II | 6 | Group A rotavirus | ND | + | + | G9 |
| 7 | 4525 | M | 10 | Maizuru | May | 2003 | II | 6 | Astrovirus/Adenovirus | ND | + | ND | Ast1 |
| 8 | 4531 | M | 65 | Maizuru | May | 2003 | II | 6 | Group A rotavirus | ND | + | + | Mix G3G9 |
| 9 | 4544 | M | 119 | Maizuru | June | 2003 | II | 6 | Group A rotavirus | ND | + | + | Mix G3G9 |
| 10 | 4611 | M | 14 | Maizuru | Dec | 2002 | II | 4 | Adenovirus | ND | + | ND | ND |
| 11 | 4656 | M | 22 | Maizuru | May | 2003 | I | 1 | Group A rotavirus | ND | + | + | G3 |
| 12 | 4811 | M | 13 | Saga | Oct | 2002 | II | 4 | Adenovirus | ND | + | ND | ND |

Note: M, male; F, female; *, Only used to detect group C rotavirus; #, Only used to detect group A rotavirus and adenovirus
$, Only used to detect group A and C rotavirus; &, Only used to serotype group A rotavirus and astrovirus; ND, Not done

**Table 3: Distribution of norovirus (GI and GII) genotypes based on the sequencing genetic analysis among infants and children with acute gastroenteritis in five different places of Japan**

| Date of fecal specimen collection | Fecal specimens positive for norovirus by RT-PCR | Norovirus genogroup I genotype (%) | | Norovirus genogroup II genotype (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 4 | 2 | 3 | 4 | 5 | 6 | 12 |
| July 2002-June 2003 | 106 | 5 (4.7) | 1 (0.9) | 1 (0.9) | 4 (3.8) | 80 (75.6) | 1 (0.9) | 12 (11.3) | 2 (1.9) |

## MATERIALS AND METHODS

### Fecal specimens

A total of 557 fecal specimens were collected from infants and children with acute gastroenteritis in five different places (Maizuru, Tokyo, Sapporo, Saga and Osaka), Japan during the period of July 2002 to June 2003. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 x g for 10 min. The supernatants were collected and stored at –30 °C until use for the detection of diarrheal viruses.

### Extraction of viral genome

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

### Viral detection and RT-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 (22, 23). Using RT- multiplex PCR with specific primers as previously reported performed the identification of two groups of diarrheal viruses. In multiplex PCR, four pairs of specific primers published (PreCAP1 and 82b for amplifying the capsid gene of AstV; G1SKF and G1SKR for the capsid gene of NVGI; COG2F and G2SKR for the capsid gene of NVGII, SLV5317 and SLV5749 for the capsid gene of SV) were mixed. These primers were specifically generated four different sizes of amplicons of 719bp, 330bp, 387bp and 434bp for astrovirus, norovirus (GI, GII) and sapovirus, respectively In order to detect the second group of viruses, four pairs of published primers (Beg9 and VP7-1', B5-2 and B3-3, G8NS1 and G8NA2 for amplifying the VP7 gene of human group A , B and C

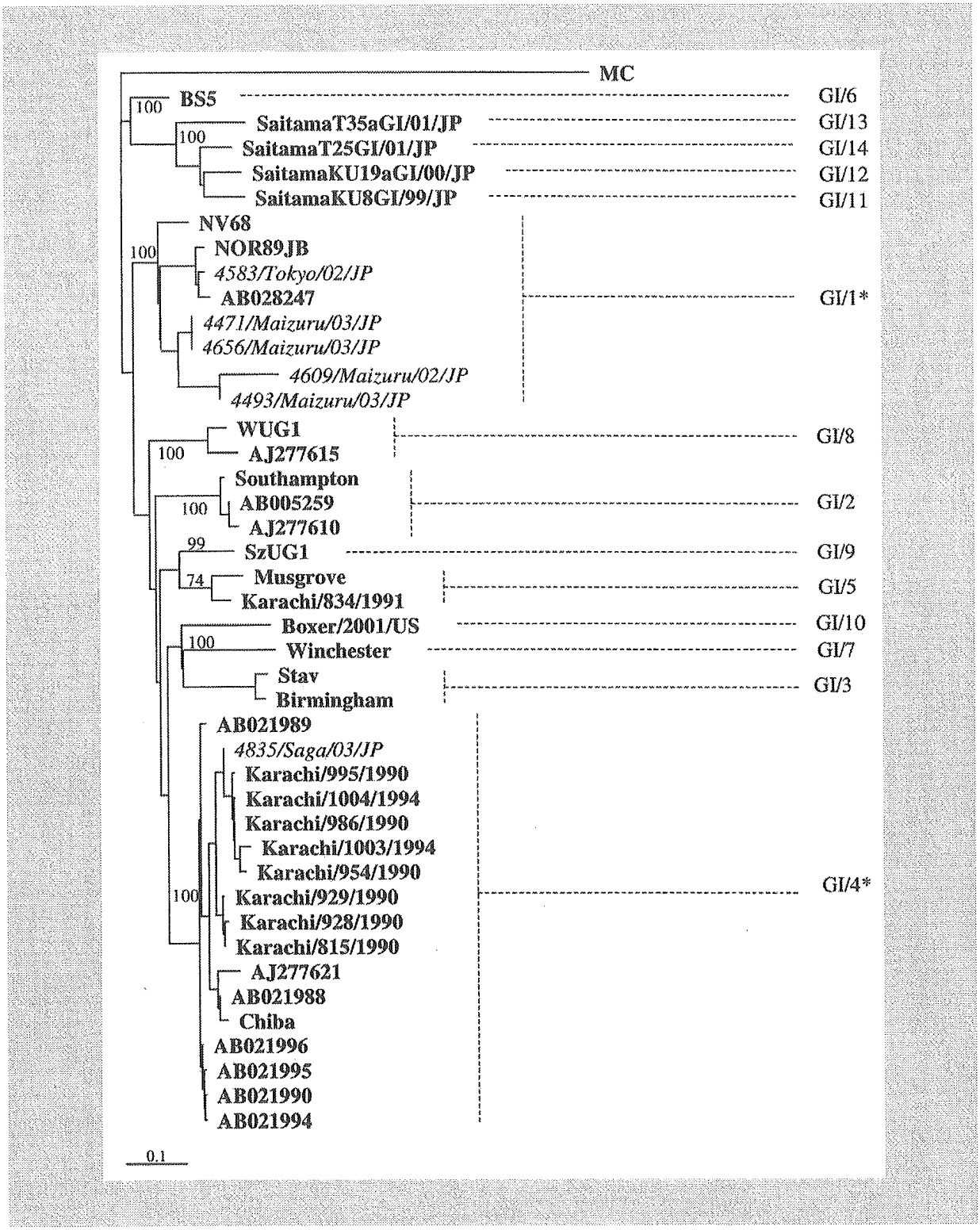



**Figure 2: Phylogenetic tree of Japanese norovirus GI isolates. The numbers in the branches indicate the bootstrap values. Manchester (MC) was used as an out-group. Note. *, Genotype contains NV GI isolates detected in the present study**

rotaviruses, respectively; Ad1 and Ad2 for the hexon gene of all species from A to F adenoviruses) were used. These primers specifically generated four different amplicon sizes of 395bp, 814bp, 352bp and 482bp for group A, B and C rotaviruses and adenovirus, respectively in one PCR tube. PCR was performed at 94 °C for 3min followed by 35 cycles of 94 °C 30s, 55 °C 30s, 72 °C 60s, and a final extension at 72 °C for 7min, and then held at 4 °C.

**Astrovirus serotyping**
Astrovirus serotyping was performed using RT-PCR analysis of extracted viral RNA with published serotype-specific primers (1 to 8) presented by Sakamoto et al. [2000] (24).

**Group A rotavirus serotyping**
Serotyping of group A rotavirus was conducted using the serotype-specific primers from the method previously described by Das et al in 1994 (25). The full length of the VP7 gene was reversely transcribed and then further amplified. The second amplification was performed using the first PCR product as the template with G-serotype-specific mixed primers to amplify the VP7 genes of G1-G4 and G9, respectively.

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

**Reverse passive hemagglutination test**
Fecal specimens positive for group C rotavirus by multiplex PCR were further screened for group C rotavirus by another method of different principle, a reverse passive hemagglutination (RPHA) test. The techniques were carried out according to the manufacturer's instructions (Denka Seiken Co., Ltd, Japan). Fixed sensitized red blood cells with purified anti-group C rotavirus antibody can be agglutinated with group C rotavirus in fecal supernatant. Based on this principle, 25 µl of the supernatant of the mixture containing 1 ml saline and 40 µl of 10% fecal suspension were reacted with sensitized red blood cells in microplates. Hemagglutination titers were observed after 2 h.

**Latex agglutination test**
The Diarlex test (Orion Diagnostica, Espoo, Finland), a commercial latex agglutination test, was used for the detection of both group A rotavirus and adenovirus. A drop of the fecal supernatant was mixed with a drop of test latex on a slide, and reaction was observed in 2min. Development of distinct agglutination in Diarlex reagent was treated as positive. If agglutination was seen in the negative control latex, the test was considered uninterpretable.

**Polyacrylamide gel electrophoresis**
The rotavirus RNA genome was subjected to polyacrylamide gel electrophoresis (PAGE) to detect viral genomic RNA. The PAGE was performed using a 10% polyacrylamide separating gel with a 4% stacking gel. Migration of RNA genome fragments was detected by silver staining (26).

**Nucleotide sequencing and phylogenetic analysis**
The nucleotide sequences of PCR products (DNA) positive for norovirus 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 norovirus strains and accession numbers used in this study were as follows:
Birmingham (AJ277612), Musgrove (AJ277614), Chiba (AB022679), NV68 (M87661), Saitama T35aGI/01/JP (AB112132), Saitama T25GI/01/JP (AB112100), Saitama T53GII/02/JP (AB112260), WUG1 (AB081723), SzUG1 (AB039775), Stav (AF145709), Hawaii (U07611), Girllington (AJ277606), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Alphatron (AF195847), Toronto (U02030), Seacroft (AJ277620), Leeds (AJ277608), Lordsdale (X86557), London/92 (U95645), Idaho Falls/96/US (AY054299), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761).

## RESULTS

**Epidemiology of viral infections**
A total of 557 fecal specimens collected from infants and children with acute gastroenteritis in five different places (Maizuru, Tokyo, Sapporo, Saga and Osaka), Japan during the period of July 2002 to June 2003 were examined for the presence of diarrheal viruses. Diarrheal viruses were detected in 314 out of 557 (56.4%) specimens tested. Among the diarrheal viruses detected, group A rotavirus was the most prevalent (43.6%) followed by norovirus (29.9%). The next was adenovirus with 7.6%. Group C rotavirus and sapovirus were closely behind with 6.4% and 5.1%, respectively. Astrovirus was the last and accounted for 1.6%. No group B rotavirus was found in these subjects. Interestingly, the high rate (7.4%) of viral mixed infections was shown in this study (Table 1).
The highest incidence of norovirus was in the 12-23 months old group (39.6%), and the lowest was in the infants aged less than 6 months (1.9%). It was also found that infants and children aged less than 3 years had a rather high rate of norovirus infection (84%).

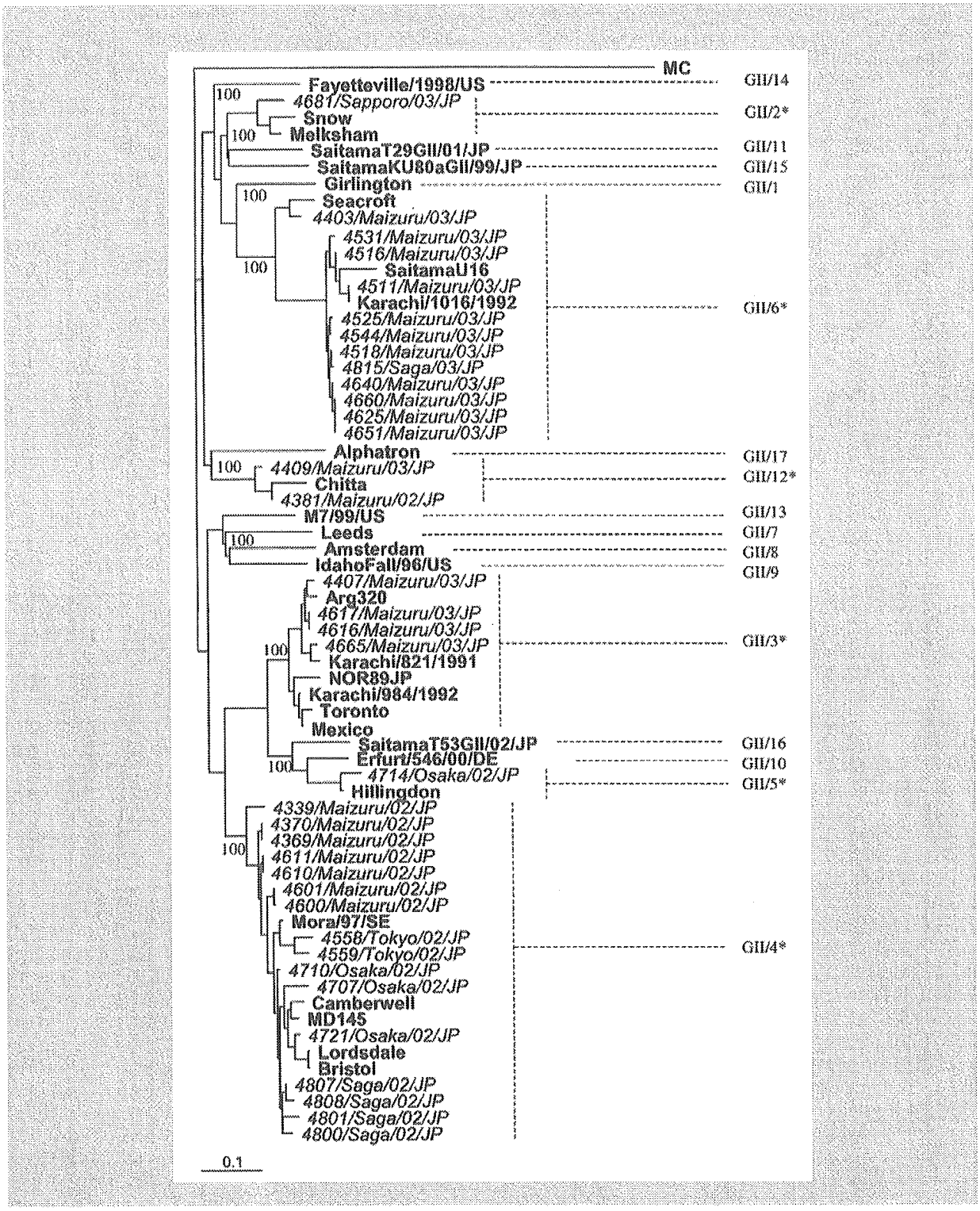



**Figure 3: Phylogenetic tree of Japanese norovirus GII isolates. The numbers in the branches indicate the bootstrap values. Manchester (MC) was used as an out-group. Note. *, Genotype contains NV GII isolates detected in the present study**

**Norovirus seasonal distribution**

The norovirus detection rate was analyzed between July 2002 and June 2003. Norovirus was identified throughout the 12-month period. The norovirus incidence was found highest in September (20.8%), followed by November (19.8%) and December (17%). The lowest norovirus detection rate was in June (1.9%). Surprisingly, there was another peak of norovirus in March (9.4%). (see Figure 1).

**Mixed infection with norovirus and other enteropathogens**

Out of 557 fecal specimens, norovirus was detected in 106 (19%) of which norovirus GII was responsible for 100 cases (94.3%) and norovirus GI for 6 cases (5.7%). Of these, 12 cases (11.3%) had mixed infection with different enteropathogens (see Table 2). It was found that almost all mixed infections were detected in Maizuru city (91.7%, 11 of 12) and only one in Saga city (8.3%, 1 of 12). The majority of mixed infection was between rotavirus and norovirus (66.7%, 8 of 12). Interestingly, one triple mixed infection with norovirus and two other diarrheal viruses (adenovirus and astrovirus) was identified in an infant aged 10 months in Maizuru City, Japan.

All diarrheal viruses (group A rotavirus, group C rotavirus, adenovirus and astrovirus), which had mixed infection with norovirus, were confirmed and further characterized by other methods such as RT-PCR serotype, PRHA, RNA-PAGE and latex agglutination test. Remarkably, group A rotaviruses detected in 3 patients (No 3, 8 and 9) were identified with mixed serotypes G3 and G4 (8.3%, 1 of 12), as well as G3 and G9 (16.7%, 2 of 12), respectively. Apparently, these specimens represented the triple mixed infections.

**Nucleotide sequencing and phylogenetic analysis of norovirus**

The PCR products of norovirus were sequenced in order to further characterize the genetic relationship among the norovirus isolates detected in infants and children with acute gastroenteritis in Japan. Their partial nucleotide sequences were compared to each other as well as to those of reference norovirus strains available in the DDBJ DNA /GenBank database by BLAST. A total of 106 norovirus nucleotide sequences including 6 norovirus GI isolates and 100 norovirus GII isolates were analyzed by phylogenetics and grouped using the recent norovirus capsid region classification schemes of Kageyama et al. [2004] (17). The norovirus GI sequences clustered into two distinct GI genotypes (see Table 3). Most of the norovirus GI sequences (83.3%, 5 of 6) belonged to genotype 1 (NVGI/1) (typified by the NOR96JP virus cluster). Only one norovirus GI sequence (16.7%, 1 of 6) clustered into NVGI/4 (known as the Chiba virus cluster). Among Japanese NVGI strains, the identity at nucleotide level was 69%-99%. The homologies between them with other norovirus reference strains in the same genogroup about 61%-99% were also noted.

All 100 norovirus GII sequences were classified into six distinct genotypes. Eighty (80%) of these norovirus sequences were similar to the genotype 4 (known as the Lordsdale virus cluster). Twelve (12%) norovirus sequences belonged to the SaitamaU16 virus cluster (genotype 6), four (4%) to genotype 3 (typified by the Toronto), two to genotype 2 (known as the Melksham cluster), two more to genotype 12 (known as the Chitta cluster), one to genotype 2 (Melksham cluster) and one in the Hillingdon cluster (GII/5). The homology of the nucleotides between the NVGI strains detected in this study with other reference strains previously registered in the DDBJ DNA database ranged from 59% to 99%.

## DISCUSSION

Viral gastroenteritis is still a health burden in developed and developing countries (9, 16, 30). In this study, diarrheal viruses were detected in 56.4% of fecal specimens tested. These findings suggested that about 56.4% of acute gastroenteritis in infants and children in five places of Japan might be due to diarrheal viruses and 33.6% caused by other etiologic agents. Group A rotavirus was the most important one, being a major cause of severe gastroenteritis in infants and young children worldwide (2). Among the diarrheal viruses detected, group A rotavirus was the most prevalent and became a leading cause of viral gastroenteritis in infants and children in Japan, followed by norovirus, adenovirus, group C rotavirus, sapovirus and astrovirus. These viruses are associated with sporadic cases and outbreaks of gastroenteritis in such settings as kindergartens, schools, hospitals, cruise ships, restaurants, nursing homes for the elderly, and among military recruits (6, 8, 11, 13, 14).

Norovirus is one of the global causes of viral gastroenteritis and also is associated with sporadic cases and outbreaks of gastroenteritis worldwide. Norovirus infection causes acute gastroenteritis in all age groups, though it occurs predominantly in young children (8, 27). In this study, it was found that infants and children aged less than 3 years had a high rate of norovirus infection, and this accounted for 84%. Out of 557 fecal specimens tested, 19% were found to be positive for norovirus by RT-multiplex PCR. These results were consistent with previously published reports on norovirus epidemiology worldwide, in which its prevalence was shown to be much lower than that of rotavirus (8, 11, 27). Our findings also confirmed norovirus as one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. In some reports, norovirus was prevalent in the cold season, and several studies did not find a seasonal correlation (8, 9, 11). According to surveillance of pediatric

cases of viral gastroenteritis in Japan, the main peak of norovirus infection was between November, December and January (27). Interestingly, norovirus in our study was identified year-round and was highest in the autumn month of September. This meant that there was a shift of norovirus infection in Japan. This might be due to the co-existence of multiple factors such as changes of climate, water, eating habits, and others. However, further research should be conducted in order to investigate this phenomenon.

To date, mixed infections with different enteric viruses have been reported by several groups of investigators (4, 11, 28). Interestingly, a high incidence (7.4%) of mixed infections with different enteric viruses was detected. Of these, mixed infections with norovirus and other viral pathogens in a high percentage of 63.2% (12 of 19) were identified. In addition, the presence of rotavirus, adenovirus and astrovirus associated with mixed infections with norovirus was also determined by other methods with different principles, PRHA, the Diarlex test and RNA-PAGE. These group A rotaviruses were further characterized for serotype by using previously published specific primers presented by Das et al. 1994 (5). Interestingly, three samples with a triple mixed infection between group A rotavirus (G3/G4 and G3/G9) and genogroup II norovirus were found. These results were in good agreement with other previously published reports in which mixed infection among norovirus and other enteropathogens is not rare (8, 29).

The results in this study showed all Japanese norovirus to belong to two distinct norovirus genogroups, I and II (GI, GII) and these represented 5.7% and 94.3%, respectively. The findings clearly indicated that NVGII was the dominant group to cause acute gastroenteritis among Japanese pediatric populations. In line with other reports published by different groups of investigators, norovirus belonging to the Lordsdale cluster (GII/4) represented the highest detection in sporadic gastroenteritis among infants and children (8, 27, 29). These strains were isolated throughout the year except in May. Other strains belonging to a wide range of norovirus genogroup I and II genotypes (GI/1, GI/2, GII/2, GII/3, GII/5, GII/6 and GII/12) were also identified as being co-circulating. These noroviruses had a low identity of the nucleotides with each other as well as with other reference strains in the same genogroup previously registered in the DDBJ DNA database. Taken together, it was noteworthy to point out that norovirus strains detected among Japanese pediatric populations with acute gastroenteritis demonstrated a great genetic diversity among them. Another interesting feature of the present study was the increase of NVGII/6 strains, which became the second predominant norovirus genotype causing illness among infants and children in Japan during 2002 and 2003. These findings were in discordance with the previous report by Irritant et al., 2003 in Japan, in which the incidence of NVGII/3 (known as the Toronto virus cluster) was found in the second place behind only NVGII/4 (Lordsdale virus cluster) (27). We found 11 (91.7%) out of 12 strains in NVGII/6 that closely matched the SaitamaU16 strain. These results suggested that the SaitamaU16-like strains were also an important cause of sporadic cases of acute gastroenteritis. Further epidemiologic studies should be conducted to determine whether strains from GII/6 including SaitamaU16-like strains continue to be dominant in Japan in the next years.

Based on the partial capsid sequences, it was noticed that four strains from GII/3 were in fact Arg320-like strains. These four Arg320-like strains in our study were detected in January, February and May 2003. Interestingly, a similar study of sporadic gastroenteritis conducted in Japan from 1996 to 2000 reported that Arg320-like strains suddenly appeared and spread between October 1999 and February 2000 (27). Likewise, we found that SaitamaU16-like strains belonging to GII/6 suddenly appeared during a short period (January 2003 to June 2003). This sudden appearance and disappearance of strains indicated that the pediatric population might lack antibody protection to these strains, and after some time they might have acquired viral immunity (30). However, several studies reported that dominant strains could persist in one region over a number of years, which might suggest that some other uncommon strains could be more virulent (31, 32).

**Acknowledgements**

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

**References**

1. Mulholland EK. Global control of rotavirus disease. Adv Exp Med Biol 2004; 549: 161-8
2. Parashar UD, Hummelman EG, Miller MA, Glass RI. Global illness and deaths caused by rotavirus disease in children. Emerg Infect Dis 2003; 9: 565-72
3. Thapar N, Sanderson IR. Diarrhoea in children: an interface between developing and developed countries. Lancet 2004; 363: 641-53
4. Bon F, Fascia P, Dauvergne M, Tenenbaum D, Planson H, Petion AM, Pothier P, Kohli E. Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France. J Clin Microbiol 1999; 37: 3055-8
5. Carter MJ, Willcocks MM. The molecular biology of astroviruses Arch Virol 1996; 12: 277-85
6. Chiba S, Sakuma Y, Kogasaka R, Akihara M, Horino K, Nakao T, Fukui S An outbreak of gastroenteritis associated with calicivirus in an infant home. J Med Virol 1979; 4: 249-54

7. Chiba S, Nakata S, Numata-Kinoshita K, Honma S. Sapporo virus: history and recent findings. J Infect Dis 2000; 181: 303-8

8. Lopman BA, Brown DW, Koopmans M. Human caliciviruses in Europe. J Clin Virol 2002; 24: 137-60

9. Marks PJ. Vipond IB, Carlisle D, Deakin D, Fey RE, Caul EO. Evidence for airborne transmission of Norwalk-like (NLV) in a hotel restaurant. Epidemiol Infect 2000; 120: 481-7

10. Noel J, Cubitt D. Identification of astrovirus serotypes from children treated at the Hospitals for Sick Children, London 1981-1993. Epidemiol Infect 1994; 113: 153-9

11. Oh DY, Gerhard G, Eckart S. Viral agents of acute gastroenteritis in German children. Prevalence and molecular diversity. J Med Virol 2003; 71: 82-93

12. Saito K, Ushijima H, Nishio O, Oseto M, Motohiro H, Ueda Y, Takagi M, Nakaya S, Ando T, Glass R. Detection of astrovirus from stool samples in Japan using reverse transcription and polymerase chain reaction amplification. Microbiol Immunol 1995; 39: 825-8

13. McEvoy M, Blake W, Brown D, Green J, Cartwright R. An outbreak of viral gastroenteritis on a cruise ship. Commun Dis Rep CDR Rev 1996; 6: 188-92

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

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

16. Matson DO. Viral gastroenteritis in day-care settings: epidemiology and new developments. Pediatrics 1994, 94: 999-1001

17. Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, Takai R, Oka T, Takeda N, Katayama K. Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan. J Clin Microbiol 2004; 42: 2988-95

18. Glass PJ, White LJ, Ball JM, Leparc GI, Hardy ME, Estes MK. Norwalk virus open reading frame 3 encodes a minor structural protein. J Virol 2000; 74: 6581-91

19. Jiang X, Wang M, Graham DY, Estes MK. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J Virol 1992; 66: 6527-32

20. Jiang X, Matson DO, Ruiz-Palacios GM, Hu J, Treanor J, Pickering LK. Expression, self-assembly, and antigenicity of a snow mountain agent-like calicivirus capsid protein. J Clin Microbiol 1995; 33: 1452-55

21. Jiang X, Matson DO, Velazquez FR, Calva JJ, Zhong WM, Hu J, Ruiz-Palacios, Pickering LM. Study of Norwalk-related viruses in Mexican children. J Med Virol 1995; 47: 309-16

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

23. Yan H, Tuan AN, Phan TG, Okitsu S, Ushijima H. Development of RT-multiplex PCR assay for detection of adenovirus and group A and C rotavirus in diarrheal fecal specimens from children in China. Kansenshougaku Zasshi 2004; 78: 699-709

24 Sakamoto T, Negishi H, Wang QH, Akihara S, Kim B, Nishimura S, Kaneshi K, Nakaya S, Ueda Y, Sugita K, Motohiro T, Nishimura T, Ushijima H. Molecular epidemiology of astroviruses in Japan from 1995 to 1998 by reverse transcription-polymerase chain reaction with serotype-specific primers (1 to 8). J Med Virol 2000; 61: 326-31

25. Das BK, Jon RG, Helen GC, Patricia AW, Aarti G, Madhumati R, Ramesh K, Bhan MK. Characterization of rotavirus strains from newborns in New Delhi, India. J Clin Microbiol 1994; 23: 1820-22

26 Theil KW, McCloskey CM, Saif LJ, Redman DR, Bohl EH, Hancock DD, Kohler EM, Moorhead PD. Rapid, simple method of preparing rotaviral double stranded ribonucleic acid for analysis by polyacrylamide gel electrophoresis. J Clin Microbiol 1981; 14: 273-80

27. Iritani N, Seto Y, Kubo H, Murakami T, Haruki K, Ayata M, Ogura H. Prevalence of Norwalk-like virus infections in cases of viral gastroenteritis among children in Osaka City, Japan. J Clin Microbiol 2003 41: 1756-9

28. Okada M, Shinozaki K, Ogawa T, Kaiho I. Molecular epidemiology and phylogenetic analysis of Sapporo-like viruses. Arch Virol 2002; 147: 1445-51

29. Phan TG, Okame M, Nguyen TA, Maneekarn N, Nishio O, Okitsu S, Ushijima H. Human astrovirus, norovirus (GI, GII), and sapovirus infections in Pakistani children with diarrhea. J Med Virol 2004; 73: 256-61

30. Hansman GS, Doan LT, Kguyen TA, Okitsu S, Katayama K, Ogawa S, Natori K, Takeda N, Kato Y, Nishio O, Noda M, Ushijima H. Detection of norovirus and sapovirus infection among children with gastroenteritis in Ho Chi Minh City, Vietnam. Arch Virol 2004; 149: 1673-88

31. White PA, Hansman GS, Li A, Dable J, Isaacs M, Ferson M, McIver CJ, Rawlinson WD. Norwalk-like virus 95/96-US strain is a major cause of gastroenteritis outbreaks in Australia J Med Virol 2002; 68: 113-8

32. Noel JS, Fankhauser RL, Ando T, Monroe SS, Glass RI. Identification of a distinct common strain of "Norwalk-like viruses" having a global distribution. J Infect Dis 1999; 179: 1334-44

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