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

Hideaki Shimizu [a], Tung Gia Phan [a], Shuichi Nishimura [a], Shoko Okitsu [a], Niwat Maneekarn [b], Hiroshi Ushijima [a,*]

[a] *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*
[b] *Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*

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Abstract

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

Keywords: *Adenovirus*; Outbreak; Maizuru

1. Introduction

Viral gastroenteritis is a common disease with a high morbidity reported worldwide especially in infants and the elderly. The mortality among children due to gastroenteritis is greater in developing than in developed countries. Acute gastroenteritis ranks consistently as one of the principal six causes of all deaths (Murray and Lopez, 1997). Among different kinds of diarrheal viruses, *rotavirus* is the most important, being a major cause of severe gastroenteritis in infants and young children worldwide (Mulholland, 2004). *Adenovirus*, however, is also considered to be a significant enteropathogen in association with sporadic cases as well as outbreaks of gastroenteritis in such settings as kindergartens, schools, and hospitals (Chiba et al., 1983; Van et al., 1992; Akihara et al., 2005).

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

* Corresponding author. Tel.: +81 3 5841 3590; fax: +81 3 5841 3629.
E-mail address: ushijima@m.u-tokyo.ac.jp (H. Ushijima).

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

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

2. Materials and methods

2.1. Fecal specimens

A total of 337 fecal specimens were collected from infants and children with acute gastroenteritis in a clinic in Maizuru City, Japan during the period of July 2004 to June 2005. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatants were collected for the detection of diarrheal viruses.

2.2. Extraction of viral genome

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

2.3. Reverse transcription (RT)

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

2.4. Detection of diarrheal viruses by polymerase chain reaction (PCR)

The first group of viruses, including *astrovirus*, *norovirus* (*GI*, *GII*), and *sapovirus* and the second group including *group A*, *B*, and *C rotaviruses*, and *adenovirus* were detected by RT-PCR with primers as previously reported by Phan et al. (2005b). The identification of the first group of viruses was performed with specific primers Beg9 and VP7-1, B5-2 and B3-3, G8NS1 and G8NA2, Ad1 and Ad2, for *group A*, *B*, and *C rotaviruses*, and *adenovirus* with four different amplicon sizes of 395 bp, 814 bp, 352 bp and 482 bp, respectively. For the detection of the second group of viruses, specific primers PreCAP1 and 82b, G1SKF and G1SKR, COG2F and G2SKR, SLV5317 and SLV5749 were utilized to specifically generate four different sizes of amplicons of 719 bp, 330 bp, 387 bp and 434 bp for *astrovirus*, *norovirus* (*GI*, *GII*), and *sapovirus*, respectively. PCR was carried out with 1 μl of cDNA in 10 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (2.5 mM/μl), primers (33 μM/μl), Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA) and MilliQ water. PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 7 min, and then held at 4 °C.

2.5. Electrophoresis

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

2.6. Serotyping of adenovirus by PCR and sequence analysis

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

Seven hypervariable regions of the hexon gene of *adenovirus* were amplified by specific primers S29 (for sense 5′-GCCAGCACRTWCTTTGACAT-3′) and S53 (for antisense 5′-CCCATGTTGCCAGTGCTGTTGTARTACA-3′) to generate the amplicon size of 1286 bp (Takeuchi et al., 1999). PCR was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 2 min, 72 °C for 3 min, and a final extension at 72 °C for 7 min, and then held at 4 °C.

2.6.2. Nucleotide sequencing and phylogenetic analysis of HVRs

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

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

3. Results

3.1. Molecular epidemiology of diarrheal viruses

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

3.2. Nucleotide sequencing and phylogenetic analysis of adenovirus

The PCR products of *adenovirus* were sequenced in order to further characterize the genetic relationship among the *adenovirus* isolates detected in infants and children with acute gastroenteritis in Maizuru City, Japan. Their nucleotide sequences containing seven hypervariable regions of the hexon gene were compared to each other as well as to those of reference *adenovirus* strains available in GenBank by BLAST. A total of 27 *adenovirus* sequences were analyzed by phylogenetic analysis using the recent seven-hypervariable regions of the hexon gene-based classification scheme of Li et al. (2004). *Adenoviruses* detected in the present study were classified into five serotypes, Ad1, Ad2, Ad3, Ad5 and Ad41. Of these, Ad41 predominated over other serotypes and represented 85.2% (23 of 27) while one of each was Ad1, Ad2, Ad3 and Ad5, respectively (Fig. 1). Using CLUSTAL X, it was also noticed that these *adenoviruses* had a high identity on the nucleotide level as well as on the amino acid level with corresponding *adenovirus* reference strains previously registered in GenBank ranging from 94% to 100%.

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

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

3.3. Outbreak of adenovirus serotype 41

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

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

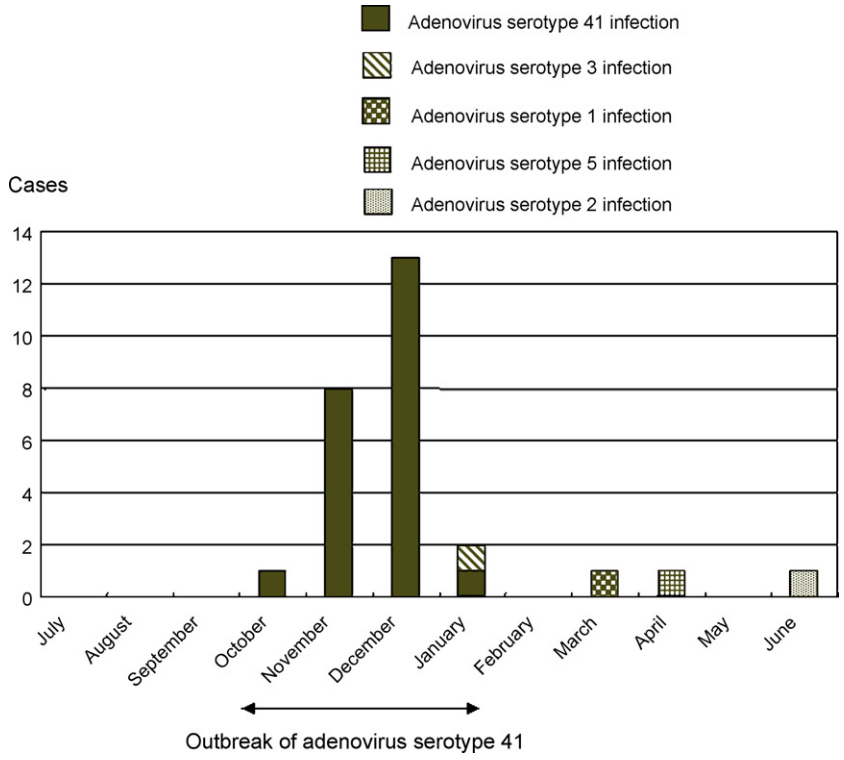

Fig. 2. Monthly distribution of *adenovirus* infection in infants and children with acute gastroenteritis in Maizuru City, Japan during the period of July 2004 to June 2005. The duration of outbreak of Ad41 infection was shown.

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

4. Discussion

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

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

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

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

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

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

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

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

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

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