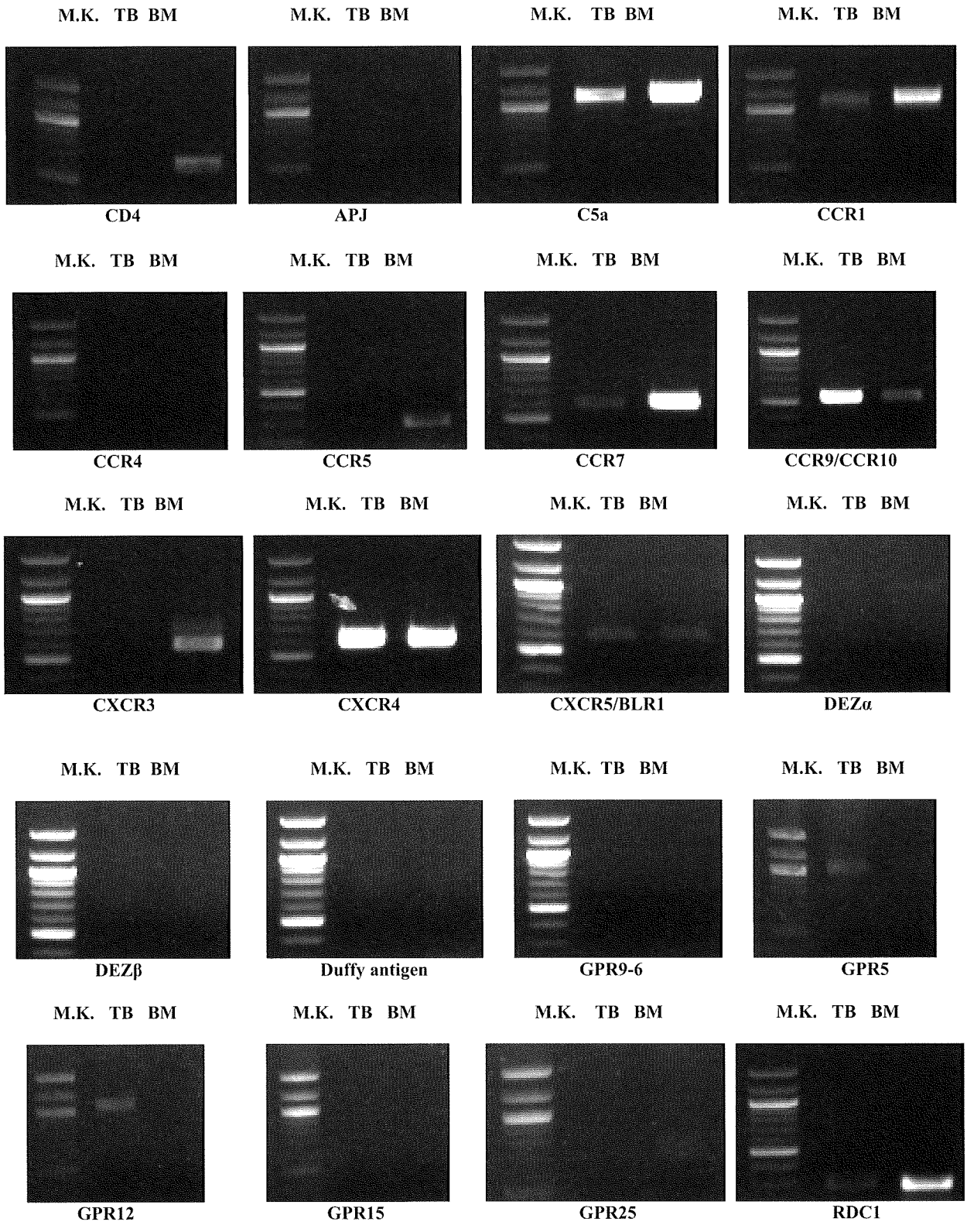

Figure 2: Electrophoresis of RT-PCR products of mRNA of G-protein coupled receptors in trophoblasts and breast milk cells: TB, trophoblast; BM, breast milk.

However, other groups of investigators could find expression of CXCR4 in early-term trophoblasts [Ishii et al., 2000; Wu et al., 2004].

In conclusion, this study investigated the existence of mRNA expressions of nineteen GPCRs and CD4 in three different cell lines, choriocarcinoma cells, trophoblasts, and breast milk cells. It was noteworthy that mRNAs of multiple GPCRs were identified in choriocarcinoma cell, trophoblasts, and breast milk cells for the first time. This study improved our current knowledge about the cellular mechanism of mother-to-child transmission of HIV infection. However, further research on the functions of these GPCRs is definitely necessary.

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Molecular epidemiology of adenovirus infection among infants and children with acute gastroenteritis in Dhaka City, Bangladesh

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ABSTRACT

A total of 917 fecal specimens collected from infants and children with acute gastroenteritis in Dhaka City, Bangladesh during 2004–2005 were examined for the presence of adenoviruses by PCR. Adenoviruses were identified in 17 of 917 (1.9%) specimens. Detected adenoviruses were subjected to molecular genetic analysis by sequencing method. Adenoviruses detected in this study were classified into three serotypes, namely Ad9, Ad10 and Ad40. Of these, Ad40 was predominant, followed by Ad9 and accounted for 42% (7 of 17) and 36% (6 of 17), respectively. This is the first report of acute gastroenteritis attributed to Ad9 and Ad10 in Dhaka City, Bangladesh. Another interesting feature of the study was absence of Ad41 serotype. Our results clearly indicated that adenovirus infections were most commonly observed in winter season (October 2004 through January 2005) and in rainy season (May 2005 through July 2005) in Dhaka City. The most common clinical symptoms of adenovirus-infected patients were dehydration (94%), abdominal pain (59%) and vomiting (30%).

To our knowledge, this is the first 1-year molecular epidemiological research of adenovirus infection in Bangladesh.

1. Introduction

Acute gastroenteritis, mainly diarrhea, is one of the most common diseases in human, and remains a leading cause of morbidity and mortality worldwide. It is reported that about 3–5 billion cases of acute gastroenteritis occur each year in children under 5 years, resulting in nearly 2 million deaths (Elliott, 2007; Mulholland, 2004; Parashar et al., 2003). Acute gastroenteritis consistently ranks as one of the top six causes of all deaths (Murray and Lopez, 1997). Among different kinds of diarrheal viruses, Group A rotaviruses (GARVs), family *Reoviridae*, are major causes of acute infantile diarrhea worldwide (Dey et al., 2009; Parashar et al., 2003). Recently, the associations of other enteric viruses such as caliciviruses, astroviruses and enteric adenoviruses have also been reported in sporadic and outbreak cases of diarrhea (Shimizu et al., 2007; Akihara et al., 2005). Enteric 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 (Akihara et al., 2005; Van et al., 1992; Chiba et al., 1983).

Human adenoviruses belong to the Mastadenovirus of family *Adenoviridae* and are double stranded DNA viruses without envelope. 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. Adenoviruses are responsible for a wide range of disease symptoms. To date, more than 51 human adenovirus serotypes have been identified and classified into six subgenera A–F based on their biological and genetic characteristics (Davison et al., 2003; De Jong et al., 1993). Among these subgenera, subgenera "F" represented by two serotypes, that is, adenovirus type

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40 and 41 has been found to be associated with acute gastroenteritis and occupied 1–20% of diarrheal cases (Akihara et al., 2005; Shimizu et al., 2007).

Neutralization test, ELISA, or virus isolation had been used for the detection and identification of adenovirus serotypes over the past decades. However, these methods are relatively complicated, labor intensive, time consuming, of low sensitivity, and sometimes require the cell culture techniques. Isolation of adenovirus is sometimes unsuccessful because of the low viral titer in clinical specimens (Li et al., 2004; Takeuchi et al., 1999; Van der Avoort et al., 1989). Now a day, PCR assay has been introduced as a convenient and powerful alternative method for molecular diagnosis. Highly sensitive and specific PCR assay is currently available for the detection of adenovirus. Additionally, genome amplification allows further characterization of the adenovirus serotype by sequence analysis (Phan et al., 2004; Takeuchi et al., 1999).

The objectives of this study were to determine the incidence of adenovirus infection in infants and young children with acute gastroenteritis in Dhaka City, Bangladesh; to characterize the detected adenoviruses according to serotypes. Additionally, the clinical symptoms and seasonal pattern of adenovirus infection were also described.

2. Materials and methods

2.1. Study population and fecal specimens

A total of 917 fecal specimens were collected from infants and children with acute gastroenteritis in Dhaka City, Bangladesh during the period of October 2004 to September 2005. For the pediatric population, the lowest age was 2 months, the highest age was 3.2 years (38 months), and the average age was 1.1 years (13 months). One stool sample was collected from each patient with acute gastroenteritis. Stool samples were collected from those patients when viral gastroenteritis was clinically suspected and stored at −20 °C. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant was heated at 65 °C for 10 min before using in experiment.

2.2. Extraction of viral genome

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

2.3. Polymerase chain reaction

We used published methods and primers for the detection of group A, B and C rotaviruses and adenoviruses (Shimizu et al., 2007; Yan et al., 2004). For the detection of these viruses, specific primers Beg9 and VP7′-1, ADG9-1F and ADG9-1R, G8NS1 and G8NA2, Ad1 and Ad2 specifically generated four different sizes of amplicons of 395, 814, 351 and 462 bp for group A, B and C rotaviruses and AdV, respectively. The 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 for 60 s and a final extension at 72 °C for 7 min and then held at 4 °C.

2.4. Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (0.5 μg/ml) for 20 min then visualized under ultraviolet (UV) light. The bands were recorded by photography.

2.5. Serotyping of adenovirus by PCR and sequence analysis

Seven hypervariable regions of the hexon gene of adenovirus were amplified by specific primers S29 (for sense 5′-GCCAG-CACRTWCTTTGACAT-3′) and S53 (for antisense 5′-CCCATGTTGCCA-GTGCTGTTGTARTACA-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. Nucleotide sequence analysis

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). The genetic distance was calculated using Kimura's two-parameter method (PHYLIP).

2.7. Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 3.2 software package (Kumar et al., 2001). The reference adenovirus strains and accession numbers used in this study were as follows: adenovirus serotype 8 (X74663), adenovirus serotype 41-subtype 1 (AB103349), adenovirus serotype 41-subtype 2 (AB103344), adenovirus serotype 9 (AB023547 and X74664), adenovirus serotype 10 (DQ149615 and AB023548), adenovirus serotype 40 (DQ115441 and X51782) adenovirus serotype 37 (X98360), adenovirus serotype 7 (X76551), adenovirus serotype 6 (X67710), adenovirus serotype 4 (X76550) and adenovirus serotype 1 (X67709).

2.8. Nucleotide sequence submission

The nucleotide sequence data of all Bangladeshi adenovirus strains had been submitted to the GenBank (NCBI, Bethesda, MD) and had been assigned accession number EU099384 to EU099399 and FJ666898, respectively.

2.9. Statistical analysis

SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The chi-squared test (χ^2) was used to compare the severity of clinical symptoms between adenovirus-positive patients and adenovirus-negative patients. A p value less than 0.05 was considered to be significant.

3. Results

3.1. Detection of adenoviruses

Adenoviruses were detected in 17/917 of the fecal specimens collected from infants and children with acute gastroenteritis. All fecal specimens were tested for the presence of group A, B and C rotaviruses. Among diarrheal viruses detected, group A rotavirus was the most prevalent (33.5%) (Dey et al., 2009), followed by 1.9% of adenoviruses. Adenoviruses detected in this study were classified into three serotypes, namely Ad9, Ad10, and Ad40. Of these, Ad40 was predominant, followed by Ad9, Ad10 and accounted for 42% (7 of 17), 36% (6 of 17) and 22% (4 of 17), respectively.

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

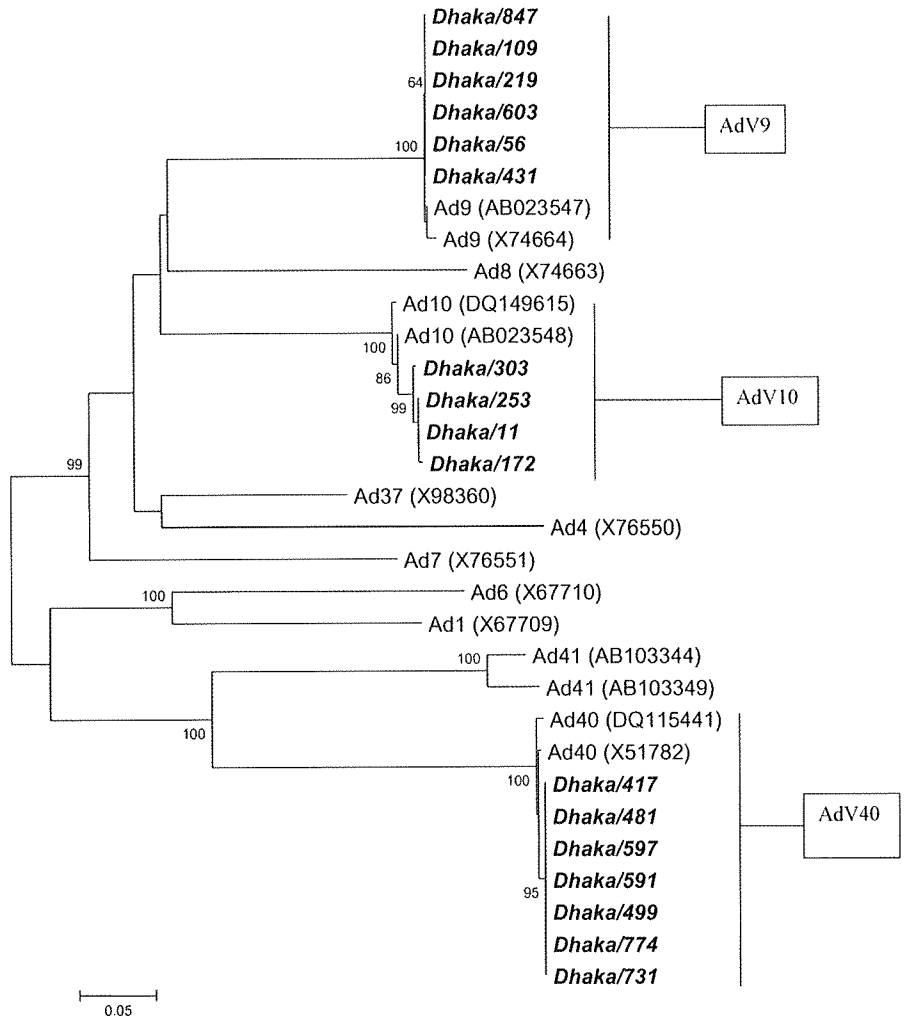

Fig. 1. Phylogenetic tree of nucleotide sequences of adenoviruses detected in acute gastroenteritis infants and children in Dhaka City, Bangladesh during 2004–2005. The tree was constructed from partial nucleotide sequences of seven hypervariable regions of the hexon gene (550 bp) of adenovirus isolates detected in Dhaka City, Bangladesh. 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.

isolates detected in infants and children with acute gastroenteritis in Dhaka City, Bangladesh. 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 17 adenovirus sequences were analyzed by phylogenetic analysis using the recent seven-hypervariable regions of the hexon gene-based classification scheme (Li et al., 2004). Adenoviruses detected in the present study were classified into three serotypes, Ad9, Ad10 and Ad40. Of these, Ad40 predominated over other serotypes and represented 42% (7 of 17) (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 95 to 99%.Clinical features of adenovirus-infected patients

The clinical symptoms of the 17 patients infected with adenovirus are listed in Table 1. It was found that the common clinical symptoms of adenoviruses infected patients were dehydration (94%), abdominal pain (59%) and vomiting (30%). All adenovirus-infected patients were hospitalized. Fever was also frequently seen among adenovirus-infected patients and the prevalence of fever seems to be 24%. The common clinical features

Table 1
Clinical features of adenovirus-positive and adenovirus-negative patients.

Sign and symptoms	Adenovirus-positive, $N = 17$ (%)	Adenovirus-negative, $N = 900$ (%)	p value
Dehydration	16 (94%)	604 (60%)	0.01
Vomiting (≥3 times per day)	5(30%)	303(33%)	0.80
Abdominal pain	10 (59%)	238 (25%)	0.00
Fever (>100 F)	4 (24%)	213 (23%)	0.60
Hospitalization	17/17 (100%)	849(92%)	0.60
3–5 times loose stools within 24 h	4 (24%)	624 (70%)	0.30
Six or more loose stools within 24 h	13 (76%)	276 (30%)	0.00
Age at hospitalization (≥24 months)	13 (76%)	811 (88%)	0.08

p value < 0.05 means "significant".

of adenovirus-negative patients were dehydration (60%), vomiting (33%), abdominal pain (25%) and fever (23%). The percentage of dehydration ($p < 0.01$) and abdominal pain ($p < 0.001$) in adenovirus-positive patients were higher than adenovirus-negative patients.

3.4. Seasonal pattern of adenovirus serotype 40 and adenovirus serotype 9

Number of stool samples collected in each month from infants and children with acute gastroenteritis in Dhaka City, Bangladesh during October 2004 to September 2005 was shown in Fig. 2A. The results shown in Fig. 1 revealed that although the fecal specimens were collected over the period of 12 months (October 2004 to September 2005), the Ad40 infection was apparently confined within a period of 4 months (October 2004 through January 2005). By contrast, AdV9 infection was confined within a period of 3 months (May 2005 through July 2005). The highest prevalence of adenovirus infection was found in January (4/17) followed by May (3/17). No adenovirus was found in February, April, August and September.

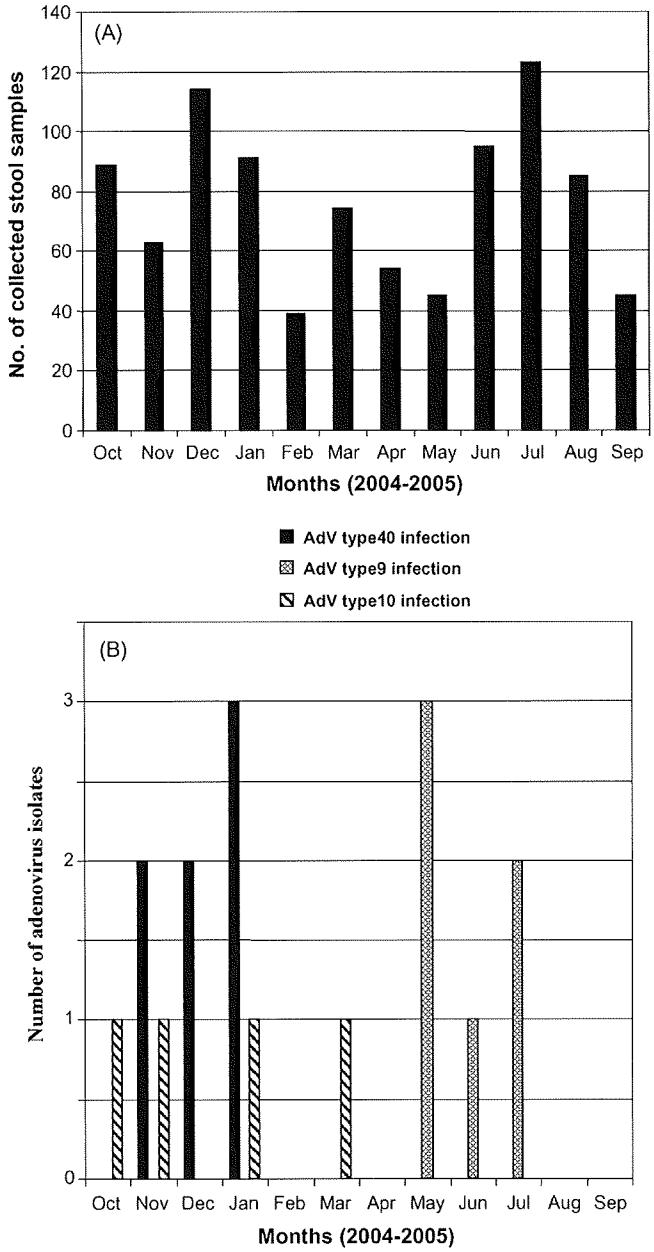

Fig. 2. (A) Number of stool samples collected in each month from infants and children with acute gastroenteritis in Dhaka City, Bangladesh during October 2004 to September 2005. (B) Seasonal pattern of adenovirus infection in infants and children with acute gastroenteritis in Dhaka City, Bangladesh during October 2004 to September 2005.

4. Discussion

Molecular epidemiological studies are increasingly popular in the field of clinical adenovirus research (Phan et al., 2004; Li et al., 2004). Epidemiological studies detected adenoviruses in stool samples collected from infants and young children with acute gastroenteritis, in the developed and developing world (Shimizu et al., 2007; Phan et al., 2005; Akihara et al., 2005; Wadell et al., 1994; Uhnoo et al., 1990; Van der Avoort et al., 1989).

Out of 917 fecal specimens tested, 17 (1.9%) was positive for adenoviruses. This result was consistent with the previous findings on adenovirus epidemiology in Indian sub-continent and Asia-Pacific region in which its prevalence was around 0.8–3.4% (Broor et al., 2007; Tang et al., 2008; Jarecki-Khan et al., 1993; Jarecki-Khan and Unicomb, 1992; Herrmann et al., 1988).

The molecular characteristics of adenovirus serotypes are only beginning to be addressed in Bangladesh. Molecular characterization of adenovirus serotypes in sporadic cases of acute gastroenteritis from Bangladesh is documented for the first time in this study. Sequence analysis showed that adenovirus detected in this study belonged to two distinct species (D and F) with three serotypes (Ad9, Ad10 and Ad40). Of these, Ad40 predominated over other serotypes and represented 42% (7 of 17). Of note, high prevalence (42%) of Ad40 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 Ad40 in Dhaka City. By contrast, the AdV9 infection was confined within a period of 3 months (May 2005 through July 2005).

Although it has been reported that the prevalence of Ad40 and Ad41 were approximately equal (Phan et al., 2004; Takeuchi et al., 1999; Shinozaki et al., 1991), but interestingly none of Ad41 was detected in the present study. The reasons for the absence of Ad41is not clear in this study. The insufficient antibody protection from acquired viral immunity against AdV41 infection among Bangladeshi pediatric population may be an explanation.

In this study, the majority of infants and children with adenovirus-infection (89%) were aged less than 24 months ($p < 0.001$). 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 (Shimizu et al., 2007; Akihara et al., 2005; Chiba et al., 1983; Jarecki-Khan and Unicomb, 1992). The percentage of hospitalization among adenovirus-infected patients was 100% in this study.

The most common sign and symptoms of infants and children infected with adenoviruses were dehydration (94%), abdominal pain (59%) and vomiting (30%). We found more patients with severe dehydration (90%) than with vomiting (75%), abdominal pain (59%). Fever (body temperature more than 100 F) was also frequently seen among our patients and the prevalence of fever seems to be 24%. Number of loose stool per day was rather extended with most of the individuals having 6–8 times (76%) ($p < 0.001$) stool per day. The most common clinical features of the negative patients were dehydration (60%), vomiting (33%), abdominal pain (25%) and fever (23%).

Although the importance of viral gastroenteritis as a prime cause of morbidity and mortality in developing countries is well recognized, very few studies have been conducted to evaluate the role of viral agents in childhood diarrhea in Bangladesh.

In conclusion, Ad9 and Ad10 infection in humans are very rare in the world. To our knowledge, this is the first 1-year molecular epidemiological research of adenovirus infection in Bangladesh. Our findings also confirmed adenoviruses as one of enteropathogen responsible for viral gastroenteritis among Bangladeshi infants and children. Future studies are needed in which systematic surveillance of gastroenteritis is done for prolonged period of time and with harmonized methods in order to find explanations for the apparent emergence of adenovirus variants in populations.

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Intergenogroup Recombinant Sapovirus in Japan, 2007–2008

Wisoot Chanit, Aksara Thongprachum, Pattara Khamrin, Shoko Okitsu, Masashi Mizuguchi, and Hiroshi Ushijima

We investigated the incidence of sapovirus (SaV)–associated gastroenteritis in infants and children in Japan during 2007–2008 and characterized the diversity of SaV-positive strains. SaV was detected in 19 (4%) of 477 fecal specimens. The leading genogroup (79%, 15 cases) comprised intergenogroup recombinant SaVs (GII/GIV).

Sapovirus (SaV) is now considered a notable global enteropathogen of acute gastroenteritis in persons of all ages (*1–3*). As a member of the family *Caliciviridae*, SaV has a single-stranded positive-sense RNA genome of ≈7.3–7.5 kb that contains either 2 or 3 main open reading frames (ORFs 1–3). SaV ORF1 encodes for nonstructural proteins and the major capsid protein (VP1), and ORF2 (VP2) and ORF3 encode proteins of yet unknown functions. On the basis of VP1 nucleotide sequences, SaVs are divided into 5 genogroups (GI–GV), of which GI, GII, GIV, and GV strains are known to infect humans; SaV GIII infects porcine species. We investigated the incidence of SaV-associated gastroenteritis in infants and children in Japan during 2007–2008 and characterized the diversity of SaV-positive strains.

The Study

We collected 477 fecal specimens from nonhospitalized children with acute gastroenteritis in pediatric clinics in 5 localities in Japan (Tokyo, Sapporo, Saga, Osaka, and Maizuru) during July 2007–June 2008. Of these, 14 specimens were from Tokyo, 30 from Sapporo, 77 from Saga, 91 from Osaka, and 265 from Maizuru. We defined diarrhea as at least 3 passages of unformed (loose and watery) feces a day. We defined acute gastroenteritis as diarrhea and other symptoms, such as vomiting, fever, and abdominal pain. Children studied ranged in age from 1 month to 14 years (median 25 months). The Ethical Committees of The University of Tokyo and Aino University approved the study. A parent or guardian of each child provided informed consent.

RNA was extracted and purified by using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription (RT) was performed with 5 μL of RNA template by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). By using multiplex RT-PCR, 2 groups of diarrheal viruses were identified: 1) astrovirus, norovirus, and sapovirus and 2) rotavirus and adenovirus (*2*). The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by staining with SYBR Safe (Invitrogen). Nucleotide sequences were determined by using a Big-Dye terminator cycle sequencing kit and ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were aligned by using ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX), and distances were calculated by using the 2-parameter Kimura method. Phylogenetic trees with bootstrap analysis from 1,000 replicas were generated by using the neighbor-joining method. The sequences of SaV strains detected in the study have been submitted to GenBank under accession nos. FJ445092–FJ445110.

Of the viruses isolated from diarrhea samples, group A rotavirus was the most prevalent (20.5%), followed by norovirus (19.3%), adenovirus (4.4%,) sapovirus (3.9%), group C rotavirus (0.8%), and astrovirus (0.2%). In addition, we found viral mixed infections in 1.8%.

Sapovirus was detected in 19 (4%) fecal specimens. The highest incidence of SaV infection was in the 1-year-old group (9 [47%]), and most (13 [68%]) of these infections occurred in infants and children <3 years of age. Infections increased slightly during December through February (12 cases). The most common signs and symptoms in SaV-infected children were diarrhea (19 children [100%]), fever >100°F (5 [26%]), and vomiting ≥3 times a day (3 [15%]).

Nineteen SaV sequences were analyzed by phylogenetic analysis and grouped by using the recent SaV capsid region classification scheme (*4*). Most SaV sequences belonged to genogroup IV (15 cases [79%]), followed by GI/4 (3 cases [16%]), and GI/1 (1 case [5%]). Three sequences of GI/4 genotype had 98%–100% nucleotide identity with each other and grouped with the Karachi/872/91/PK and Osaka/5836/JP strains known to belong to GI/4 genotype. One GI/1 sequence had 97% nucleotide identity and clustered with Manchester sequence (Figure 1).

Nucleotide sequence comparison of the identified 15 GIV shared little or almost no divergence among themselves (98%–100% identity), even when they were detected in regions of Japan distant from each other. They are likely to represent a single strain, 8208/Maizuru/08/JP. The 8208/Maizuru/08/JP sequence closely matched

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Ehime1107 and SW278 (*5*), and Yak2 (*6*) sequences, which were previously established as intergenogroup recombinant SaV strains with the GII polymerase region and GIV capsid region, with 97% and 96% nucleotide identities, respectively. To determine whether our GIV strains were the recombinant SaV, 5 of the 15 GIV strains were randomly selected as representative, and long genomic fragments that included part of the RNA polymerase and part of the capsid genes were amplified by using primers SR80/2 (5′-TGGGATTCTACACAAAACCC-3′) and SLV5749 (5′-CGGRCYTCAAA VSTACCBCCCCA-3′), which generated a 1,151-bp product. The products were directly sequenced, and capsid- and polymerase-based phylogenetic trees confirmed these strains as the recombinant SaVs (Figure 2). We suggest the GIV strains isolated in our study were intergenogroup recombinants. Also, these recombinant strains were detected in 4 locations distant from each other: Maizuru city (10 cases), Sapporo and Saga (2 cases each), and Osaka (1 case), which suggests that the recombinant strains were widely spread through the country.

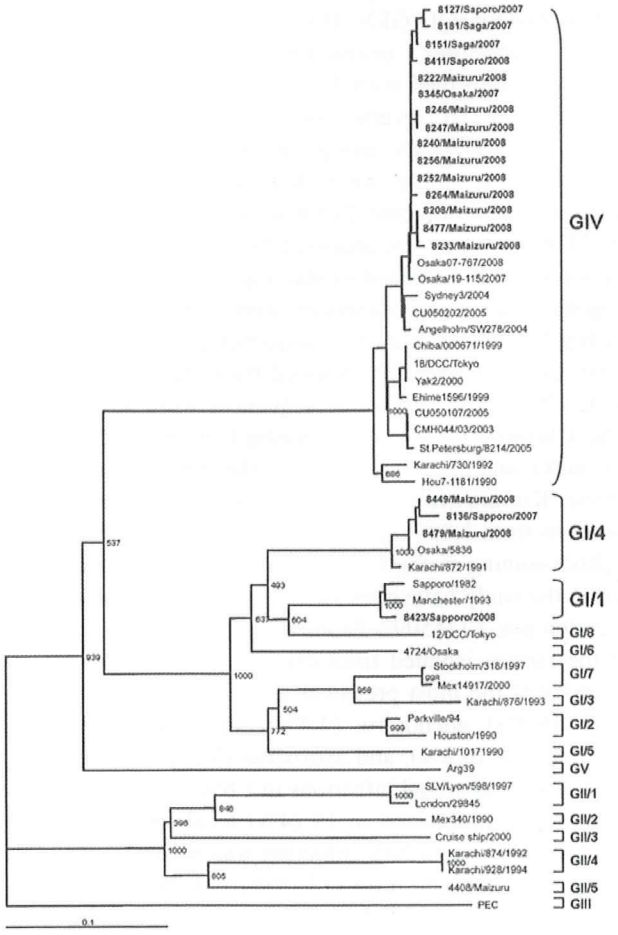

Figure 1. Phylogenetic tree of nucleotide sequences of sapovirus (SaV) strains (shown in **boldface**). The tree was constructed from partial nucleotide sequences of the capsid region by using PEC strain (a porcine SaV) as an outgroup. The numbers on each branch indicate the bootstrap values. Scale bar indicates nucleotide substitutions per position. GenBank accession numbers of reference strains are as follows: Osaka07-767/08/JP (AB433785), Osaka/19-115/07/JP (AB327280), Sydney3/04/AU (DQ104357), CU050202/05/HK (DQ155647), Angelholm/SW278/04/SE (DQ125333), Chiba/000671/99/JP (AJ786349), 18/DCC/Tokyo/43/JP (AB236378), Yak2/00/JP (AB046353), Ehime1596/99/JP (AM049952), CU050107/ 05/HK (DQ155646), CMH044/03/03/THA (EF600796), St. Petersburg/8214/05/RUS (FJ214057), Karachi/730/92/PK (AB126249), Hou7-1181/90/USA (AF435814), Osaka/5836/04/JP(AB242324), Karachi/872/91/PK (AB181231), Sapporo/82/JP (U65427), Manchester/ 93/UK (X86560), 12/DCC/Tokyo/44/JP (AB235380), 4724/Osaka/02/JP (AB180212), Stockholm/318/97/SE (AF194182), Mex14917/00/USA (AF435813), Karachi/867/93/PK (AB181132), Parkville/94/UK (U73124), Houston/90/USA (U95644), Karachi/1017/90/PK (AB181227), Arg39/95/ARG (AY289803), Lyon/598/97/F (AJ271056), London/29845/92/UK (U95645), Mex340/90/USA (AF435812), Cruise ship/00/USA (AY289804), Karachi/874/92/PK (AB181129), Karachi/928/94/PK (AB181128), 4408/Maizuru/03/JP (AB180209), and PEC (AF182760).

Conclusions

According to the past 5 years of SaV surveillance conducted in the same setting and population in Japan, SaV GI/1 was the most common genotype during 2003–2004, and thereafter genotype GI/6 dominated over the GI/1 in 2004–2005 (*2*). Then, the GI/6 genotype was replaced by the predominant SaV GI/1 since 2005 until 2007 (*7*; S.K. Dey, unpub. data). Although GIV SaV strains had been isolated in some countries, including Japan, Thailand, Pakistan, and Hong Kong Special Administrative Region of the People's Republic of China (*4,8–11*), they were detected less often than strains from the other genogroups, and whether they are the recombinant strains has not been confirmed. These findings demonstrate the changing epidemiology of SaV genogroup with the emergence of intergenogroup recombinant SaVs (GII/GIV) and the sudden decrease of predominant SaV GI in Japan during 2007–2008.

Recently, several types of recombinant SaV strains, including intergenogroup and intragenogroup, have occurred frequently in Japan (*5,12–14*), which indicates that recombination between SaV genomes is another important feature of the evolution of SaV. Although factors contributing to the emergence of these recombinant stains during the period of surveillance are not known, the recombinant strains possibly appeared when the pediatric population might have lacked antibody protection to these strains.

Our results suggest that recombinant strains may be underestimated. Characterization of SaVs usually is based on the capsid gene sequence only, whereas both the capsid gene and RNA polymerase sequences are needed to identify such viruses. Furthermore, constant surveillance is important to successfully monitor the emergence of these strains.

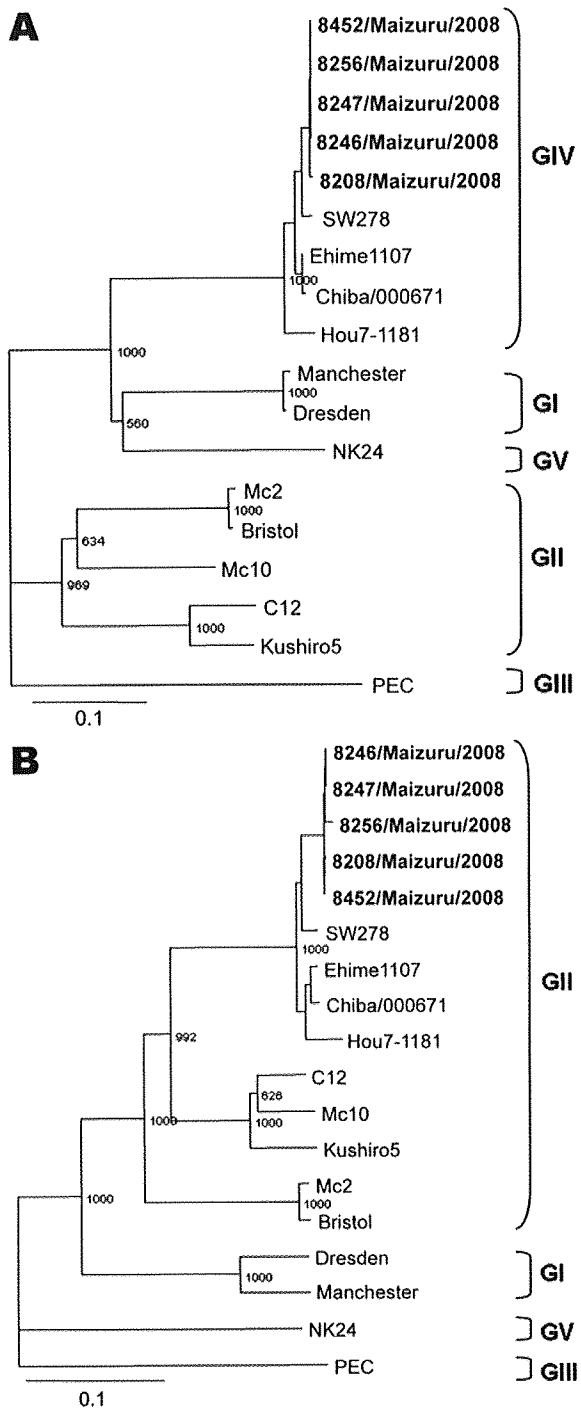

Figure 2. Phylogenetic analysis of the polymerase region (A) and the capsid region (B), showing the different genogroups (GI–GV). The sapovirus (SaV) isolates detected in the study are highlighted in **boldface**. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. GenBank accession numbers of reference strains are as follows: C12 (AY603425), Mc10 (AY237420), Kushiro5 (AB455793), Mc2 (AY237419), Bristol (AJ249939), Dresden (AY694184), NK24 (AY646856), Ehime1107 (DQ058829), Yak2 (AB046353), and Yokohama/16/2007/JP (AB305049).

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Short communication

Molecular analysis of G3 rotavirus among infants and children in Dhaka City, Bangladesh after 1993

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ABSTRACT

Between 2004 and 2005, 917 fecal specimens were collected from children below age 5 who presented to Child Health Institute for treatment of diarrhoea in Dhaka City, Bangladesh. The specimens were screened by RT-PCR for the presence of group A rotavirus and positive stools genotyped. Group A rotavirus was detected in 307 stools and serotype G3P[8] strains were detected in nine specimens. Sequence analysis clustered the G3 strains into one distinct lineage (lineage I) with other Asian G3 strains. In addition, one amino acid change at position 96 in antigenic region A, similar to lineage II G3 Chinese strains, was noted. To our knowledge this is the first report of serotype G3 strains in Bangladesh since 1993 and the first report of the molecular characterization of these strains.

In Bangladesh, rotaviruses cause 6000–14,000 deaths each year in children <5 years of age (ICDDR, B, 2006). Rotaviruses, which comprise a genus in the family *Reoviridae*, are spherical in appearance and measure about 70 nm in diameter. Rotaviruses contain 11 segments of double-stranded RNA. Each segment encodes only one protein, except segment 11 encodes two distinct proteins (Estes and Kapikian, 2007). Rotaviruses are classified into seven groups (A–G) on the basis of their distinct antigenic and genetic properties. Human infection has been reported with group A, B and C rotaviruses. Of these, group A rotavirus is the most important, being a significant cause of severe gastroenteritis in children worldwide (Dey et al., 2009; Parashar et al., 2006). The two outer capsid proteins, VP4 and VP7, allow the rotavirus classification into P and G genotypes, respectively. In rotavirus, at least 20 G and 28 P genotypes have been identified (Solberg et al., 2009). Rotaviruses bearing G1, G2, G3, and G4 are the most common rotaviruses worldwide (Santos and Hoshino, 2005). G3 is one of the most prevalent rotavirus types worldwide but G3 strain has not been detected in Bangladesh since 1993 (Rahman et al., 2007; Kang et al., 2005; Unicomb et al., 1999). The objectives of the study were to investigate the molecular character of G3 strains detected in Dhaka City, Bangladesh in 2005.

Between October 2004 and September 2005, 917 stool specimens were collected from infants and children below 5 years of age presenting with diarrhoea to hospitals in Dhaka City, Bangladesh. In addition, demographic, hospital admission and diarrhoeal symptoms data were also collected. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000 × g for 10 min. The supernatant was collected and viral genomes were extracted from fecal specimens using the QIAamp viral RNA Mini Kit (QIAGEN, Germany). Using RT-PCR with specific primers as previously reported resulted in the identification of diarrhoeal viruses including group A, B, C rotaviruses and adenovirus (Phan et al., 2006).

Group A rotavirus (RAV) positive samples were then subjected to G and P genotyping by nested PCR with previously published primers (Das et al., 1994; Gouvea et al., 1990; Gentsch et al., 1992). The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with SYBR Safe (Invitrogen, Carlsbad, CA, USA) for 30 min then visualized under ultraviolet (UV) light. The bands were recorded by photography.

The nucleotide sequences of PCR products (1025 bp) positive for the rotavirus G3 VP7 genes were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic

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Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Their VP7 nucleotide sequences were compared to each other as well as to those of reference rotavirus strains available in GenBank by BLAST. Sequence analysis was performed using CLUSTAL X software (Version 1.6). The phylogenetic tree with 100 bootstrap replicates of the nucleotide alignment datasets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 3.2 software package (Kumar et al., 2001).

Reference group A rotavirus strains and their accession numbers used in this study were as follows—RAV G3 strains:

J-12/1990/Japan (D86279), TK08/1995/Japan (D86281), TK28/1991/Japan (D86283), TK15/1991/Japan (D86282), CMH054/Thailand (AY707794), CMH229/Thailand (AY707791), CMH272/Thailand (AY707790), RMC437/India (AY603153), CS02-01/Taiwan (AY165009), MP126/India (AF386915), MaCH09004/Malaysia (AY900173), E579/07/China (EU708586), L478/06/China (EU708580), E885/07/China (EU708578), L695/06/China (EU708575), MaCH09404/Malaysia (AY870661) 107E1B/93/India (AB081594), ITO/80/Japan (D86278), CHW17/92/China (D86276), CH-55/90/China (D86274), AU-17/80/Japan (D86272), M/USA (AB180974), Rus-44/Russia (DQ904504), Rus-47/Russia (DQ904505), CMH055/Thailand (AY707793), CMH301/Thailand (AY707789), and SA11 (K02028).

The sequences of VP7 genes of rotaviruses detected in the study had been submitted to GenBank (NCBI, Bethesda, MD) and had been assigned accession numbers FJ713712–FJ713720.

Group A rotavirus was detected in 307 stools and G2 (43.3%) was detected at the highest frequency, followed by G4 (19.5%), G9 (13.7%), G1 (12.7%), and G3 (2.6%). P[8] was the most predominant genotype (53.2%), followed by P[4] (42.9%) (Dey et al., 2009). G2P[4] combination was the most predominant genotype (39%), followed by G4P[8] (18.2%), G9P[8] (13%), G1P[8] (11.8%), and G3P[8] (2.9%) (Dey et al., 2009). In this study, we found nine samples were positive for G3 group A rotavirus strains. All of the genotype G3 strains were found associated with P[8]. Genotype G3 rotavirus infections were only detected during 2005 in the months of January (2/9), February (1/9), March (3/9) and April (3/9). The sequences of the VP7 gene of the nine G3 strains detected in Bangladesh were compared with the VP7 sequences of international G3 strains.

The most common clinical sign and symptoms of G3 rotavirus infected infants and children were dehydration (100%) ($p < 0.001$), abdominal pain (77%) ($p < 0.001$) and vomiting (55%). The majority of children infected with G3 strains demonstrated 5–8 stools (66%; $p > 0.05$) per day. In addition, all infected children were hospitalized and less than 2 years of age (Table 1).

Group A rotavirus infections still contribute a substantial burden to overall diarrhoeal diseases and are the most common enteropathogen responsible for viral gastroenteritis among infants and children in Bangladesh (Dey et al., 2009; Rahman et al., 2007). Similarly, RAV is the most common cause of diarrhoea in the developing countries like Bangladesh, India, Thailand, China, Malaysia, Indonesia, Brazil, Myanmar, and Vietnam (Dey et al., 2009; Nguyen et al., 2007; Kang et al., 2004; Parashar et al., 2006).

G3 is one of the most prevalent rotavirus types worldwide but G3 strain has not been detected in Bangladesh since 1993 (Rahman et al., 2007; Kang et al., 2005; Unicomb et al., 1999). Recently, different surveys showed that the distribution of rotavirus G-types seems to be changing. Rotavirus G1 has decreased, while rotavirus G3 has gradually increased and dominated over other G-types in some countries, such as Japan, China, Russia, and Ireland (Fang et al., 2005; Santos and Hoshino, 2005; Lo et al., 2005; Reidy et al., 2005; Phan et al., 2007; Yoshinaga et al., 2006).

The VP7 genes of the human G3 strains identified in this study and international G3 strains clustered into two distinct branches. Lineage I included the Bangladeshi G3 strains and G3 strains detected in Japan, Thailand, Russia, India, Vietnam and the USA while lineage II comprised Chinese G3 strains isolated between 1990 and 1992 (Wen et al., 1997) (Fig. 1).

Distance matrix analysis of the VP7 gene sequences of these strains combined with their phylogenetic distribution demonstrated two major lineages (designated as I and II) (Fig. 1). Within lineage I, the G3 strains from Bangladesh were more closely related to the G3 strains CMH054/Thailand, CMH272/Thailand, CMH229/Thailand and Russ-44/Russia, demonstrating nucleotide homologies of more than 99% at nucleotide and amino acid level. In addition, nucleotide and amino acid homology of greater than 94% was noted between the Bangladeshi G3 strains and other G3 strains in lineage I. Homology between lineage I G3 strains and the Chinese lineage II strains ranged from 88.5% to 93.1% at an amino acid level and from 84% to 89.5% at a nucleotide level (data not shown). The same lineages were defined by 2.8% and 10.2% nucleotide distances, while distances between lineages were higher than 16.7%. Interestingly, we found one amino acid changes at position 96 in antigenic region A from aspartic acid (Asp) to asparagine (Asn). By amino acid substitutions at this position, with or without glycosylation change, human rotaviruses can change their antigenicity and escape host immunity (Trinh et al., 2007). This amino acid substitution, with others, was thought to be responsible for the emergence of G3 strains in China and Russia. Therefore, the emergence of these G3 variants in Bangladesh should be monitored to examine this trend.

The G3 strains isolated in this study could not be compared to the previous Bangladeshi data, because molecular characterization of the G3 VP7 genes strain in sporadic cases of acute gastroenteritis from Bangladesh were not sequenced. Unlike the emergence of G3 strains in China and Russia, no further G3 strains were detected during the surveillance period and, therefore, a similar pattern of emergence was not noted in the Bangladeshi population. Future surveillance for G3 strains in Bangladesh will allow the investigation of the possible emergence of this serotype after an absence of a decade.

Table 1
Clinical data and P genotypes of selected Bangladeshi G3 rotavirus strains isolated from infants and children in Dhaka City, Bangladesh, 2004–2005.

Isolate	Date of collection	P type	Age (months)	Dehydration	Vomiting	Fever	AP	H	Loose stool/day
BD-73	January, 05	P[8]	14	+	+	+	+	+	5–8
BD-89	January, 05	P[8]	7	+	–	–	–	+	5–8
BD-91	February, 05	P[8]	1	+	+	–	+	+	3–8
BD-211	March, 05	P[8]	5	+	–	+	+	+	5–8
BD-257	March, 05	P[8]	16	+	+	+	+	+	5–8
BD-278	March, 05	P[8]	2	+	–	–	–	+	3–5
BD-283	April, 05	P[8]	11	+	+	–	+	+	5–8
BD-304	April, 05	P[8]	6	+	+	–	+	+	5–8
BD-312	April, 05	P[8]	4	+	–	–	+	+	3–8

AP means abdominal pain. Fever means more >100 °F body temperature. H means hospitalization (>24 h stay in hospital). + means positive. – means negative.

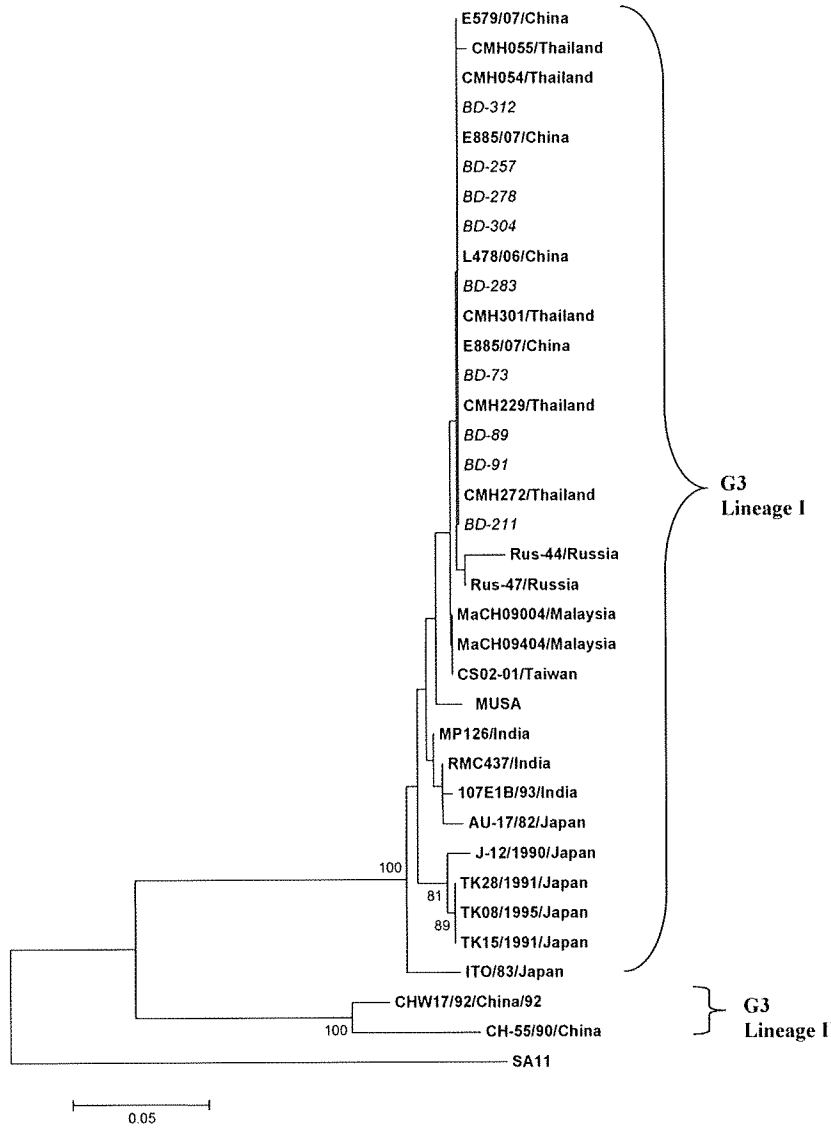

Fig. 1. Phylogenetic analysis of VP7 nucleotide sequences of the Bangladeshi G3 RAV strains. The tree was constructed from nucleotide sequences of VP7 of rotavirus G3 detected in Bangladesh in 2004–2005. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. Percentage bootstrap values above 70% are shown at the branch nodes. Reference strains of RAV G3 rotavirus were selected from DNA Data Bank of Japan (DDBJ/GenBank) under the accession number indicated in bold. Bangladeshi G3 strains in 2004–2005 were highlighted in italic. The Genbank accession numbers of this study are FJ713712–FJ713720.

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Molecular and epidemiological trend of rotavirus infection among infants and children in Japan

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ABSTRACT

This study investigated the prevalence of group A, B, and C rotavirus (RAV, RBV, RCV) and adenovirus (AdV) infections in infants and children with acute gastroenteritis in Japan from July 2006 to June 2007. A total of 628 fecal specimens collected from infants and children with acute gastroenteritis in five different places (Maizuru, Tokyo, Sapporo, Saga and Osaka) in Japan during the period of July 2006–June 2007 were examined for RAV, RBV, RCV and AdV by RT-multiplex PCR. RAV was further characterized to G-typing and P-typing by RT-multiplex PCR and sequencing method. It was found that 123 (19.6%) fecal specimens were positive for RAV followed by AdV of 4.5%. RBV and RCV could not be detected in this study. Serotype G1 (58.5%) was identified at high levels followed by G9 (20.3%), G2 (11.4%), and G3 (7.3%). P genotyping revealed P[8] as predominant (84.6%) followed by P[4] (13.8%) and P[6] (1.6%). During the 2006/2007 rotavirus season, G1P[8] strains were most common with G9P[8], G2P[4], G3P[8], G1P[4] and G9P[6] also detected. It is clear from this study that RAV is still the cause of diseases in Japan. To our knowledge, this is the first report of RV P[6] strain in humans in Japan.

1. Introduction

Viral gastroenteritis is a common disease with a high morbidity reported worldwide, especially in the infants and the elderly. The mortality among children due to acute gastroenteritis is greater in developing countries than in developed countries (Dey et al., 2009b; Parashar et al., 2003).

In the 1940s, viruses were suspected of being important causes of gastroenteritis; however, the etiology remained unknown in most cases. Since then, group A rotavirus (RAV) has been identified as the leading cause of severe gastroenteritis in infants and young children worldwide and is estimated to account for 600,000 deaths in children under 5 years of age (Parashar et al., 2006; Kapikian, 1996).

RAVs are members of the *Reoviridae* family (Matthews, 1979), and are characterized by their non-enveloped icosahedral structure and 70-nm diameter. When observed under an electron microscope, they have a 'wheel' shape (Kapikian and Chanock, 1996; Offit and Clark, 1995). The capsid consists of three protein layers; the outer capsid is composed of the structural proteins VP7 and VP4, and the inner capsid mainly of VP6. The core is found inside the inner capsid, and encloses the rotavirus genome, composed of 11 segments of double-stranded RNA. Given the segmented nature of the RNA genome, co-infection of cells with two different strains of rotavirus may result in reassortant viruses, with RNA segments from each of the progenitors (Desselberger, 1996).

RAVs are classified into seven groups (A–G) on the basis of distinct antigenic and genetic properties (Bridger et al., 1986). Human infections have been reported with group A, B, and C rotaviruses. Of these, RAVs are the most important, being a major cause of severe gastroenteritis in infants and young children worldwide (Kapikian et al., 2001). On the basis of reactivity with monoclonal antibodies and/or sequence analysis, the VP7 and VP4 proteins and/or corresponding encoding genes of RAVs have been classified into G- and/or P-serotypes and/or genotypes. A total of 20 G and 28 P genotypes have been identified (Solberg et al., 2009). Although numerous G/P combinations have been reported, a recent review revealed G1P[8], G2P[4], G3P[8] and G4P[8] as four globally important rotavirus G/P combinations (Santos and Hoshino, 2005). The major human G-types including G1, G2, G3, G4, and G9, which, combined with P-types P[8], P[4], and P[6],

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account for >80% of rotavirus-associated gastroenteritis episodes worldwide (Dey et al., 2009b; Gentsch et al., 2005).

The G1, G3, G4, and G9 types are usually associated with P[8] specificity, while G2 is associated with P[4]. The role of type-specific versus cross-protective (heterotypic) immunity for protection against rotavirus infection and disease is still debated. Taking into account the antigenic heterogeneity of rotaviruses, recently, a promising monovalent G1P1A[8] human attenuated vaccine, Rotarix, which is reported to induce good heterotypic protection, has been licensed. This formulation is derived from the fact that both P1A[8] and G1 specificities are unanimously acknowledged as the most prevalent and ubiquitous (Arista et al., 2006).

G- and P-genotyping is performed using type-specific primer-based RT-PCR. Two primer sets, designed by Das et al. (1994) and Gouvea et al. (1990), are routinely utilized for G genotyping while one primer set, designed by Gentsch et al. (1992), is used for P-typing.

Enteric adenovirus 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 (Dey et al., 2009a).

The objective of this study was to describe the molecular and epidemiological trends in the circulation of rotavirus strains among infants and young children with acute gastroenteritis in Japan during the 2006/2007 seasons.

2. Materials and methods

2.1. Study population and fecal specimens

Between July 2006 and June 2007, 628 stool specimens were collected from infants and children below ten years of age presenting with diarrhoea to hospitals in five different places (Maizuru, Tokyo, Sapporo, Saga and Osaka), Japan. Most of the stool samples collected from Tokyo (252/628), followed by Maizuru (211/628), Saga (92/628), Osaka (48/628) and Sapporo (25/628). The stool specimens were stored at −30 °C until use for the detection of diarrheal viruses.

2.2. Extraction of viral genome

Fecal specimens were thawed, diluted with distilled water to 10% suspensions, and centrifuged at 10,000 × g for 10 min. Viral RNA was extracted from 140 μl of the supernatant using a spin-column technique (QIAamp Viral RNA kit; QIAGEN®, Hilden, Germany) according to the manufacturer's instructions.

2.3. Reverse transcription

For reverse transcription (RT), 4 μl of extracted viral genome was added with 4 μl of a reagent mixture consisting of 1 μl of 5× first strand buffer (Invitrogen, Carlsbad, CA, USA), 0.4 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.4 μl of 10 mM DTT (Invitrogen, Carlsbad, CA, USA), 0.4 μl of superscript reverse transcriptase III (Invitrogen, Carlsbad, CA, USA), 0.2 μl of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.3 μl of RNase inhibitor (Toyobo, Osaka, Japan) and 1.3 μl MilliQ water. The total of reaction mixture was 8 μl. The RT step was carried out at 50 °C for 1 h, followed by 99 °C for 5 min and then held at 4 °C (Phan et al., 2005).

2.4. Screening for target viral pathogens by multiplex PCR

A group of viruses including RAV, RBV, RCV and AdV were detected by RT-PCR with previously reported primers (Phan et al., 2005). For the detection of group A, B, C rotaviruses and adenovirus, primers sets Beg9 and VP7-1, B5-2 and B3-3, G8NS1 and G8NA2, Ad1 and Ad2 were used, respectively. The relative amplicon sizes for RAV, RBV, RCV and AdV detection were 395 bp, 814 bp, 352 bp and 482 bp, respectively.

For polymerase chain reaction (PCR), 1 μl of cDNA was added with 10 μl of a reagent mixture consisting of 2.6 μl 10× Taq DNA polymerase buffer (Promega, MADISON WI USA), 1 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 1.6 μl of primers, 0.06 μl of Taq DNA polymerase (Promega, MADISON WI USA) and 4.74 μl MilliQ water. The total of reaction mixture was 11 μl. 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.

2.5. Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with SYBR Safe (Invitrogen, Carlsbad, CA, USA) for 30 min then visualized under ultraviolet (UV) light. The bands were recorded by photography.

2.6. Rotavirus VP-7 gene analysis

G-typing of RAV was performed by using previously published specific primers (Das et al., 1994). The nearly full-length of VP7 was amplified with primers 9con1 and End9. The size of PCR product generated from the nearly full-length VP7 was 1025 bp. The second amplification was performed using the first PCR product as the template with G-type-specific mixed primers (9T1-1, 9T1-2, 9T1-3P, 9T-4 and 9T-B) for downstream priming and 9con1 for upstream priming in amplification of VP7 genes of G1–G4 and G9, respectively. The amplicon sizes for G1, G2, G3, G4 and G9 strains detection are 158 bp, 224 bp, 466 bp, 403 bp and 110 bp, respectively.

2.7. Rotavirus VP-4 gene analysis

P-typing of RAV was performed by using previously published specific primers (Gentsch et al., 1992). The VP4 gene was amplified with primers Con2 and Con3. The size of PCR product generated from the VP4 gene was 877 bp. The second amplification was performed using the first PCR product as the template with P-type-specific mixed primers (2T-1, 3T-1, 1T-1, 4T-1 and 5T-1) for downstream priming and con3 for upstream priming in amplification of VP4 genes of P [4], P [6], P [8], P [9] and P [10], respectively. The amplicon sizes for P[4], P[6], P[8], P[9] and P[10] strains detection are 484 bp, 268 bp, 346 bp, 392 bp and 584 bp, respectively.

2.8. Nucleotide sequence and phylogenetic analyses of RAV

The nucleotide sequences for RAV VP7 (1025 bp) and VP4 (877 bp) genes were determined by using the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc. Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 3.2 software package (Kumar et al., 2001).

2.9. Phylogenetic analysis

Reference strains and accession numbers used in this study were as follows:

RAV G1 strains: PA73R-/04 (DQ377601), PA17c/86 (DQ377567), PA51R-/04 (DQ377599), PAG128/95 (DQ377580), PAG161/95 (DQ377581), PA11/89 (DQ377570), PA33/89 (DQ377571), PAF166/94 (DQ377578), PA378/00 (DQ377589), PA430/00 (DQ377591), PA5/90 (DQ377573), PA10/90 (DQ377587), PA103/02 (DQ377595), AU19 Japan (AB018697), C60Porcine (L24164), T44Bovine (M92651), Kor-64 (U26378), Fin-220 (Z80294), Fin-110 (Z80277), Wa-USA/74 (K02033), D (AB118022), Brazil4 (U26365), PA3c (DQ377566), PA32 (DQ377574), PA8/01 (DQ377592), PA19/01 (DQ377593), PA2/04 (DQ377598), PA5/03 (DQ377596), and 407-B4-Russia (S83903).

RAV P[8] strains: BP785/00/Hungary (AJ605315), VA70/USA (AJ540229), WA/USA (L34161), HOCHI/Japan (AB039943), ODELIA/Japan (AB039942), MW670/Malawi (AJ302146), OP530/Malawi (AJ302152), AI-75/Japan (AB008285), MW258/Malawi (AJ302143), OP511/Malawi (AJ302151), CH927B/China (AB008273), MO/Japan (AB008278), Kagawa/90-513/Japan (AB039944), CU132P8/Thailand (DQ235955), DK V00-2138/Denmark (AY509910), S8/Porcine/Brazil (AF052449), CU90P8/Thailand (DQ235978), WH-1194/China (AY856445), Dhaka25-02 (DQ146652), CU90P8 (DQ235978), and WH-624/China (AY856444).

3. Results

3.1. Diversity of viral agents causing acute gastroenteritis

Among diarrheal viruses detected, RAV was the most common viral enteropathogen, which could be identified in 123 samples (19.6%). AdV was responsible for 28 cases (4.5%). Co-infection between RAV and AdV was identified in 7 out of 628 samples. The co-infection was confirmed by monoplex-PCR with specific primers. RBV and RCV could not be detected in this study.

3.2. Distribution of age and gender

For the pediatric population, the lowest age was 1-month, the highest was 6 years, and the average age was 4-month. Most of the patients were in the 1–12-month age group.

The distribution of gender in RAV positive cases was 55% in male and 45% in female.

3.3. Distribution of rotavirus G- and P-types

The distributions of G- and P-types of RAV during the study period from June 2006 to July 2007 are shown in Table 1. Genotype G1 strains were the most common (61%) followed by G9 (20.3%), G2 (11.4%), and G3 (7.3%) consecutively (Table 1). The 123 rotavirus positive specimens were P genotyped and P[8] was predominant (84.6%) followed by P[4] (13.8%) and P[6] (1.6%) at lower levels. Strains bearing G1P[8] antigens were predominant throughout the study period (58.5%) although G9P[8] (18.7%), G2P[4] (11.4%), G3P[8] (7.3%), G1P[4] (2.5%) and G9P[6] strains were also detected.

Table 1
Distribution of Group A rotavirus G and P Genotypes among infants and children with acute gastroenteritis in Japan during 2006 to 2007.

	P[4]	P[8]	P[6]	Total
G1	3	72	0	75
G9	0	23	2	25
G2	14	0	0	14
G3	0	9	0	9
Total	17	104	2	123

3.4. Nucleotide sequence and phylogenetic analyses of RAV

In an attempt to understand the molecular basis of RAV genetic diversity within G1, 21 rotavirus G1 isolates were randomly chosen and their VP7 genes were sequenced. As shown in Fig. 1, the G1 RAV segregated into six lineages (I–VI) and the Japanese G1 RV clustered into two such genetic lineages, lineage I with 24% (5 of 21) and lineage II with 76% (16 of 21). Within lineage II, all 16 strains belonged to sublineage IIa and closely related to PA36/92, PA19/92, PA11/89 and PA33/89 strains. Within lineage I, 3 RAV G1 strains, belonged to sublineage Ia and closely related to PA8/01 and PA19/01 strains which had great identities at the nucleotide as well as amino acid level. On the other hand, other 2 strains belonged to sublineage Ic. The degree of nucleotide identity among the Japanese G1 strains in each lineage was >96.5% within lineage I and >94.5% within lineage II.

In an attempt to understand the molecular basis of RAV strain genetic diversity within P[8], 21 rotavirus P[8] isolates were randomly chosen and their VP4 genes were sequenced. As shown in Fig. 2, the P[8] RAV segregated into four lineages (I–IV) and the Japanese P[8] RAV sequences in this study were classified into one distinct lineage, lineage II and sublineage IIa. P[8] strains analyzed in this study belonged to Asian cluster and were most closely related to Kagawa/90-513/Japan, Dhaka25-02, CU132P8/Thailand, WH-1194/China, WH-624/China and CU90P8/Thailand strains which had great identities at the nucleotide level each other (96–99%).

3.5. Seasonal pattern of RAV and adenovirus infection

Number of stool samples collected in each month from infants and children with acute gastroenteritis in Japan during July 2006 to June 2007 was shown in Fig. 3A. The seasonal pattern of RAV infection in this study is shown in Fig. 3B. The rotavirus detection rate was analyzed between July 2006 and June 2007. RAV was detected continuously in the 7-month period lasting from December to June (Fig. 3B). Rotavirus was detected most frequently in March–May ($P < 0.002$). The presence of rotavirus remained low from 0% to 5.2% in August–November. From this study, it is clear that rotavirus infection is common in late winter and spring season in Japan. The highest prevalence of RAV was found in April (27/123) followed by May (23/123) and December (19/123) respectively. On the other hand, adenovirus was detected continuously in the 6-month period lasting from December to May. Adenovirus was most frequently detected in February to May ($P < 0.001$).

3.6. Nucleotide sequence submission

The sequences of VP7 and VP4 genes of rotaviruses detected in the study had been submitted to GenBank and had been assigned accession numbers FJ713721-FJ713743.

4. Discussion

RAV is the most common cause of non-bacterial gastroenteritis, not only in the developing countries like Bangladesh, India, Thailand, China, Malaysia, Indonesia, Brazil, Myanmar, Vietnam but also in developed countries like Japan, Korea and Germany (Dey et al., 2009b; Nguyen et al., 2007; Kang et al., 2004; Oh et al., 2003; Parashar et al., 2003; Santos and Hoshino 2005; Song et al., 2003). A total of 628 fecal specimens were tested for rotavirus and 19.6% were positive. These results are consistent with previous findings on rotavirus prevalence in Japan ranging from 15% to 25% (Phan et al., 2007a; Phan et al., 2007b; Yoshinaga et al., 2006). While approximately 20% of the diarrhoeal cases were due to rotavirus and 4.5% were due to enteric adenovirus, 75.5% were

Fig. 1. Phylogenetic tree of nucleotide sequence of Japanese rotavirus G1 isolates. The tree was constructed from nucleotide sequences of VP7 of rotavirus G1 isolates detected in Japan in 2006–2007. Reference strains of rotavirus G1 were selected from DNA Data Bank of Japan (DDBJ/GenBank) under the accession number indicated in bold. Japanese G1 strains in 2006–2007 were highlighted in italic. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values.