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Development of genotype-specific primers for differentiation of genotypes A and B of Aichi viruses

Ngan Thi Kim Pham[a], Quang Duy Trinh[a], Tuan Anh Nguyen[a], Shuvra Kanti Dey[a], Tung Gia Phan[a], Le Phuc Hoang[b], Pattara Khamrin[c], Niwat Maneekarn[d], Shoko Okitsu[e], Masashi Mizuguchi[a], Hiroshi Ushijima[c,*]

[a] Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[b] Department of Gastroenterology, Children's Hospital 1, Ho Chi Minh City, Viet Nam
[c] Aino Health Science Center and Aino University, Tokyo, Japan
[d] Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand
[e] Aino Science Center and Aino College, Tokyo, Japan

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ABSTRACT

A nested polymerase chain reaction method using genotype-specific primers based on the capsid gene was developed to differentiate between genotypes A and B of Aichi viruses. Results of the study showed that the PCR using newly designed genotype-specific primers could generate appropriate PCR products from all 17 samples tested, the newly developed primers could differentiate genotype A from genotype B, and all matched those obtained by nucleotide sequencing of the capsid regions. The nested PCR method using genotype-specific primers is useful and can be used for genotyping of Aichi viruses isolated from epidemiological studies.

1. Introduction

Aichi virus was first recognized in 1989 as the likely cause of oyster-associated gastroenteritis in Japanese patients. It is a small round virus about 30 nm in diameter (Yamashita et al., 1991). The virus was classified into a new genus named Kobuvirus of the family *Picornaviridae* which contains nine genera: Aphthovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus (which includes Aichi virus and bovine kobuvirus), Parechovirus, Rhinovirus, and Teschovirus according to the International Committee on Taxonomy of Viruses (Yamashita et al., 1998; Pringle, 1999).

The complete Aichi virus genome was first determined in 1998 and shown to be a single-stranded positive-sense RNA molecule with 8280 bases excluding a poly (A) tail (Yamashita et al., 1998; Sasaki et al., 2001). In the year 2000, a reverse transcription-polymerase chain reaction (RT-PCR) method for the detection of Aichi virus was developed and genetic analysis was performed based on the 519 base RNA sequences at the putative junction between the C terminus of 3C and the N terminus of 3D. As a result, Aichi virus isolates have been divided into two groups: group 1 (genotype A) and group 2 (genotype B) (Yamashita et al., 2000). Recently, Aichi virus was detected in Brazil, Germany, France, Thailand, Bangladesh, Vietnam, and Tunisia (Oh et al., 2006; Pham et al., 2007; Ambert-Balay et al., 2008; Sdiri-Loulizi et al., 2008). A new Aichi virus genotype (genotype C) which contains a sole strain detected in France was also identified (Ambert-Balay et al., 2008).

Aichi virus virions contain three capsid proteins (VP0, VP3, and VP1), not four as other picornaviruses (VP1, VP2, VP3, and VP4), capsid proteins of 42, 30, and 22 kDa. No protein band corresponding to VP4 (usually 7–8 kDa) was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Yamashita et al., 1998). In comparison to genotype A strains, the capsid gene sequences of genotype B strains (including a sole strain from Brazil and three others from Bangladesh) have a triple nucleotide insertion within the VP0 region (Yamashita et al., 1998; Oh et al., 2006; Pham et al., 2008). In a previous study, phylogenetic and sequence analyses of the capsid gene were undertaken and a high correlation of Aichi virus genotype to the 3CD junction region was demonstrated (Pham et al., 2008), and the genetic diversity of the capsid sequences of Aichi virus strains within each genotype was determined. The results showed that nucleotide sequence diversity within genotype A was higher than that of genotype B. In addition, the nucleotide distances between the two genotypes were equal to or greater than 12.7%, and the genomic differences between

* Corresponding author. Tel.: +81 3 3486 8481; fax: +81 3 3486 8481.
E-mail address: ushijima-hiroshi@jcom.home.ne.jp (H. Ushijima).

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them could be found throughout the capsid sequences. At least 14 different deduced amino acids which were considered to be genotype-specific, especially the different triple of amino acids located within the VP0 region, were found and can be considered as the reference region for developing primers for genotyping (Pham et al., 2008).

For identification, Aichi virus genotype has been determined based only on sequence analysis of the 3CD junction region or the capsid gene, a critical method for genotyping of this virus. However, these procedures are complex and time consuming, requiring expensive reagents, equipment, and well-trained personnel. Therefore, the aim of this study was to develop a nested PCR method using genotype-specific primers based on the capsid gene for genotyping of Aichi virus.

2. Materials and methods

2.1. Primer designation

Reference nucleotide sequences of the capsid genes of 15 Aichi virus strains were obtained. Three of the fifteen capsid sequences were derived from Aichi virus reference sequences available in the GenBank database under the accession numbers AB040749, AY747174, and DQ028632. The 12 remaining nucleotide sequences of the capsid gene were from our previous study and their accession numbers are the following: EU143271 to EU143279, EU143282, EU143286, and EU143287 (Pham et al., 2008). Alignment of the 15 capsid gene sequences using Clustal X software was performed to find the divergent and conserved regions and to create genotype-specific primers. Genotype-specific primers for A and B were designed based on the different sequences between A and B genotypes.

2.2. Specimens tested

Seventeen stored extracted RNAs known to be positive for Aichi virus were used in this study. The samples were collected from epidemiological and surveillance studies conducted in the following countries: Japan (10 samples), Bangladesh (3 samples), Thailand (1 samples), and Vietnam (3 samples) (Phan et al., 2005a; Khamrin et al., 2006; Dey et al., 2007; Nguyen et al., 2007). The genotypes of all 17 Aichi strains isolated from the above samples were known as genotype A or B from previous studies by sequence analysis (Pham et al., 2007, 2008).

2.3. Reverse transcription

For reverse transcription, 5 μl of the stored, extracted RNA was added to a reagent mixture consisting of 5× First Strand Buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannheim, Germany), 0.1 M 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 distilled water. The total volume of reaction mixture was 15 μl. RT reaction was carried out at 50 °C for 1 h, followed by 95 °C for 5 min and then rapid cooling on ice (Yan et al., 2004; Phan et al., 2005b).

2.4. Differentiation of genotypes A and B by nested PCR with genotype-specific primers

After adding 2 μl of cDNA into 23 μl of the reagent mixture containing 5× Taq DNA polymerase buffer (Promega, Madison, WI, USA), dNTPs (10 mM), primers (20 μM), Taq DNA polymerase (5 U/μl) (Promega, Madison, WI, USA), and distilled water, the first PCR was conducted using primers AiV-Cap-F and AiV-Cap-R (Table 1). The PCR protocol was 95 °C for 1 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Next, the nested PCR was carried out using three primers: AiV-Cap-A, AiV-Cap-B, and AiV-Cap-ABR (Table 1) with the same thermal cycler program to generate a 336 bp product, which is a partial segment of VP3, specific for Aichi virus genotype A or an 879 bp product, which is a partial segment of VP0 and VP3, specific for Aichi virus genotype B (Fig. 1). Analysis of the amplification products was performed by 1.5% agarose gel electrophoresis, and the bands were visualized by SYBR Safe (Invitrogen, Tokyo, Japan) staining under ultraviolet light. The results of genotyping by PCR were compared to the results determined by sequence analysis of the capsid gene.

Table 1
Oligonucleotide sequences of the newly developed primers and positions. Y means C or T, and M means C or A. Sequence position (*) is based on the full genome sequence of the Japanese reference strain with the accession number of AB040749.

Primer	Sequence 5′ to 3′	Sense	Position*
AiV-Cap-F	GGC GAY CGC ACC TGG TTG	+	1765–1782
AiV-Cap-R	GAG ATG AAG GGG ATG GT	–	2837–2821
AiV-Cap-A	TGC CTA CTA CCC CCC MAA TG	+	2448–2467
AiV-Cap-B	TCY ACA AAC TCT CCC ACC GC	+	1905–1924
AiV-Cap-ABR	GTG TAT GTT CCG CGC AT	–	2783–2767

3. Results

The oligonucleotide sequences of the new primers are shown in Table 1. The genotype A-specific primer (AiV-Cap-A) was designed based on six significant nucleotide mismatches between the capsid genes of the two genotypes at positions 2448–2467. The genotype B-specific primer (AiV-Cap-B) was developed at the positions 1905–1924 containing 8–10 nucleotide mismatches, especially including an insertion of CCC or TCC triple at positions 1917–1919 (located within the VP0 region) of the capsid sequences of genotype B strains when compared to those of genotype A strains. The primer binding sites of the two genotype-specific primers and the expected PCR products are shown in Fig. 1.

Using 17 genotype-known reference strains, PCR genotyping was done using the newly developed primers. PCR products were successfully generated from all 17 samples; 14 were 336 bp segments identified as genotype A, and the 3 remaining were 879 bp segments identified as genotype B. In other words, 14 genotype A and 3 genotype B Aichi virus strains were recognized by using this genotyping PCR. The electrophoresis result is shown in Fig. 2. These results matched those obtained by analyses of the capsid gene sequenced previously.

To test the specificity of the new genotype-specific primers, an additional PCR was carried out using genotype-determined samples with different genotype-specific primer for the second round of the genotyping PCR. For example, using genotype A-specific primer (AiV-Cap-A) for genotype B sample, or conversely, genotype B-specific primer (AiV-Cap-B) for genotype A sample. By such a PCR, no cross-reaction was observed. All samples were negative, except for two positive control reactions by using the right matches of the genotype-specific primers and genotype-known samples: sample A with primer AiV-Cap-A, and sample B with primer AiV-Cap-B (data not shown).

4. Discussion

These results demonstrate that nested PCR using the new primers generated appropriate PCR products from all 17 samples tested, and the new primers can separate genotype A from genotype B. As demonstrated in this study, the new primers are sensitive and specific enough for differentiation between genotypes A and

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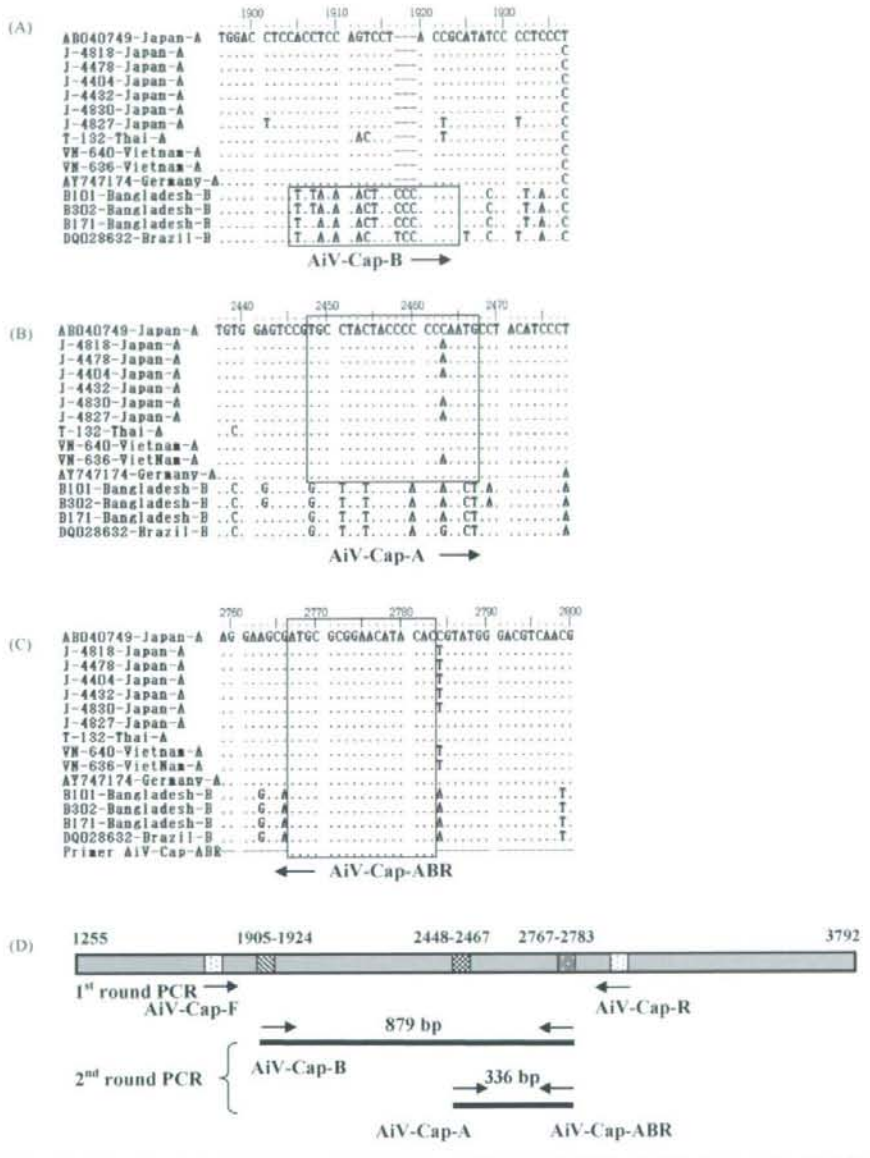

Fig. 1. (A–C) Extracts of alignment results of the 15 reference capsid gene sequences. The identical nucleotides in each Aichi reference strain with the Japanese reference strain under the accession number AB040749 are identified by dots. The primer binding areas are in boxes. (D) Schematic representation of the capsid gene of Aichi virus, and the expected PCR products.

B of Aichi viruses. The new primers functioned accurately and conclusively. Obviously, without the need for sequencing, the genotyping method by PCR is easier, faster, and more cost effective when compared to the critical method of sequence analysis of the 3CD junction region or the capsid region.

Briefly, nested PCR using genotype-specific primers is useful and can be used for genotyping of Aichi viruses isolated during epidemiological studies. However, although the new primers were developed based on all available full lengths of the capsid gene of Aichi viruses in GenBank up to the time when the study was conducted, it is noted that the number of reference capsid genes was limited to 15 sequences. Globally, there have been only 3 full lengths of the capsid gene which were derived from 3 full genomic sequences of reference strains (each of these strains isolated from

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Fig. 2. Results of genotyping using the reference strains. The PCR fragments possess the expected molecular weights when compared to the molecular weight marker: (lanes 1–14) genotype A, (lanes 15–17) genotype B, (lane M) 100 bp ladder marker.

Japan, German, and Brazil) and 12 others which were obtained in a previous study (Pham et al., 2008). Therefore, the limitation of primer designation regarding reference strains of this study is also the limitation of global studies on the capsid gene of Aichi viruses. In addition, because of the low detection rate of Aichi virus from specimens, the number of samples positive for Aichi virus available for primer testing was small. Consequently, despite this study showing a strong capacity for differentiating between genotypes A and B of Aichi viruses, these new primers should be evaluated further with larger numbers of Aichi virus strains.

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Molecular Characterization of Rotaviruses, Noroviruses, Sapovirus, and Adenoviruses in Patients With Acute Gastroenteritis in Thailand

Leera Kittigul,[1]* Kannika Pombubpa,[1] Yuthana Taweekate,[1] Thanapat Yeephoo,[2] Pattara Khamrin,[3] and Hiroshi Ushijima[3]

[1]*Faculty of Public Health, Department of Microbiology, Mahidol University, Bangkok, Thailand*
[2]*Medical Pathology Department, Lopburi Hospital, Lopburi Province, Thailand*
[3]*Aino Health Science Center, Aino University, Tokyo, Japan*

Outbreaks of viral gastroenteritis occur worldwide including Thailand. Unfortunately, there is limited information since etiologic agents have not been identified in several outbreaks of nonbacterial gastroenteritis. The genotype of enteric viruses causing acute gastroenteritis in Thailand was determined using reverse transcription-multiplex polymerase chain reaction and DNA sequencing. From January 2006 to February 2007, stool samples were collected from patients with acute gastroenteritis of all age groups attending a hospital in Thailand, and patients with nonbacterial acute gastroenteritis (262 patients) were tested for enteric viruses. The overall positive detection rate of enteric viruses was 14.9%; group A rotaviruses (6.1%), noroviruses (6.5%): GI (0.8%) and GII (5.7%), adenoviruses (1.5%), and sapoviruses (0.8%) were found. Group B and C rotaviruses, and astroviruses were not detected in the enrolled patients. Viral acute gastroenteritis occurred in children less than 15 years of age (25.2%, 33/131) with higher frequency than in adults (4.6%, 6/131), *P*-value <0.001. Rotavirus G1 was the most predominant genotype, followed by G3, and G9. Among noroviruses, GI-2 was identified; whereas, GII was predominant with a high frequency of GII-4 observed, followed by GII-16, GII-2, GII-3, and GII-12. Sapovirus GII-3 and human adenoviruses were identified. This study suggests that enteric viruses play an essential role in patients with acute gastroenteritis attending hospital and mainly in children who have a higher prevalence of group A rotaviruses and noroviruses. The genetic analyses provide molecular epidemiological data for viruses important to public health. ***J. Med. Virol. 81: 345–353, 2009.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: rotaviruses; noroviruses; sapoviruses; adenoviruses; acute gastroenteritis; Thailand

INTRODUCTION

Acute gastroenteritis or acute diarrhea is one of the most significant diseases causing morbidity and mortality worldwide [Clark and McKendrick, 2004]. In Thailand, acute diarrhea is the primary cause of morbidity among diseases documented in the annual report of epidemiological surveillance [http://epid.moph.go.th]. Based on routine bacterial cultures of stool samples, only a few patients have been identified in the past with an underlying viral cause. However, a large proportion of patients with gastroenteritis were not identified with enteric viruses because virus identification has not been undertaken routinely in the country. Using improved molecular techniques, the etiologic role of viruses causing acute gastroenteritis has been established in various outbreaks [Koopmans, 2005; Siebenga et al., 2007; Svraka et al., 2007] and in studies on hospitalized children [Chen et al., 2007; Fabiana et al., 2007]. Enteric viruses that have been reported as a cause of nonbacterial acute gastroenteritis include group A rotaviruses, noroviruses, sapoviruses, astroviruses and enteric adenoviruses.

Rotaviruses (RVs) are members of the Reoviridae family, which consists of seven groups (A through G). Group A rotavirus causes acute diarrhea and is most commonly found among infants and young children [Glass et al., 2006]. Group B and C rotaviruses, however, infect children to a lesser extent [Phan et al., 2004; Barman et al., 2006]. RV is a nonenveloped virus containing 11 segments of double-stranded RNA [Estes

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*Correspondence to: Leera Kittigul, Faculty of Public Health, Department of Microbiology, Mahidol University, 420/1 Rajvithi Road, Bangkok 10400, Thailand. E-mail: phlkt@mahidol.ac.th

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and Kapikian, 2007]. There are 19G genotypes and 27P genotypes in RVs recovered from humans and animals [Matthijnssens et al., 2008]. The most prevalent human RVs are G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] [Santos and Hoshino, 2005]. Noroviruses (NoVs), which belong to the genus Norovirus in the family Caliciviridae, are the most common viral cause of acute gastroenteritis in all ages but are known to have more severity in young children and the elderly [Estes et al., 2006]. NoVs are nonenveloped, positive-sense, single-stranded RNA viruses and are classified into five distinct genogroups. Human NoV strains have been found in genogroups I (GI), II (GII), and IV (GIV) and are subdivided further into at least 8 genotypes for GI, and 19 genotypes for GII [Zheng et al., 2006]. Sapoviruses (SaVs) are members in the genus Sapovirus within the same Caliciviridae family as NoVs. Among those admitted to hospital, acute diarrhea caused by SaVs is more widespread in infants and young children. SaVs have a lower detection rate than NoVs [Hansman et al., 2004b; Khamrin et al., 2007a]. Human astroviruses (AsVs) belong to the Astroviridae family. The virion is nonenveloped and contains a positive single-stranded RNA genome [Mendez and Arias, 2007]. Besides rotaviruses and caliciviruses, AsVs are one of the major causes of acute gastroenteritis within young children and the elderly [Guix et al., 2005]. Human adenoviruses (AdVs) are only one DNA virus among other enteric RNA viruses and members of the family Adenoviridae, which consists of six subgenera (A through F). AdVs subgenus F are divided into two serotypes (AdV40 and AdV41) both of which are associated with diarrhea in children [Fabiana et al., 2007].

Rotaviruses and noroviruses are important enteric viruses in Thailand because they are often causes of acute gastroenteritis in infants and children admitted to hospital. In two studies, RVs were reported to be associated with approximately 37–43% of children with acute gastroenteritis admitted to hospital [Jiraphongsa et al., 2005; Khamrin et al., 2007c]. In other studies, NoVs were detected in hospitalized infants and children at a rate of 8.6–14.1% [Guntapong et al., 2004; Hansman et al., 2004b; Khamrin et al., 2007a]. Nevertheless, genetic analysis of the enteric viruses causing acute gastroenteritis has not been studied extensively. This particular study was carried out to determine the presence of gastroenteritis viruses in patients of all age groups attending hospital with acute gastroenteritis. The study used reverse transcription-multiplex polymerase chain reaction (RT-multiplex PCR) for screening and DNA sequencing for confirmation. Molecular characterization of the enteric viruses found in the stool samples of patients with acute gastroenteritis is included.

MATERIALS AND METHODS

Stool Samples

A total of 273 stool samples were collected from patients attending Lopburi Hospital, Lopburi Province, Thailand, and who had a clinical diagnosis of acute gastroenteritis or acute diarrhea. The study period was from January 2006 to February 2007, a 14-month period. The patients enrolled in this study had watery diarrhea for less than 7 days and one or more of the following symptoms; nausea, vomiting, abdominal cramps, headache, muscle pain and/or fever (≥38.0°C). Written informed consent was obtained from each patient or, if the patient was a child, their parent. The study was conducted with the approval of the Ethical Committee for Human Rights Related to Human Experimentation, Mahidol University, and of Lopburi Hospital. Stool samples were tested routinely for pathogenic bacteria, that is, *Salmonella*, *Shigella*, and *Vibrio* species. All stool samples were diluted in a ratio of 1:3 in 0.05 M phosphate-buffered saline, at pH 7.2. The suspensions were centrifuged at 500*g* for 15 min and the supernatant was stored at −70°C. Samples were sent to the laboratory and diluted 1:10 prior to RNA extraction.

RNA Extraction and Reverse Transcription-Multiplex Polymerase Chain Reaction (RT-Multiplex PCR)

Viral RNA was extracted from 140 μl of diluted supernatant (1:10) using a QIAamp Viral RNA Mini kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. Screening for the presence of RV groups A, B, and C, NoV GI and GII, SaV, AsV, and AdV in the stool specimens was conducted using RT-multiplex PCR. Two sets of primers were used: set A, including the primers for the detection of RV groups A, B, C, and AdV; and set B, primers for the detection of NoV GI, GII, SaV, and AsV. The RT-multiplex PCR was performed according to the methods described previously by Yan et al. [2003, 2004].

Briefly, complementary DNA (cDNA) was prepared by adding 5 μl of RNA to 5 μl of random hexamers (50 ng/μl), 10 mM dNTP mix and DEPC-treated water (Invitrogen, Carlsbad, CA) and this was followed by incubation at 65°C for 5 min. The mixture was put on ice for 5 min. RT mixture (10 μl) containing 1× RT buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 5 mM $MgCl_2$, 1 mM of each dNTP, 0.1 M DTT, Rnase OUT™ (40 Units/μl), and Superscript™ III RT (200 Units/μl), was then added to the sample. RT was carried out at 25°C for 10 min, which was followed by 50°C for 50 min. The reaction was terminated by incubation at 85°C for 5 min, and then chilling the mixture on ice. cDNA was centrifuged briefly, 1 μl of RNaseH was added and the resultant mixture was incubated at 37°C for 20 min.

In the multiplex PCR assay, 5 μl of cDNA was added to 20 μl of PCR mixture containing 1× *Taq* DNA polymerase buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 200 μM dNTP, and 33 μM each of specific primers, 5 U/μl of *Taq* DNA polymerase, and nuclease free water. Two specific primer sets were added in separate tubes. Set A contained Beg 9, VP7-1′ [group A RV]; ADG9-1F, ADG9-1R [group B RV]; G8NS1, G8NS2 [group C RV];

and Ad1, Ad2 [AdV]. While set B contained G1-SKF, G1-SKR [NoV GI]; COG2F, G2-SKR [NoV GII]; SLV 5317, SLV 5749 [SaV]; and PreCAP1, 82b [human AsV]. PCR was performed at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 3 min.

Monoplex PCR was also carried out for confirmation of virus-positive stool samples detected by RT-multiplex PCR using the procedure for RT-multiplex PCR but with only one pair of primers specific to each virus.

PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (0.5 μg/ml) for 30 min, then visualized under ultraviolet light of a transilluminator. The results were recorded by photography. Specific DNA bands that corresponded to the product size for enteric viruses included group A RV (395 bp), group B RV (814 bp), group C RV (351 bp), AdV (462 bp), NoV GI (330 bp), NoV GII (387 bp), SaV (434 bp), and AsV (719 bp).

DNA Sequencing and Phylogenetic Analysis

DNA products were purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced at the Bioservice Unit of the National Science and Technology Development Agency, Bangkok. The nucleotide sequences were compared with those of RV, NoV, SaV, and AdV strains deposited in the NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search Tool) program [Altschul et al., 1990]. Phylogenetic relationships of RV, NoV, and SaV were examined by aligning sequences with the ClustalX program. A phylogenetic tree was constructed according to the neighbor-joining method using MEGA version 3.1 [Kumar et al., 2004].

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the study strains have been deposited at GenBank (accession nos., EU603407-EU603420 for RVs, EU603421 for NoV GI, EU603422-EU603436 for NoVs GII, EU603437 for SaV, and EU603438-EU603441 for AdVs).

RESULTS

Characteristics of Patients

From January 2006 to February 2007, a total of 273 patients with acute gastroenteritis or acute diarrhea were enrolled in this study. One hundred and ninety eight (72.5%) and 75 (27.5%) cases attended the Inpatient Department and Outpatient Department in Lopburi Hospital, respectively. The ratio of patients attending the Inpatient Department to Outpatient Department was 2.6:1. The ratio of males to females was 1.1:1. The median age was 12.5 years old (range 2 months–86 years old).

Virological Testing

In this study, enteropathogenic bacteria were identified in 11 patients and the samples from these patients were therefore excluded from the analysis. Among the 262 patients with nonbacterial acute gastroenteritis, 39 (14.9%) cases of enteric viruses were detected by RT-multiplex PCR and confirmed by RT-monoplex PCR. These enteric viruses included group A RVs (6.1%), NoVs (6.5%); GI (0.8%) and GII (5.7%), SaVs (0.8%) and AdVs (1.5%), as shown in Table I. After DNA amplification and gel electrophoresis with ethidium bromide staining, positive DNA bands were shown at 395 bp for group A RV, 330 bp for NoV GI, 387 bp for NoV GII, 434 bp for SaV, and 462 bp for AdV (Fig. 1). Group B RV, group C RV and AsV were not detected. The ratio of patients with viral gastroenteritis attending the Inpatient Department to Outpatient Department was 12:1. The patients with acute gastroenteritis in the age group 0–4 years had the highest frequency of viral infection. The median ages of patients infected with RVs and NoVs GII were 18 months (range 2 months–48 years) and 22 months (range 4 months–59 years), respectively.

TABLE I. Enteric Viruses in Patients With Acute Gastroenteritis Detected Using RT-Multiplex PCR

	Nonbacterial gastroenteritis no.	Viral infection (%)[a]	Rotavirus (%)	Norovirus		Sapovirus (%)	Adenovirus (%)
				GI (%)	GII (%)		
Patients	262	39 (14.9)	16 (6.1)	2 (0.8)	15 (5.7)	2 (0.8)	4 (1.5)
Age group (years)							
0–4	106	30 (28.3)	12 (11.3)	1 (0.9)	12 (11.3)	1 (0.9)	4 (3.8)
5–9	16	3 (18.8)	2 (12.5)	—	—	1 (6.3)	—
10–14	9	—	—	—	—	—	—
15–30	10	—	—	—	—	—	—
>30	121	6 (5.0)	2 (1.7)	1 (0.8)	3 (2.5)	—	—
Male:female	1.1:1	1.3:1	3:1	All female	0.7:1	All male	1:1
Days after onset							
1–3	206	21 (10.2)	5 (2.4)	1 (0.5)	10 (4.8)	2 (1.0)	3 (1.5)
4–6	51	16 (31.4)	9 (17.6)	1 (2.0)	5 (9.8)	—	1 (2.0)
7–9	4	2 (50)	2 (50)	—	—	—	—
>9	1	—	—	—	—	—	—

[a]Percentage of virus-infected patients in all nonbacterial gastroenteritis patients in each row.

Infections with NoVs GI were found in one child aged 4 years and one adult aged 47 years. SaV infections occurred in children at the age of 6 and 8; whereas, all patients with AdV infections were less than 5 years old. Analysis by the Chi-square test was used to compare the two variables, the results showed that viral acute gastroenteritis occurred in children less than 15 years of age (33/131, 25.2%) with higher frequency than in adults (6/131, 4.6%) at P-value <0.001. Viral gastroenteritis occurred in males at a greater rate than in females. RV infection was observed in males at a rate three times greater than in females but for NoV GII, females were infected at a greater rate than males. The stool samples collected from the patients with acute gastroenteritis on days 1–3 after onset of illness were positive for RVs, NoVs, SaVs, and AdVs. Group A RVs were still detected in the stool samples collected on days 7–9 (Table I).

Season

Patients with rotaviral diarrhea occurred in the winter season, from January to March. A monthly analysis of cases with acute gastroenteritis, in order to determine the proportion of virus-infected patients, revealed a peak of RV infection in January. The patients infected with NoVs GI were found in October, while the patients with NoVs GII appeared between February and March and between August and November; the peak NoV GII infections was August and September. All patients with acute gastroenteritis caused by SaVs or AdVs were diagnosed in the early summer months (March and April), data not shown.

Genetic Analysis

Based on screening using RT-multiplex PCR, 16 group A RV (6.1%) cases were detected in 262 stool samples. Fourteen DNA products (87.5%) of the RVs detected were subjected to DNA sequencing of the partial VP7 capsid region. The genotypes of RVs were classified according to the phylogenetic analysis proposed by Khamrin et al. [2007b]. DNA amplification products were associated with RV reference strains and belonged to RVs G1 (11 samples), G3 (2 samples), and G9 (1 sample), as shown in Figure 2 and Table II. It was found that in this study G1 was the most predominant genotype, followed by G3 and G9. RVs G1 were only found in adults with acute gastroenteritis. Using the BLAST program and phylogenetic analysis, one sample positive for RV G1 showed 98% nucleotide sequence identity with human RV G1 isolate Chi-87 (DQ512998) within the same cluster as the reference KU strain; whereas, nearly all other RVs G1 showed 98–99% identity with rotavirus A strain 7265/JP (EF079066) within the same cluster as reference AU007 strain.

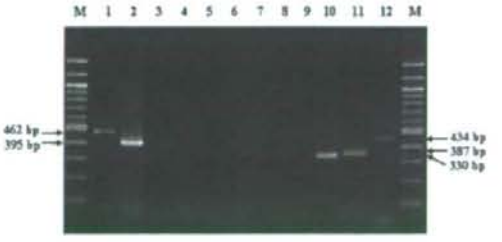

Fig. 1. Detection of enteric viruses in stool samples. Lanes: M, DNA marker (100-bp DNA Ladder); 1, adenovirus-positive sample (No. 057); 2, rotavirus-positive sample (No. 048); 3–9, virus-negative stool samples; 10, norovirus GI-positive sample (No. 105); 11, norovirus GII-positive sample (No. 085); 12, sapovirus-positive sample (No. 039). Gel electrophoresis of the RT-multiplex PCR products of adenovirus showed 462 bp; group A rotavirus, 395 bp; norovirus GI, 330 bp; norovirus GII, 387 bp; and sapovirus, 434 bp.

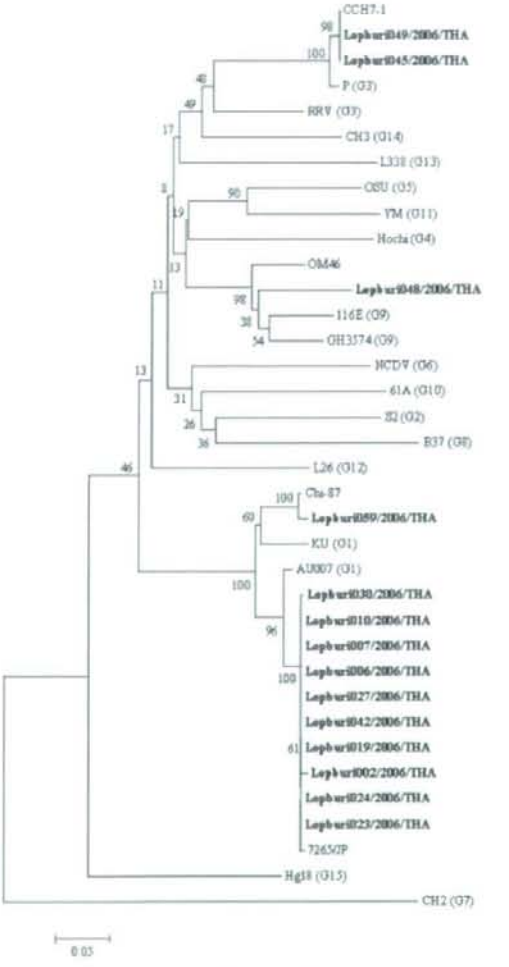

Fig. 2. Phylogenetic analysis of partial VP7 nucleotide sequences of group A rotavirus detected in patients with acute gastroenteritis (Lopburi002-059/2006/THA). The GenBank accession numbers for 15 known G genotypes include: G1; KU (D16343), AU007 (AB081799), G2; S2 (M11164), G3; P (AB118024), RRV (AF295303), G4; Hochi (AB012078), G5; OSU (X04613), G6; NCDV (M12394), G7; CH2 (X56784), G8; B37 (J04334), G9; GH3574 (AY211068), 116E (L14072), G10; 61A (X53403), G11; YM (M23194), G12; L26 (M58290), G13; L338 (D13549), G14; CH3 (D25229), and G15; Hg18 (AF237666). The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

TABLE II. Genotypes of Norovirus GI, Norovirus GII, Group A Rotavirus, and Sapovirus Derived From Phylogenetic Analysis of Enteric Viruses in Patients With Acute Gastroenteritis

Etiologic virus[a]	Genotype[b]	Sample code	Date of collection	Age (years)	Gender
Norovirus GI	GI-2	Lopburi105/2006/THA	October 20, 2006	4	Female
Norovirus GII	GII-2	Lopburi026/2006/THA	February 14, 2006	27	Female
	GII-3	Lopburi020/2006/THA	February 8, 2006	<1	Male
	GII-4	Lopburi084/2006/THA	August 19,2006	<1	Male
		Lopburi095/2006/THA	September 28, 2006	2	Female
		Lopburi102/2006/THA	October 20, 2006	2	Male
		Lopburi103/2006/THA	October 20, 2006	<1	Male
		Lopburi109/2006/THA	October 20, 2006	2	Female
		Lopburi117/2006/THA	October 21, 2006	2	Female
		Lopburi127/2006/THA	October 22, 2006	59	Female
		Lopburi146/2006/THA	October 30, 2006	48	Female
		Lopburi159/2006/THA	November 15, 2006	2	Female
	GII-12	Lopburi043/2006/THA	March 9, 2006	2	Female
	GII-16	Lopburi085/2006/THA	August 19, 2006	1	Male
		Lopburi147/2006/THA	October 30, 2006	<1	Female
		Lopburi155/2006/THA	November 8, 2006	53	Female
Rotavirus	G1	Lopburi002/2006/THA	January 5, 2006	<1	Male
		Lopburi006/2006/THA	January 10, 2006	1	Female
		Lopburi007/2006/THA	January 10, 2006	4	Female
		Lopburi010/2006/THA	January 18, 2006	3	Male
		Lopburi019/2006/THA	January 30, 2006	48	Female
		Lopburi023/2006/THA	February 8, 2006	1	Male
		Lopburi024/2006/THA	February 10, 2006	<1	Male
		Lopburi027/2006/THA	February 15, 2006	46	Male
		Lopburi030/2006/THA	February 15, 2006	<1	Male
		Lopburi042/2006/THA	March 9, 2006	2	Male
		Lopburi059/2006/THA	March 26, 2006	<1	Male
	G3	Lopburi045/2006/THA	March 9, 2006	2	Male
		Lopburi049/2006/THA	March 16, 2006	5	Male
	G9	Lopburi048/2006/THA	March 15, 2006	1	Male
Sapovirus	GII-3	Lopburi039/2006/THA	March 7, 2006	8	Male

[a]Enteric virus detected using RT multiplex-PCR.
[b]Genotype determined by phylogenetic analysis.

Seventeen NoVs (6.5%) were detected in 262 stool samples; GI appeared in 0.8% of the samples (two NoVs GI) and GII appeared in 5.7% of the samples (15 NoVs GII). DNA products were sequenced and identified by phylogenetic analysis of the partial capsid region according to the phylogenetic clustering method described by Kageyama et al. [2004]. Only one sample of a DNA product from NoV GI was available for nucleic acid sequencing. Using the BLAST program and phylogenetic analysis, the DNA sequence showed 97% nucleotide sequence identity with human calicivirus SRSV/MI1/94/JP (AB005259), within the same cluster as the reference Southampton strain. This sequence belonged to GI-2 (Fig. 3 and Table II). A total of 15 NoVs GII were classified further into five genotypes: GII-2 (one sample), GII-3 (one sample), GII-4 (nine sample), GII-12 (one sample), and GII-16 (three samples), as shown in Figure 4 and Table II. The most common genotype was NoV GII-4. Two of these NoV GII-4 strains exhibited 99–100% nucleotide identity with NoV Hu/GII.4/Terneuzen70/2006/NL (EF126964); seven of the samples had 99–100% nucleotide identity with NoV Hu/GII.4/Nijmegen115/2006/NL (EF126966). NoV GII-16 was the second most frequent genotype found in this study. The distribution and genetic diversity of NoV GII genotype was observed in all ages.

Of 262 stool samples, two (0.8%) were positive for SaVs, as determined by RT-multiplex PCR and one DNA product was sequenced on the partial capsid region. The genotype was classified according to the clustering method mentioned by Phan et al. [2007b]. Using the BLAST program and phylogenetic analysis, this DNA product showed 94% nucleotide sequence identity with the cruise ship reference strain and classified into GII-3 (Fig. 5 and Table II). Four stool samples (1.5%) were positive for AdVs and all amplified DNA products specific to the hexon gene were analyzed by DNA sequencing. Using the BLAST program, two samples showed 96% and 97% nucleotide sequence identity with human adenovirus type 41 Tak prototype strain (X51783.1). The other two samples showed 96% nucleotide sequence identity with human adenovirus 2 (EU 128938.1) and 97% identity with human adenovirus type 38 (DQ 149633.1).

DISCUSSION

Outbreaks of gastroenteritis caused by enteric viruses have been recognized and reported from all over the world. The disease burden and severity of viral acute gastroenteritis in Thailand has not been studied extensively since methods for detection of these viruses

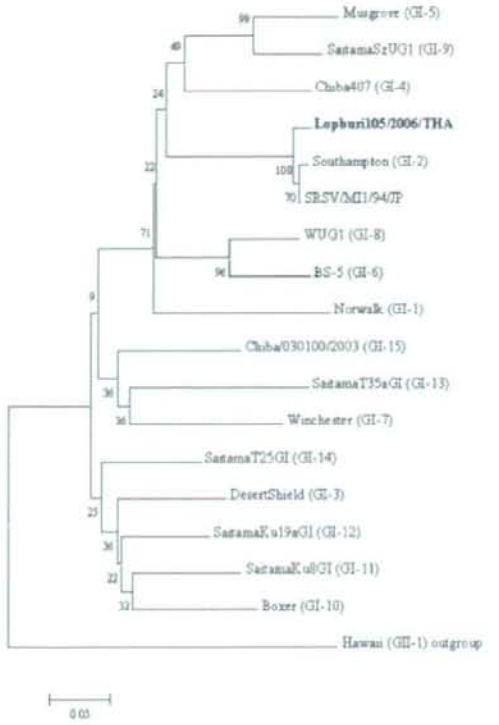

Fig. 3. Phylogenetic analysis of partial capsid sequence of norovirus GI detected in a patient with acute gastroenteritis (Lopburi105/2006/THA). The GenBank accession numbers for 15 known GI strains include: GI-1; Norwalk (M87661), GI-2; Southampton (L07418), GI-3; DesertShield (U04469), GI-4; Chiba407 (AB042808), GI-5; Musgrove (AJ277614), GI-6; BS-5 (AF093797), GI-7; Winchester (AJ277609), GI-8; WUG1 (AB081723), GI-9; Saitama SzUG1 (AB039774), GI-10; Boxer (AF538679), GI-11; Saitama KU8GI (AB058547), GI-12; Saitama KU19aGI (AB058525), GI-13; Saitama T35aGI (AB112132), GI-14; Saitama T25GI (AB112100), GI-15; Chiba/030100/2003 (AJ844469), and for GII-1; Hawaii (U07611) as outgroup. The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

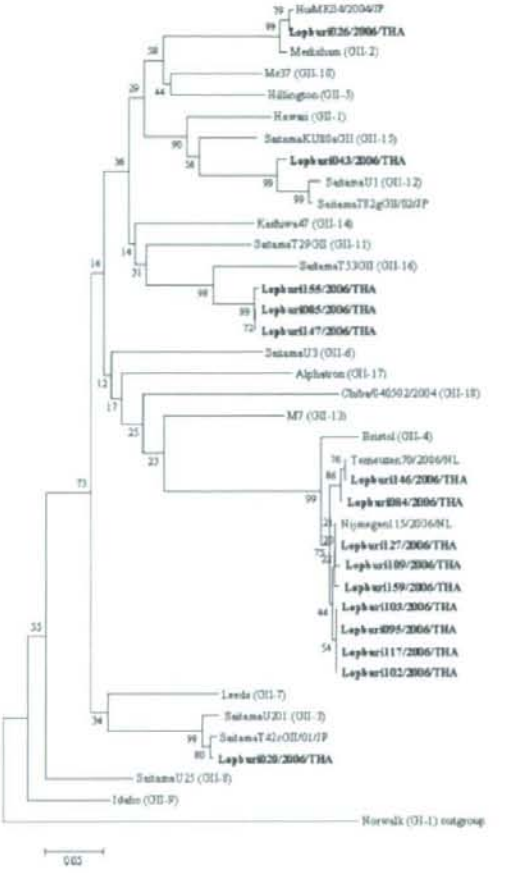

Fig. 4. Phylogenetic analysis of partial capsid sequences of norovirus GII detected in patients with acute gastroenteritis (Lopburi020-159/2006/THA). The GenBank accession numbers for 18 known GII strains include: GII-1; Hawaii (U07611), GII-2; Merksham (X81879), GII-3; Saitama U201 (AB067542), GII-4; Bristol (X76716), GII-5; Hillington (AJ277607), GII-6; Saitama U3 (AB039776), GII-7; Leeds (AJ277608), GII-8; Saitama U3 (AB067543), GII-9; Idaho Fall (AY054299), GII-10; Mc37 (AY237415), GII-11; Saitama T29GII (AB112221), GII-12; Saitama U1 (AB039775), GII-13; M7 (AY130761), GII-14; Kashiwa47 (AB078334), GII-15; Saitama KU80aGII (AB058582), GII-16; Saitama T53GII (AB112260), GII-17; Alphatron (AF195847), GII-18; Chiba/040502/2004 (AJ844470), and for GI-1; Norwalk (M87661) as outgroup. The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

have not been used routinely in a hospital setting. In this study, the RT-multiplex PCR method has been used to investigate the role of enteric viruses in acute gastroenteritis in all age groups attending a hospital in Thailand. Identification and genetic analysis were undertaken by molecular technique.

Among 273 stool samples, enteropathogenic bacteria were identified only in 11 cases since most of the patients had been treated with antibiotics prior to attending the hospital. During the 14-month period study, 14.9% (39/262 patients) were detected with enteric viruses, using the RT-multiplex PCR. NoVs are the etiologic agents of acute gastroenteritis in people of all age groups but group A RVs, SaVs, AsVs, and AdVs are the cause of the infection mainly in children or the elderly [Koopmans, 2005]. The most common viruses found in the patients were NoVs as well as RVs, followed by AdVs and SaVs. Among NoV-infected patients, NoV GII was predominant. This finding is consistent with that reported previously [Hansman et al., 2004a,b; Fabiana et al., 2007; Nguyen et al., 2007; Papaventsis et al., 2007; Siebenga et al., 2007] including Thailand [Guntapong et al., 2004; Khamrin et al., 2007a]. However, the difference in NoV and SaV detection rates between this study and previous studies can be explained by the selection criteria for patients enrolled in this study. In this study, the patients enrolled were from all age groups, as opposed to the previous studies which enrolled children. Statistical significance indicates the enteric viruses caused acute gastroenteritis mainly in children less than 15 years old, as opposed to in adults.

The seasonal distribution of RV infection demonstrated a peak in January and occurred continually through March; this finding is consistent with the study

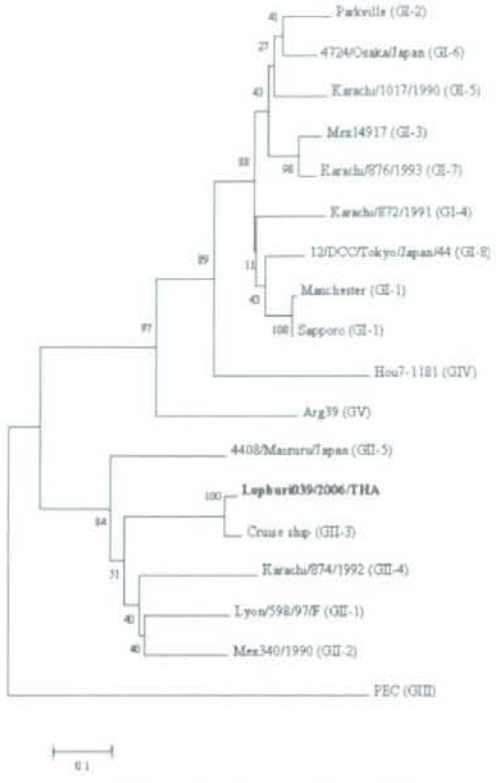

Fig. 5. Phylogenetic analysis of partial capsid sequence of sapovirus detected in a patient with acute gastroenteritis (Lopburi039/2006/THA). The GenBank accession numbers for eight known GI strains include: GI-1; Sapporo (U65427), Manchester (X86560), GI-2; Parkville (U73124), GI-3; Mex 14917 (AF435813), GI-4; Karashi/872/1991 (AB181231), GI-5; Karashi/1017/1990 (AB181132), GI-6; 4724/Osaka/Japan (AB180212), GI-7; Karashi/876/1993 (AB181227), GI-8; 12/DCC/Tokyo/Japan/44 (AB236380), for 5 known GII strains include: GII-1; Lyon/598/97/F (AJ271056), GII-2; Mex 340/1990 (AF435812), GII-3; Cruise ship (AY289804), GII-4; Karashi/874/1992 (AB181129), GII-5; 4408/Maizuru/Japan (AB180209), for GIII; PEC (AF182760), for GIV; Hou 7-1181 (AF435814), and for GV; Arg39 (AY289803). The tree was generated based on the neighbor-joining method and the numbers on each branch indicate the bootstrap values.

by Jiraphongsa et al. [2005]. NoV GI infection took place in October and NoV GII had a peak in August and September, which was earlier than the peak of RV infection, which could be found throughout the year. SaV and AdV infections, however, were only observed in children and in early summer. The seasonal pattern should be interpreted carefully since this study has some limitations regarding the collection of stool samples; the distribution of gastroenteritis patients in age groups and in each month is very different and a number of patients with gastroenteritis who had HIV infection were excluded from the study. Although viral RNA extraction kit (QIAGEN GmbH, Hilden, Germany) was used to extract total RNA from the viruses, identification of AdVs provided the feasibility of co-extracting DNA with total RNA. Using RT-multiplex PCR and DNA sequencing, AdVs found in this study were associated with human AdVs type 41, type 2 or type 38. AdV type 41 is the predominant serotype in Asian countries and associated with acute gastroenteritis in infants and children [Li et al., 2005].

In this study, RV G1 strains were the most prevalent, followed by G3 and G9. RV G9 strains were predominant in Thailand during the year 2001–2003 [Jiraphongsa et al., 2005] and declined with a reemergence of G1 and G2 during 2002–2004 [Khamrin et al., 2007c]. In 2006, RV G1 strains were found to be predominant both in this study and in other countries such as Australia [Kirkwood et al., 2007], Germany [Mas Marques et al., 2007], and Japan [Phan et al., 2007a]. One RV G1 strain showed 98% nucleotide sequence identity with human RV G1 (Chi-87), and belonged to lineage III [Trinh et al., 2007]. Other RV G1 strains showed 98–99% identity with rotavirus A strain (7265/JP) which was found in Japan during 2005–2006 and belonging to lineage II; sublineage IIC [Phan et al., 2007a].

Interestingly, a stool sample was obtained from an infant, with a RV G9 associated with a nucleotide sequence that was a close match to the reference strain GH 3574 (AY 211068). This was in accord with a previous report on the detection of group A RV in raw oysters [Kittigul et al., 2008]. These findings confirmed the presence of human RV genotypes circulating in gastroenteritis patients and bivalve shellfish. Therefore, shellfish is likely to be one of the potential vehicles for rotavirus transmission as well as environmental water, as reported by Kittigul et al. [2005].

The predominance of NoVs GII-4 in this study (60%) is similar to other studies in Thailand [Guntapong et al., 2004; Khamrin et al., 2007a]. NoV GII-4 strains cause 80% of gastroenteritis outbreaks and emergence of the GII-4 variants in The Netherlands has been reported [Siebenga et al., 2007]. In 2006, the emergence of two GII-4 variants (2006a and 2006b) were reported worldwide as having caused outbreaks of gastroenteritis in cruise ships [Koopmans et al., 2006], and healthcare settings [Buesa et al., 2008]. NoV 2006a and 2006b have been implicated as the etiologic agents in acute gastroenteritis in Europe and also in Australia and New Zealand [Tu et al., 2008]. The present study carried out during the year 2006–2007, identified both NoV GII-4 strains; two samples similar to NoV Hu/GII.4/Terneuzen70/2006/NL (2006a variant) and seven samples similar to NoV Hu/GII.4/Nijmegen115/2006/NL (2006b variant). Therefore, these NoV GII-4 strains might be associated with 2006a and 2006b variants, respectively. Further characterization of the viruses would provide information of outbreak strains circulating globally. Using the screening RT-multiplex PCR, NoVs GI were identified in one child (4 years old) and one adult (47 years old). The child infected with NoV GI had the GI-2 genotype. NoVs GI were detected in the year 2000–2001 [Hansman et al., 2004b], but disappeared during 2002–2004 [Khamrin et al., 2007b] and reemerged in this study (year 2006–2007); two NoV GI-positive stool samples were collected in October 2006. NoVs GI in patients with acute gastroenteritis are detected rarely in Japan [Yan et al., 2003] or Vietnam [Hansman et al.,

2004a; Nguyen et al., 2007]. In Thailand, NoVs are circulating in humans, oysters, and environmental water, as with RVs. Of note, NoV GI is predominant genogroup in the environment (manuscript in preparation). Further studies on the ecology of NoVs GI and GII need to be elucidated.

SaVs were found at a lower frequency than NoVs. One SaV strain belonged to GII-3, with an almost 100% nucleotide sequence identity with the cruise ship reference strain. Two previous studies have demonstrated the presence of SaV GI/1, GI/2, GIV [Khamrin et al., 2007a] and GI, GII, GV strains [Hansman et al., 2004b] with genetic diversity. The differences in SaV genotypes circulating depend on the different study sites.

The hospital-based study reported above demonstrate the importance of enteric viruses as causes of gastroenteritis in Thailand. An accurate diagnosis of acute gastroenteritis would facilitate appropriate management of patients and reduce extensive or unnecessary use of antibiotics for treatment. The use of a short conserved sequence is valuable for diagnosis of enteric virus infections, however, genotyping and phylogenetic analyses using the short sequence should be determined with caution. In conclusion, by screening acute gastroenteritis using molecular methods including RT-multiplex PCR and confirmation by DNA sequencing, an etiologic identification and genetic characterization of enteric viruses can be achieved, leading to a comprehensive investigation of the disease burden caused by RVs, NoVs, SaVs, and AdVs.

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G2 Strain of Rotavirus among Infants and Children, Bangladesh

Shuvra Kanti Dey, Yuko Hayakawa, Majibur Rahman, Rafiqul Islam, Masashi Mizuguchi, Shoko Okitsu, and Hiroshi Ushijima

To determine G and P genotypes, we performed nested PCR on 307 rotavirus specimens collected in Dhaka, Bangladesh, during 2004–2005. 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%).

Group A rotavirus (RAV) is the leading cause of severe gastroenteritis in infants and young children worldwide and accounts for ≈600,000 deaths in children <5 years of age (*1*). Rotaviruses are members of the *Reoviridae* family (*2*) and are classified into 7 groups (A–G) on the basis of distinct antigenic and genetic properties (*3*). On the basis of neutralization assay and sequence analysis, a total of 15 G and 27 P genotypes of RAV have been documented (*4*). The major human G types are G1, G2, G3, G4, and G9, which, when combined with the P types P[8], P[4], and P[6], account for >80% of rotavirus-associated gastroenteritis (*5*).

The Study

A total of 917 stool specimens were collected from infants and children with acute gastroenteritis in Dhaka Children's Hospital, Bangladesh, during October 2004 to September 2005. 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 by using the QIAamp viral RNA Mini Kit (QIAGEN, Hilden, Germany). Using reverse transcription–PCR (RT-PCR) with specific primers, as previously reported (*6*), resulted in the identification of diarrheal viruses, including group A, B, and C rotaviruses and adenovirus.

RAV-positive samples were then subjected to G and P genotyping by nested PCR with previously published primers (*7–9*).The RAV isolate for which the G and P types could not be determined by RT-PCR method was subjected to nucleotide sequence analysis of PCR products positive for VP7 and VP4 genes with the BigDye Terminator Cycle Sequencing Kit and ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). Their VP7 and VP4 nucleotide sequences were compared as well as those of reference rotavirus strains available in GenBank by using BLAST (http://blast.ncbi.nlm.nih.,gov/Blast.cgi). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 3.2 software (*10*). The sequences of VP4 and VP7 genes of rotaviruses detected in this study had been submitted to GenBank under accession nos. EU855813–EU855822 and EU855823–EU855830, respectively.

Among diarrhea-causing viruses detected, RAV was the most prevalent (33.5%), followed by adenoviruses (1.9%). Rotavirus group B and rotavirus group C could not be detected in this study. Ninety-seven percent of RAV-infected patients were hospitalized. Co-infection between RAV and adenoviruses was identified in 7 of 917 samples. Most of the patients in this study were 1 to 24 months of age; RAV infection was most commonly detected in patients 6 to 23 months of age. Gender distribution of patients with RAV-positive samples was 56% male and 44% female.

We could not initially determine G type for 10 RAV and P type for 8 RAV isolates, even though their VP7 and VP4 genes were successfully amplified by RT-PCR. Sequence analysis showed that all of untypeable RAV were G1 and P[8]. Ten rotavirus G1 sequences were classified into a distinct lineage, lineage1 and sublineage 1a. G1 strains analyzed in this study belonged to the Asian cluster and were most closely related to Dhaka-02, Dhaka-03, and Thai-1602 strains, which had high identities at the nucleotide level with each other (99%–100%). Eight rotavirus P[8] sequences in this study belonged to 1 distinct lineage, lineage P[8]-II, but made a novel sublineage, sublineage P[8]-IIB, which had a high nucleotide sequence identity of 100% within lineage P[8]-II (Figure 1). These untypeable rotavirus P[8] strains contained 2–3 point mutations at the VP4 primer-binding site.

RAV was detected all year round, but 2 peaks in infections occurred: 1 peak apparently lasted 4 months (October 2004 through January 2005) and another peak lasted 3 months (June 2005 through August 2005) (Figure 2). Five different G types, G1–G4 and G9, were detected during the study period. Of these, G2 was the most common (43.3%) followed by G4 (19.5%), G9 (13.7%), G1 (12.7%), and G3 (2.6%). Mixed infections between >1 G genotypes were

Author affiliations: The University of Tokyo, Tokyo, Japan (S.K. Dey, M. Mizuguchi, H. Ushijima); Society for Rural Education and Development, Dhaka, Bangladesh (R. Islam); University of Dhaka, Dhaka (M. Rahman); Gunma Paz College, Gunma, Japan (Y. Hayakawa); and Aino College, Tokyo (S. Okitsu).

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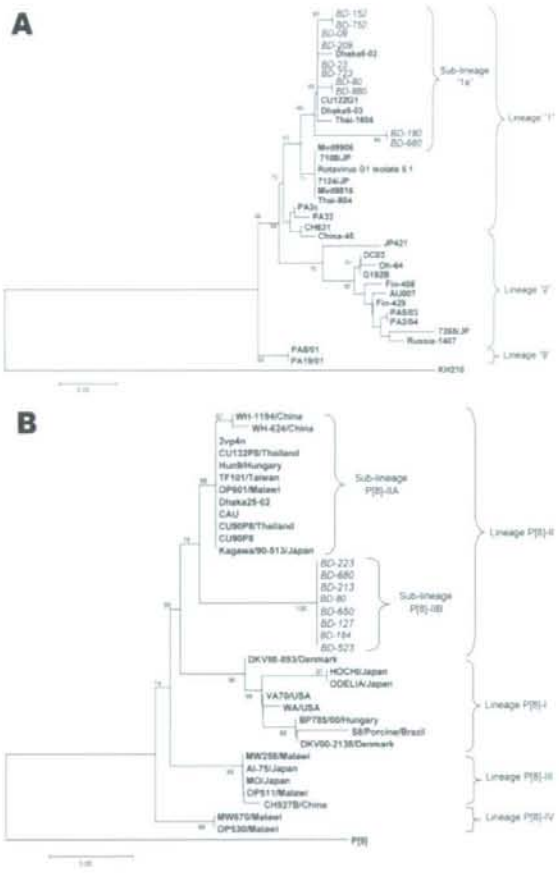

Figure 1. Phylogenetic analysis of the nucleotide sequences of the VP7 and VP4 genes of untypeable group A rotavirus strains (RAV) from Bangladesh. A) Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP7 encoding genes for untypeable RAV strains. B) Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP4 encoding genes for untypeable RAV strains. The numbers in the branches indicate the bootstrap values. Reference strains of RAV G1 and P[8] strains were selected from DNA database of Japan/GenBank under the accession number indicated in **boldface**. G1 strains from Bangladesh are highlighted in *italics*. The scale bars indicate nucleotide substitutions per position. Reference RAV strains used in this study and their accession numbers are as follows: 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), OP601/Malawi (AJ302153), CU132P8/Thailand (DQ235955), DK V98-893/Denmark (AY509908), DK V00- 2138/Denmark (AY509910), S8/Porcine/Brazil (AF052449), CU90P8/Thailand (DQ235978), TF101/Taiwan (AF183870), Hun9/Hungary (AJ605320), WH-1194/China (AY856445), Dhaka25-02 (DQ146652), CU90P8 (DQ235978), 2vp4n (DQ675009), CAU 164 (EU679398) and WH-624/China (AY856444); RAV G1 strains: Dhaka9-03 (DQ482715), CU122G1 (DQ236053), PA5/03 (DQ377596), KH210 (AB303218), 7014/JP (EF079064), rotavirus G1 isolate 5.1(DQ672628), Mvd9906 (AF480278), 7265/JP (EF079066), 7124/JP(EF079069), 7108/JP (EF079068), JP421 (D16326), Fin-408 (Z80303), PA2/04 (DQ377598), Fin-429 (Z80312), AU007 (AB081799), G192B (AF043678), DC03 (AF183859), Oh-64 (U26387), PA3c (DQ377566), PA32 (DQ377574), Thai-1604 (DQ512981), Dhaka8-02 (AY631049), Thai-804 (DQ512979), Mvd9816 (AF480293) CH631 (AF183857), China-45 (U26371), Russia-1407 (S83903), PA8/01 (DQ377592), PA19/01 (DQ377593).

identified in 4% of the specimens (Table). Genotype G2 was detected in every month with a relatively high incidence rate. Among 307 RAV-positive samples, 280 samples were P typed successfully, and P[8] was the most predominant genotype (53.2%), followed by P[4] (42.9%) and mixed infections between different P genotypes (5.0%). 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%).

Conclusions

Of 917 fecal specimens tested, 307 (33.5%) were positive for RAV. This result was consistent with the previous findings on rotavirus epidemiology in Bangladesh in which its prevalence was ≈29% (*11,12*). Our study demonstrated 2 peaks of rotavirus infection. The winter rotavirus peak is usually observed worldwide, but the monsoon peak is not common in settings with temperate climates. Why there was a relation between rainy season and viral infection in this study is not clear. We identified most of the globally common rotavirus types (G1, G2, G4, and G9) in our study. Even though G3 is one of the most prevalent rotavirus types worldwide, the G3 strain has not been detected in Bangladesh since 1993 (*13*). However, we found that 4% of the rotavirus types identified in this study were G3. Results of rotavirus diversity from this study were compared with results of previous studies in Bangladesh (*13*), and we found that G2 was a predominant rotavirus strain among infants and children in Dhaka, Bangladesh.

Rotavirus G4 genotype was the most common genotype in Dhaka from 1992 through 1997 but became a less common rotavirus strain over time; G9 was the leading genotype followed by G2, G4, and G1 in Dhaka (*12,14*). The prevalence of G9 strains was nearly the same in our study, but G2 strains showed a dramatic increase. From 2001–2004, the most common rotavirus genotype was P[8] (76%); non-P[8] strains constituted ≈20%. We also found that rotavirus P[8] (53.2%) strain was the most prevalent. We found that the 4 most common strains globally, G1P[8], G2P[4], G3P[8], and G4[8], were found in 83.9% of cas-

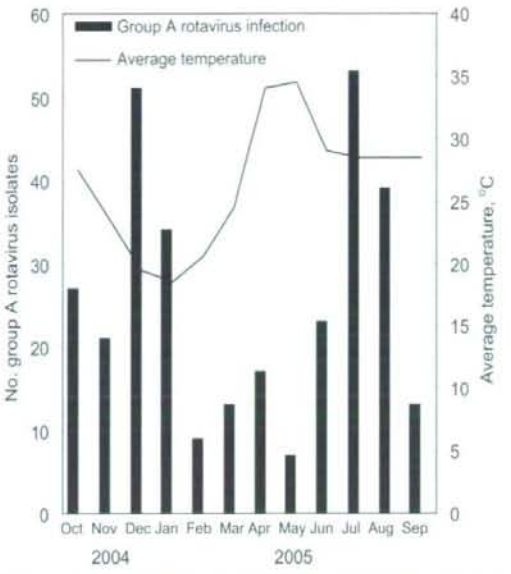

Figure 2. Seasonal pattern of group A rotavirus infection in infants and children with acute gastroenteritis in Dhaka, Bangladesh, October 2004–September 2005.

es. The G1P[8] strains, less common in 2001, became the predominant strains in the following years, but decreased again in 2005–06. Rotaviruses show great genomic diversity, and several studies in different regions of Bangladesh have identified types not targeted by candidate rotavirus vaccines (*11,14*). The frequent genomic reassortment among different rotavirus types was accelerated by mixed infection and generated huge genomic diversity (*13*).

RAV has been associated with gastroenteritis outbreaks in infants and children <5 years of age. However, less is known of the age distribution of rotavirus infection in Bangladesh. In this study, infections were most commonly detected in children <2 years of age.

Common clinical symptoms of RAV-infected patients were dehydration (84%), vomiting (69%), abdominal pain (52%), and fever (31%), which are in agreement with previous published reports (*15*). Number of loose stools per day was increased, with most patients (76%) having loose stools 3–5 times per day. Our study is limited because we could not conduct other tests such as enzyme immunoassay or polyacrylamide gel electrophoresis to confirm rotavirus illness. The incidence of rotavirus gastroenteritis identified by RT-PCR could be an overestimate because healthy controls tested by RT-PCR had a 5%–10% general incidence of rotavirus.

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Mr Dey is a PhD student at The University of Tokyo. His research interest focuses on molecular epidemiology of gastroenteritis viruses in humans.

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Table. Distribution of group A rotavirus G and P genotypes among infants and children with acute gastroenteritis in Dhaka City, Bangladesh, 2004–2005*

Genotype	P[8]	P[4]	P[6]	Mixed*	Nontypeable	Total
G1	33	4	0	1	2	40
G2	11	109	0	9	13	142
G3	8	0	0	1	0	9
G4	51	4	0	2	5	62
G9	36	2	1	2	3	44
Mixed*	10	0	0	0	0	10
Total	149	119	1	15	23	307

*>1 G or P genotype was recognized.

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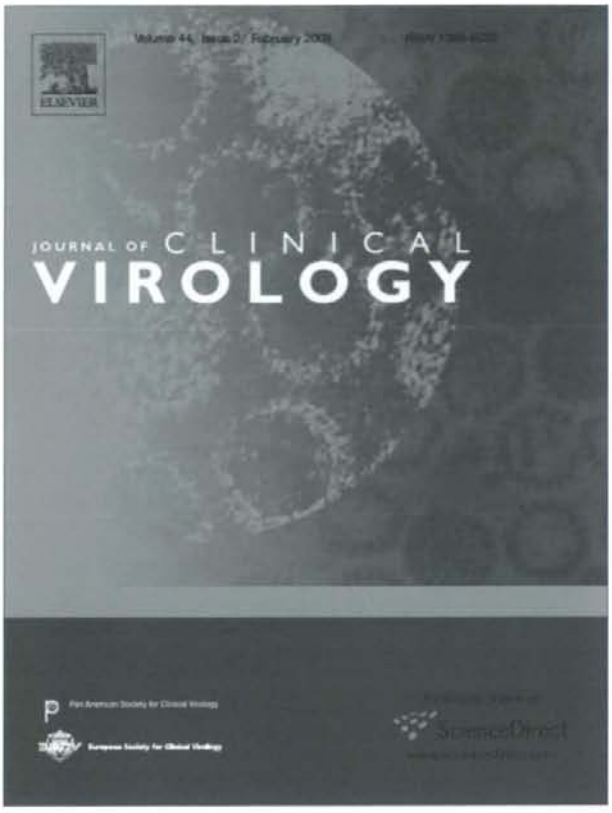
Address for correspondence: Hiroshi Ushijima, Aino Health Science Center, Aino College, 2-17-3 Shibuya, Shibuya-ku, Tokyo 150-0002, Japan; email: ushijima-hiroshi@jcom.home.ne.jp



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Short communication

Detection, genetic characterization, and quantification of norovirus RNA from sera of children with gastroenteritis

Sayaka Takanashi[a,b], Shintaro Hashira[c], Teiichi Matsunaga[d], Akira Yoshida[e], Tomoyuki Shiota[a], Phan Gia Tung[a], Pattara Khamrin[a], Shoko Okitsu[a], Masashi Mizuguchi[a], Takashi Igarashi[b], Hiroshi Ushijima[a,*]

[a] Department of Developmental Medical Sciences, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[b] Department of Pediatrics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[c] Division of Infection Control, Teikyo University School of Medicine, Tokyo, Japan
[d] Eijudo clinic, Tokyo, Japan
[e] First Department of Pediatrics, Japanese Red Cross Society Wakayama Medical Center, Wakayama, Japan

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ABSTRACT

Background: Norovirus (NoV) infection is thought to be confined to the intestines, whereas many reports suggest antigenemia and viremia occur during rotavirus gastroenteritis.
Objectives: To detect NoV RNA in sera and cerebrospinal fluids (CSF) from NoV-infected children, and to quantify and genetically characterize the NoV found in these compartments.
Study design: Semi-nested PCR was conducted on stool, serum and CSF samples from 56 patients with acute gastroenteritis. Positive samples for NoV were analyzed further by sequencing and real-time PCR.
Results: From 39 patients with NoV RNA in stools, 6 also had NoV RNA in sera and none had NoV RNA in CSF. Genotypes of the NoV in stool and serum from the same patient matched completely. The strains in this study had high homology (98.1–100%) with registered strains in the database. The median viral load in stools of the serum-positive patients was greater than that of the serum-negative patients, but this difference was not statistically significant (9.8×10^9 copies/g versus 1.1×10^9 copies/g ($p = 0.117$)).
Conclusions: NoV RNA appeared in the blood stream in 15% of the patients of NoV gastroenteritis. Although the viral load in stool was not statistically correlated with NoV appearance in serum, genetic analysis indicated that NoV RNA in sera originated from the NoV gastroenteritis.

1. Introduction

Norovirus (NoV), a member of the family *Caliciviridae* in the genus *Norovirus*, is a major cause of non-bacterial acute gastroenteritis all over the world.[1–3] The main symptoms of NoV infection are diarrhea and vomiting, which are usually mild and self-limiting. However, a recent case report demonstrated that a patient suffered from disseminated intravascular coagulation during a NoV outbreak, in association with obtundation, headache and photophobia[4] and Ito et al.[5] reported NoV-associated encephalopathy with altered consciousness. These reports indicate a potential spread of NoV to organs other than the intestines.

Many studies have been conducted seeking evidence of extra-intestinal manifestations of rotavirus infection. These included detection of rotavirus RNA in blood[6,7], CSF[7–9] and throat swabs.[7] Although early works suggested that this was due to unusual rotavirus strains or rare host genetic or immunologic defects in the infected child,[10] recent analysis revealed that rotavirus antigen is commonly detected in sera of immunocompetent children with rotavirus diarrhea (43–67%).[11–13]

Human NoV, unlike rotavirus, is not capable of growing in cell lines and has no animal model available, thus hindering study of systemic spread after intestinal infection. Detection of NoV RNA from specimens other than stools has been limited to one case in which NoV was present in serum and CSF from a previously healthy NoV-infected girl with encephalopathy.[5]

In this study, we sought to detect NoV RNA in blood and CSF from patients with NoV gastroenteritis. Genetic analyses and quantification of NoV RNA were undertaken on positive samples.

2. Materials and methods

2.1. Sample collection

From the diarrheal patients who attended Department of Pediatrics in Teikyo University Hospital, Eijudo clinic, and Red Cross

* Corresponding author. Tel.: +813 5841 3590; fax: +813 5841 3629.
E-mail address: ushijima@m.u-tokyo.ac.jp (H. Ushijima).

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Society Wakayama Medical Center from December 2005–2006, 56 cases who needed venepuncture for examination or infusion were recruited into this study. Stool samples were collected from 56 cases only once, while several blood samples were taken from patients who were required additional venepuncture in later course (total = 90). The interval between stool and the first blood sample collection was less than 2 days. CSF was also obtained from two patients who had convulsions. Ethical approvals were obtained from the Ethical Committees and informed consent was given by the guardians of the patients.

2.2. Reverse transcription (RT)-PCR

RT-PCR was performed according to the previously described protocol[14] with primers COG1F/G1-SKR (first round PCR) and G1-SKF/G1-SKR (second round (semi-nested) PCR) for NoV genogroup I (GI), and COG2F/G2-SK (first round PCR) and G2-SKF/G2-SKR (second round PCR) for NoV genogroup II (GII).[15,16] We used known-positive specimen extracts as positive controls, JP 6146 for NoV GI and JP 5235 for NoV GII, respectively. PCR amplicons were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (EtBr, 0.5 μg/ml) for 20 min and visualized under ultraviolet light.

2.3. Sequence analysis

Positive PCR products were subjected to sequencing by Big-Dye terminator cycle sequencing kit and an ABI prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). We used first round PCR products for sequencing if first round PCR gave positive results. If only second round PCR was positive, we used second round PCR products. In both cases, we sequenced with the same primers used in each round on both strands. Sequence analysis was performed using CLUSTAL X software (Version 1.81). Reference NoV strains and accession numbers used in this study were as follows: Norwalk/68/US (M87661), Melksham/89/UK (X81879), SaitamaU201/98/J (AB067542), Arg320/95/AR (AF190817), Lordsdale/93/UK (X86557), Bristol/93/UK (X76716), and Manchester_Sapovirus (X86560).

2.4. Quantification of NoV

We performed real-time PCR to quantify NoV viral RNA in PCR-positive samples, as previously described.[17] Data were corrected by using ROX passive reference as an internal standard and recovery rate of the NoV genome was tested by highly purified NoV plasmid standards containing PCR products of the ORF1–ORF2 junction of the GII strain (Saitama U201, AB039782).[17] Half of the serum samples which were positive by RT-PCR in our laboratory were selected arbitrarily and tested for real-time PCR at the National Institute of Infectious Diseases in Tokyo in order to reconfirm the existence of NoV genes in serum.

2.5. Statistical analysis

SPSS software version 12.0J was used for data analysis. A p-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. RT-PCR and patients' characteristics

Among the 56 stool samples collected, 26 were positive for NoV GII by first round PCR and 13 were positive by second round. Among the 90 serum samples collected from the 56 patients, 6 were positive for NoV GII by second round PCR and were confirmed by sequence analysis to be NoV. Neither of the two CSF samples contained NoV RNA even by second round PCR, although stool samples from these patients contained NoV GII RNA by first round PCR. All of the samples tested were negative for NoV GI.

3.2. Nucleotide sequence and phylogenetic analysis of NoV GII

The partial nucleotide sequences (282 bp) of the NoV capsid gene were analyzed by phylogenetic grouping according to the classification schemes described by Kageyama et al.[18] They were grouped into three distinct genotypes; two stool samples in genogroup II genotype 2 (GII/2), four stool samples and one serum sample in GII/3, and 33 stool and five serum samples in GII/4. The genotypes of stool and serum samples collected from the same patients were all matched and the identities of nucleotide sequences between these pairs ranged between 99.2% and 100%. The phylogenetic tree of the six pairs of stool and serum samples together with the reference strains is shown in Fig. 1.

The identities among strains in the same genotypes ranged from 96.8% to 99.6%, and each strain showed high identities (98.1–100%) with previously registered Japanese strains in the DDBJ DNA/GenBank database.

3.3. Quantification of NoV RNA

The median RNA viral load detected in the stool samples from patients with serum-positive and negative for NoV GII was 9.8×10^9 (range 4.2×10^8 to 4.0×10^{10}) and 1.1×10^9 (range 5.6×10^3 to 1.4×10^{11}) copies/g of stool, respectively. This difference was not statistically significant ($p = 0.117$, two-tailed Mann–Whitney U-test). The mean value for each group was 1.4×10^{10} (standard error, 8.1×10^9) and 1.0×10^{10} (standard error, 5.0×10^9) copies/g of stool with no statistical difference ($p = 0.722$, t-test). We also investigated any correlation between RNA viral load in the serum sample and that in the paired stool sample. Patient C was excluded from this analysis because no data by real-time PCR was available. With this small number of pairs ($n = 5$), no linear correlation between the serum and stool viral load was observed ($r = 0.071$, $p = 0.910$).

4. Discussion

Potential extra-intestinal spread is an important issue in understanding the pathogenesis of viral gastroenteritis. In this study, we observed that 15% of the NoV gastroenteritis patients (6/39) had NoV RNA in serum and could not detect NoV RNA in either of two CSF samples.

Our genetic analysis showed a very high homology between strains found in stool and serum, indicating that the viral RNA in blood had originated from the intestinal tract. The high homology between the strains in this study and those deposited in GenBank implied that no unique strains were associated with detection in blood stream.

Although the mean viral load in stool of serum-positive group was greater than that of serum-negative group, there was not statistically significant difference between these two groups. Several studies on rotavirus gastroenteritis showed that the antigen level in blood samples decreased as diarrhea subsided, implying a relationship between the presence of virus in the intestines and in the blood.[11,12] Hence, we may be able to observe a similar relationship in a larger sample sized study and/or samples collected consecutively from the same group.

Recently, experiments with murine NoV in a mouse model showed that infectious virus was detected in peripheral organs such as spleen, liver, and lung after oral inoculation, and that histopathological changes were observed not only in the intestines, but also in the spleen of immunocompetent mice.[19,20] These findings are of