

Fig. 1. Phylogenetic tree of the VP6 deduced amino acid sequences of SG I, SG II, and SG non-(I+II) human rotaviruses. The reference strains selected from the GenBank database are indicated by boldface. The tree was constructed based on the neighbor-joining method using ClustalX program and drawn with TreeView program.

strains. The percentage of amino acid identity lay within the range of sequence identity of the virus in the same SG. CMP127/01 was, therefore, designated as Genogroup I (SG II*) based on VP6 sequence analysis.

Comparison of VP6 amino acid sequence between the viruses of different SGs revealed somewhat lower sequence identities; 93.1–94.2% between SG I and SG II, 92.4–94.2% between SG (I+II) and SG II, and 91.9–94.4% between SG non-(I+II) and SG II. Surprisingly, the strains with SG (I+II) shared a high sequence identity with SG non-(I+II) at 96.2–99.7%, and SG (I+II) and SG non-(I+II) shared sequence identity with SG I strains as high as 96.4–100% and 94.7–99.7%, respectively. The degree of sequence identities lay

within the range of sequence identity of the virus in the same SG. Based on VP6 sequence analysis, the rotavirus strains with SG (I+II) and SG non-(I+II) were, therefore, designated as Genogroup I.

Alignment of VP6 deduced amino acid sequences of porcine rotavirus strains revealed that the viruses with SG I (CMP39/00, CMP34/01) were distinguishable from SG II strains in at least 13 amino acid positions (Table II). These amino acid substitutions were observed at positions 120 (Ser → Ala), 134 (Asp → Glu), 158 (Leu → Phe), 172 (Ala → Met), 182 (Ile → Leu), 211 (Val → Ile), 213 (Pro → Gln), 244 (Thr → Ala), 257 (Ser → Asn), 295 (Val → Met), 312 (Pro → Gln), 315 (Ile → Gln), and 339 (Ser → Asn). Only three amino acid

residues were identical with those of human rotaviruses, at positions, 120, 172, and 339.

It should be noted that the strains which were serologically identified as SG (I + II) and SG non-(I + II) had amino acids at these positions identical with those of SG I strains over the entire sequence of VP6 (Table II).

Therefore, based on the VP6 amino acid sequence analysis, the rotavirus with SG (I + II) and SG non-(I + II) specificity were designated as Genogroup I strains, while the rotavirus with SG I and SG II were designated as Genogroup I and Genogroup II, respectively.

Phylogenetic analysis of VP6 amino acid sequences of these porcine rotaviruses (Fig. 2) revealed that the strains of SG I, SG (I + II), and SG non-(I + II) clustered together within Genogroup I which was distinctly separated from a branch of Genogroup II. Three genetic lineages, IA, IB, and IC, were distinguished within Genogroup I, whereas two genetic lineages, IIA and IIB, were observed within Genogroup II. However, there was no correlation between serologically determined SGs and genetic lineages.

In addition, phylogenetic analysis revealed a clustering of CMP127/01, which was serologically defined as a SG II strain, in a monophyletic branch with SG I strains (Fig. 2). The data confirmed that CMP127/01 (SG II*) belongs to Genogroup I.

DISCUSSION

A number of studies have described the SG-specific amino acid residues for SG I and SG II on the VP6 protein of rotaviruses [Gorziglia et al., 1988; Lopez et al., 1994; Tang et al., 1997]. A total of 20 SG-specific amino acids have been identified and most studies have focused mainly on the VP6 region encompassing the amino acid positions previously recognized by SG-specific MAbs.

In the present study the full-length VP6 deduced amino acid sequences of human and porcine rotaviruses with different subgroup specificities, including SG I, SG II, SG (I + II), and SG non-(I + II) were examined. It was found that at least 21 amino acid positions in human rotaviruses were conserved over the entire sequence of VP6, distinguishing between the virus with SG I and SG II, whereas only 13 positions were observed in porcine rotaviruses. All these amino acid positions were located within the regions reported previously to contribute to the SG-specific epitopes [Gorziglia et al., 1988].

In addition, amino acid residues found to be conserved for SG I or SG II at six positions, 83 (Asn for SG I and Thr for SG II), 86 (Asp for SG I and Glu for SG II), 89 and 92 (Val for SG I and Ile for SG II), 101 and 217 (Val for SG I and Ala for SG II), were identical with those described by Gorziglia et al. [1988].

Our data confirmed the findings of by Tang et al. [1997] that an Ala residue was conserved at positions 172 and 305 for SG I of human rotavirus strains, while at position 315, a Gln rather than a Glu was conserved for SG II strains (Table II). However, the results were in good agreement with those of Iturriza-Gomara et al.

[2002], that is, Ala at position 305 and Gln at position 315 conserved for SG I and SG II, respectively.

The results also confirmed the findings described by Lopez et al. [1994] that an Ala residue at positions 172 and 305 is specific for SG I epitope and an Asn residue at 305 is specific for SG II epitope. Although 21 amino acids have been identified as SG I- or SG II-specific residues in our study and 20 amino acids by others [Tang et al., 1997], few amino acid positions have been demonstrated to contribute to the reactivity of SG-specific MAbs [Gorziglia et al., 1988; Lopez et al., 1994; Tang et al., 1997]. Other amino acid residues located distantly in the VP6 protein and found to be conserved for SG I or SG II may also contribute to the formation of SG-specific epitopes by the folding of VP6 or interaction between VP6 monomers [Gorziglia et al., 1988].

The present study also investigated the amino acid residues specific to SG I or SG II epitopes in porcine rotaviruses. Interestingly, three amino acid positions, 120, 172, and 339, were found to be common to both SG I or SG II in human and porcine rotaviruses, suggesting that these amino acid residues are probably specific for SG I or SG II rotaviruses regardless of whether the viruses were derived from humans or swine. However, further comprehensive investigation needs to be performed in rotavirus strains of other animal species.

The deduced amino acid sequences of human rotavirus strains serologically determined as SG II and SG non-(I + II) as well as SG I* were indistinguishable from each other. All these strains had conserved amino acid residues identical with those of SG II at positions 39, 60, 83, 86, 89, 92, 101, 109, 115, 120, 151, 172, 217, 248, 305, 310, 315, 339, 369, 342, and 348. The genetic similarity among strains serologically determined as SG II, SG non-(I + II), and SG I* suggested that subgrouping of rotaviruses by serological method, based on reactivity of SG-specific MAbs, is unreliable (Tables I and II).

Similar to human rotaviruses, the porcine rotaviruses also had amino acid residues that conserved and distinguished SG I from SG II strains at positions 120, 134, 158, 172, 182, 211, 213, 244, 257, 295, 312, 315, and 399. Amino acid residues among SG I, SG (I + II), SG non-(I + II), and SG II* at these positions were identical, suggesting that they all belonged to the same subgroup in SG I. The findings, again, demonstrate the failure of subgrouping rotavirus by using SG-specific MAbs.

A reasonable explanation for misidentification of SG non-(I + II), SG (I + II), SG I*, and SG II*, based on the reactivity of SG-specific MAbs, is still unclear. It was thought possible that a single amino acid mutation at the SG-specific epitopes might change the SG specificity [Lopez et al., 1994; Tang et al., 1997]. However, this is not the case, since human rotavirus strains with SG non-(I + II) and SG I* had SG-specific amino acid residues identical with those of SG II strains at 21 positions in the region implicated previously in recognition by SG-specific MAbs. None of the strains determined serologically as SG non-(I + II) and SG I* showed substitutions at any other SG-determining amino acid positions different from SG II strains (Table I).

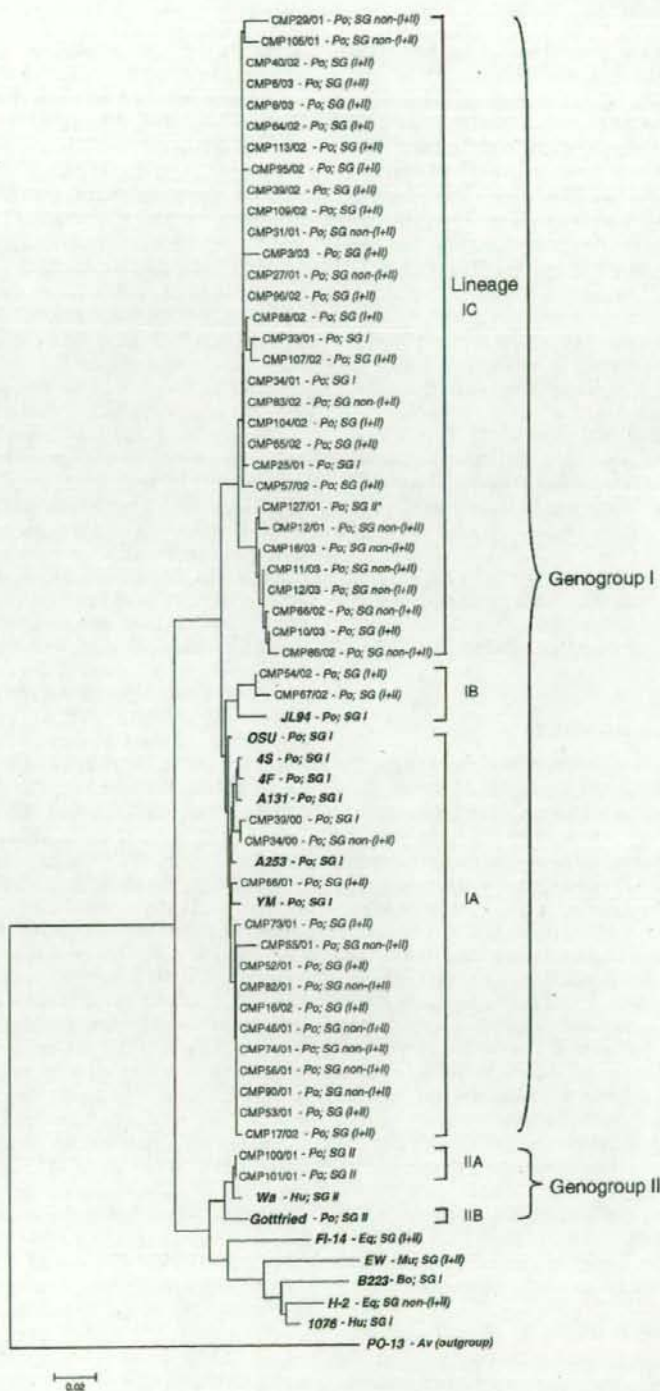


Fig. 2. Phylogenetic tree of the VP6 deduced amino acid sequences of SG I, SG II, SG (I + II), SG non-(I + II) porcine rotaviruses. The reference strains selected from the GenBank database are indicated by italic boldface. The tree was constructed based on the neighbor-joining method using ClustalX program and drawn with TreeView program.

Similar findings were also observed in porcine rotaviruses. The porcine rotaviruses with SG (I + II), SG non-(I + II), and SG II* had SG-specific amino acid residues identical to those of SG I strains at 13 positions (Table II) that conserved for SG I or SG II strains.

It is well documented that VP6 is the major structural component of rotavirus virions and plays a key role in virion structure by interacting with both the outer capsid proteins VP4 and VP7 and the core protein VP2 [Estes, 2001]. Theoretically, it is possible that interactions of VP6 with different G (VP7) and P (VP4) genotypes on the virion might somehow affect the conformational formation of trimeric SG-specific epitopes of the VP6 protein. This possibility awaits examination.

The unsatisfactory subgrouping of rotaviruses, based on reactivity of SG-specific MAbs, observed in both human and porcine origins, suggests an urgent need to look for a new approach to subgrouping rotaviruses. Molecular methods, such as DNA sequencing of VP6 genes, provide more accurate and reliable information on the diversity of VP6, but are relatively expensive and time consuming. Rapid and less expensive molecular methods such as the multiplex RT-PCR using SG-specific primers may have to be developed as an alternative method for subgrouping of rotaviruses.

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

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ABSTRACT

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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.

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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

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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 sample), 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

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	GCC GAY CGC ACC TGG TTT	+	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 CC	+	1905–1924
AiV-Cap-ABR	GTG TAT GTT CCG CGC AT	–	2783–2767

(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.

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

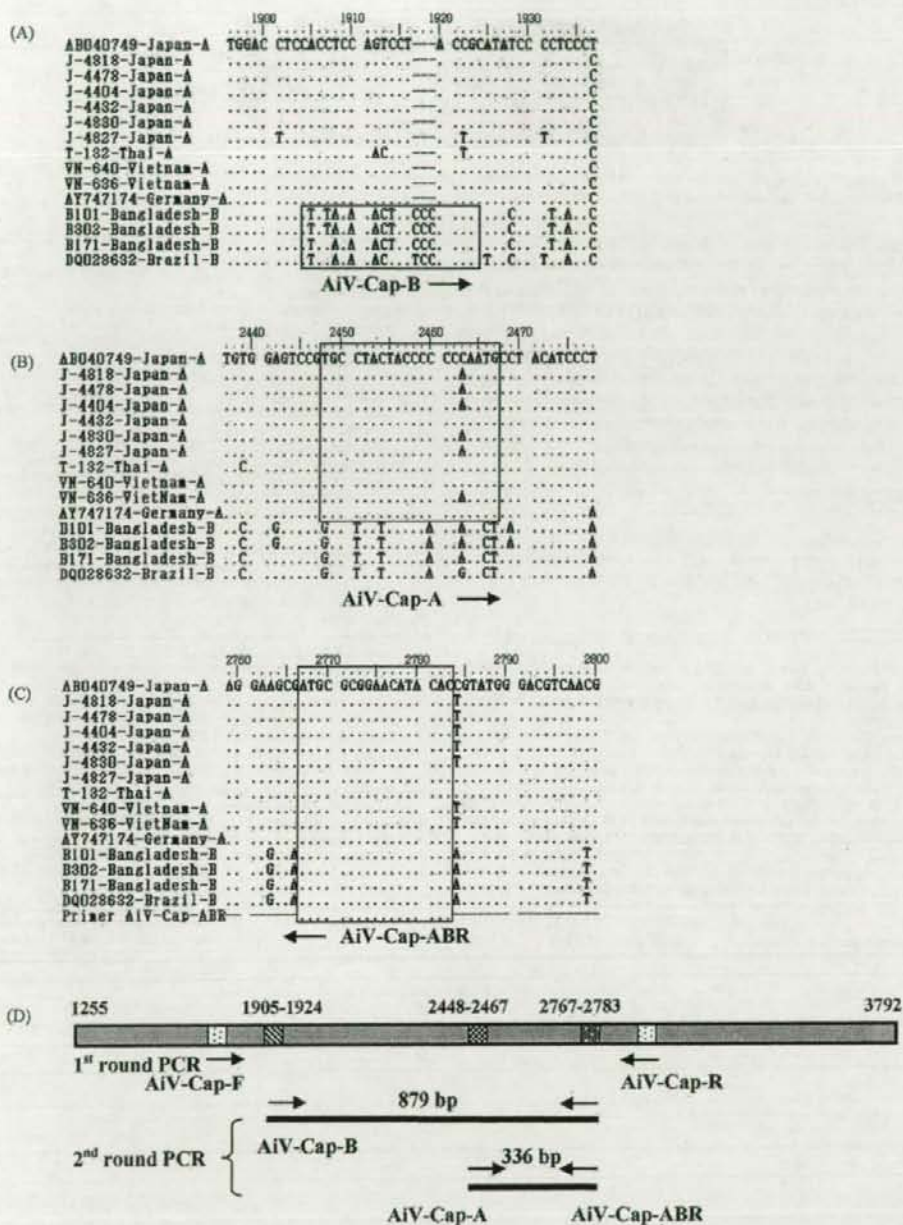


Fig. 1. (A-C) Extracts of alignment results of the 15 reference capsid gene sequences. The identical nucleotides in each Aichi reference strain with the Japanese reference strain under the accession number AB040749 are identified by dots. The primer binding areas are in boxes. (D) Schematic representation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

demiological 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



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

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Outbreaks of viral gastroenteritis occur worldwide including Thailand. Unfortunately, there is limited information since etiologic agents have not been identified in several outbreaks of non-bacterial 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 non-bacterial 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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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 calciviruses, 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 ($\geq 38.0^\circ\text{C}$). 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 500g 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 \times 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 OUTTM (40 Units/ μl), and SuperscriptTM 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 \times 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	—	—	—	—	—	—

^aPercentage 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

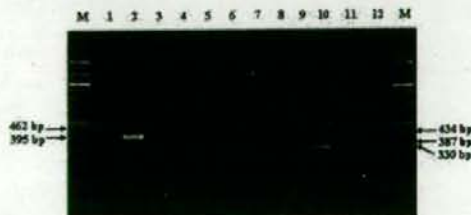


Fig. 1. Detection of enteric viruses in stool samples. Lanes: M, DNA marker (100-bp DNA Ladder); 1, adenovirus-positive sample (No. 057); 2, rotavirus-positive sample (No. 048); 3–9, virus-negative stool samples; 10, norovirus GI-positive sample (No. 105); 11, norovirus GII-positive sample (No. 085); 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.

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.

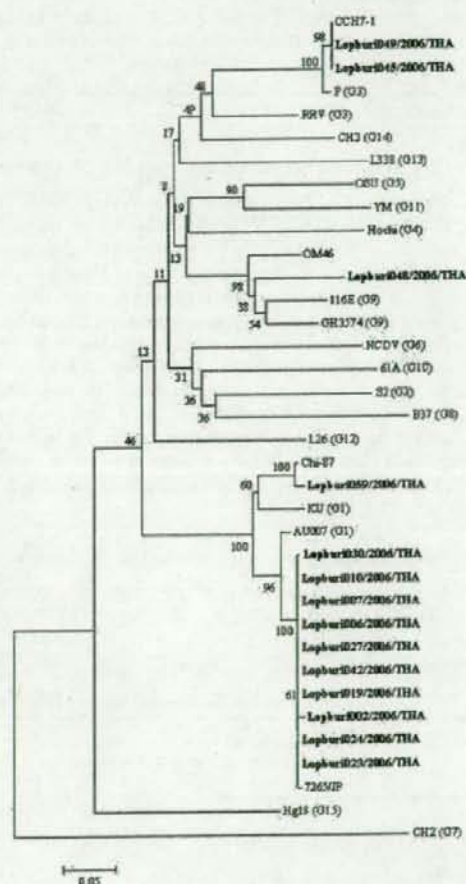


Fig. 2. Phylogenetic analysis of partial VP7 nucleotide sequences of group A rotavirus detected in patients with acute gastroenteritis (Lopburi002-059/2006/THA). The GenBank accession numbers for 15 known G genotypes include: G1; KU (D16343), AU007 (AB081799), G2; S2 (M11164), G3; P (AB118024), RRV (AF295303), G4; Hoshi (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 GII-3 GII-4	Lopburi026/2006/THA	February 14, 2006	27	Female		
		Lopburi020/2006/THA	February 8, 2006	<1	Male		
		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	
		Rotavirus	G1	Lopburi155/2006/THA	November 8, 2006	53	Female
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	
	Lopburi048/2006/THA			March 15, 2006	1	Male	
Sapovirus	GII-3			Lopburi039/2006/THA	March 7, 2006	8	Male

^aEnteric virus detected using RT multiplex-PCR.

^bGenotype 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 samples), 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

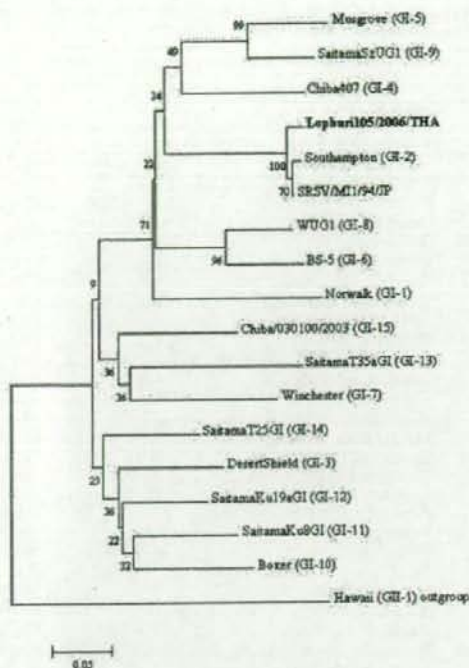


Fig. 3. Phylogenetic analysis of partial capsid sequence of norovirus GI detected in a patient with acute gastroenteritis (Lopburi105/2006/THA). The GenBank accession numbers for 15 known GI strains include: GI-1; Norwalk (M87661), GI-2; Southampton (L07418), GI-3; DesertShield (U04469), GI-4; Chiba407 (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.

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.,

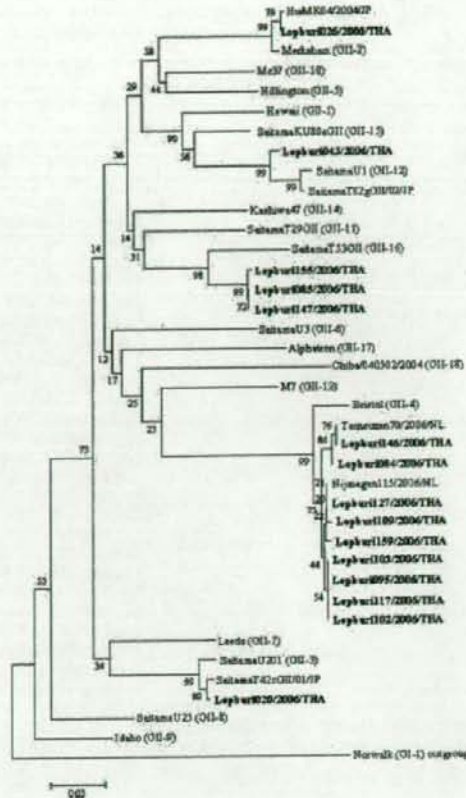


Fig. 4. Phylogenetic analysis of partial capsid sequences of norovirus GII detected in patients with acute gastroenteritis (Lopburi020-159/2006/THA). The GenBank accession numbers for 18 known GII strains include: GII-1; Hawaii (U07611), GII-2; Merkaham (X81879), GII-3; Saitama U201 (AB067542), GII-4; 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; Alphontr (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.

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

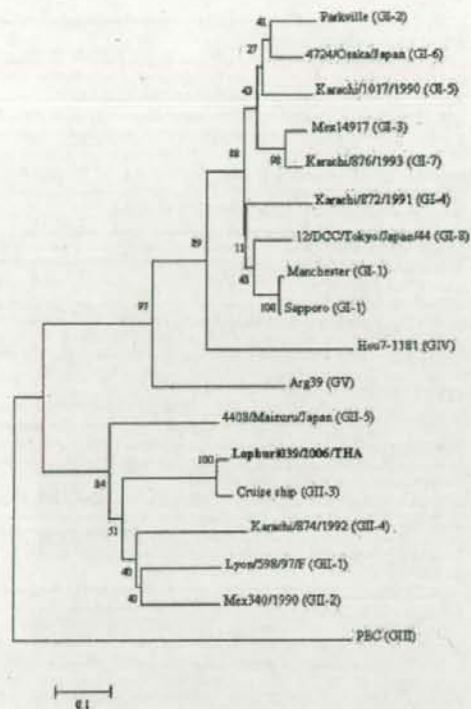


Fig. 5. Phylogenetic analysis of partial capsid sequence of sapovirus detected in a patient with acute gastroenteritis (Lopburi039/2006/THA). The GenBank accession numbers for eight known GI strains include: GI-1; Sapporo (U65427), Manchester (X86560), GI-2; Parkville (U73124), GI-3; Mex 14917 (AF435813), 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

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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 diarrheal-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

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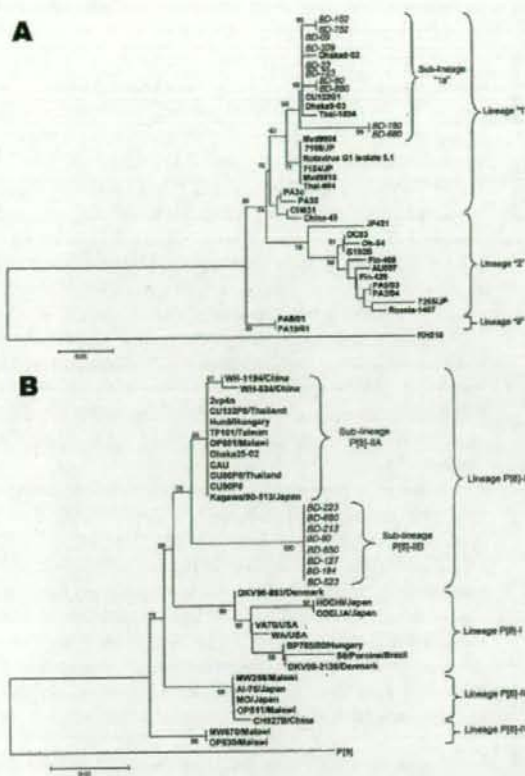


Figure 1. Phylogenetic analysis of the nucleotide sequences of the VP7 and VP4 genes of untypeable group A rotavirus strains (RAV) from Bangladesh. A) Neighbor-joining phylogenetic tree based on nucleotide sequences of the VP7 encoding genes for untypeable RAV strains. B) Neighbor-joining phylogenetic 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 (AJ805320), WH-1194/China (AY856445), Dhaka25-02 (DQ146652), CU90P8 (DQ235978), 2vp4n (DQ675009), CAU 164 (EU679398) and WH-824/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 (EF079068), 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 $\approx 29\%$ (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 $\approx 20\%$. 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-