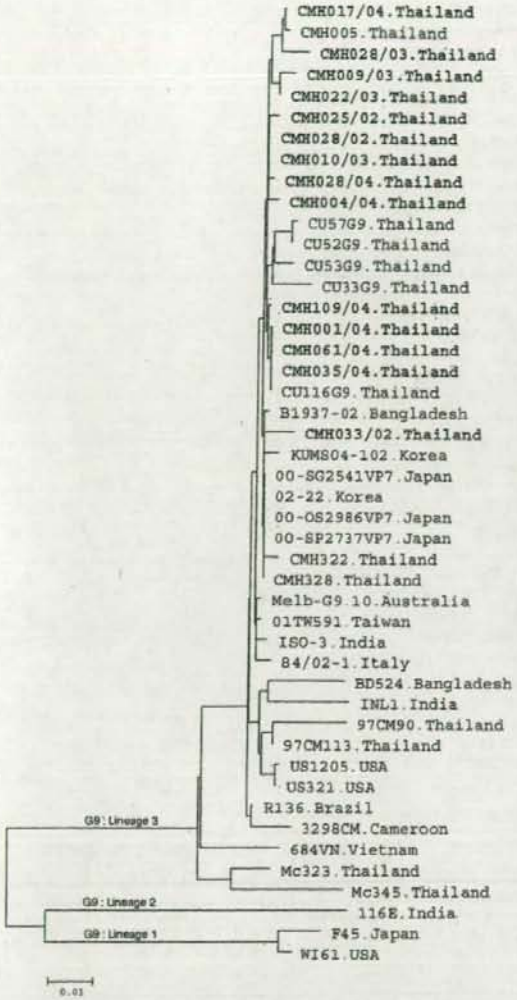

Fig. 2. Phylogenetic analyses of VP7 gene nucleotide sequences of rotavirus G9 strains. The tree was generated based on the neighbor-joining method by using the Mega 3.1 program. The G9 strains used in the analysis were obtained from the GenBank database. The accession numbers of the VP7 reference sequences are listed as follows: CMH005 (AY699290), CU57G9 (DQ236068), CU52G9 (DQ236065), CU53G9 (DQ236067), CU33G9 (DQ236060), CU116G9 (DQ236058), B1937-02 (AY487856), KUMS04-102 (DQ056300), 00-SG2541VP7 (AB091750), 02-22 (AY879296), 00-OS2986VP7 (AB091747), 00-SP2737VP7 (AB091752), CMH322 (AY699302), CMH328 (AY699304), Melb-G9.10 (AY307087), 01TW591 (DQ490173), ISO-3 (AF501580), 84/02-1 (AY184813), BD524 (AJ250543), INL1 (AJ250277), 97CM90 (AY866503), 97CM113 (AY866505), US1205 (AF060487), US321 (AJ250275), R136 (AF438228), 3298CM (DQ647423), 684VN (AB091778), Mc323 (D38053), Mc345 (D38055), 116E (L14072), F45 (AB180970), WI61 (AB180969).

Considering the G and P genotype combinations detected in this study, the three common rotavirus genotypes were G1P[8], G2P[4], and G9P[8], and they comprised nearly 98% of all the rotaviruses detected. Two strains of the uncommon rotavirus G3P[9] were identified for the first time in Thailand, as a rare type in children hospitalized with diarrhea [Khamrin et al., 2007b]. It should be noted that G4 has disappeared completely from the Chiang Mai area since 1997 and it remained undetectable in this study.

The G9 rotavirus was first reported in the United States in the early 1980s [Clark et al., 1987]. Soon after its detection, G9 disappeared for about a decade and then reemerged in the beginning of the 1990s, and currently it represents the fifth most common G genotype of rotavirus infections throughout the world [Gentsch et al., 2005; Santos and Hoshino, 2005]. Sequence analysis of G9 rotaviruses isolated globally has demonstrated the existence of at least three distinct VP7 lineages among the G9 strains [Hoshino et al., 2004, 2005]. Most G9 viruses, which have been reported recently from several countries worldwide, belong to lineage 3. In 1989, two rotavirus G9 strains (Mc323 and Mc345) were first detected in Chiang Mai, Thailand at a relatively low prevalence of 1.98% in children hospitalized with diarrhea [Urasawa et al., 1992], and both of them were in combination with P[19] [Okada et al., 2000]. About one decade later, the prevalence of G9 in Chiang Mai was shown to increase to 16.2% and found to associate with either P[4] or P[6] genotypes [Zhou et al., 2001]. Recently, during 2000 and 2001, the prevalence of G9 strains increased abruptly as high as 91.6% and all were associated exclusively with the P[8] genotype [Khamrin et al., 2006]. It is interesting to point out that the P genotype of G9 strains circulating in Chiang Mai changed over time from P[19] in 1989 to P[4] or P[6] in 1997, and recently (2000–2001) to P[8]. In this study (2002–2004) the G9 strains were detected exclusively in association with the P[8] genotype. They shared the VP7 nucleotide sequence identities among themselves (98.4–100%) at the same high level as that of the G9 strains isolated in the same epidemic season (2002–2004) in Bangkok (98.2–99.8%). The data indicate that G9 strains isolated from 2002 to 2004 in Chiang Mai and Bangkok seem to share the same evolutionary ancestor.

In addition to G1, G3, G4, and G9 genotypes, G2 has been recognized as one of the most predominant genotypes in several regions around the world, including Brazil, Uruguay, and the African continent during the late 1990s [Linhares et al., 2002; Berois et al., 2003; Page and Steele, 2004b]. In Thailand, G1 and G2 were reported as the co-dominant genotypes that reached a peak incidence at approximately 4 or 5-year intervals in the survey studied during the 1982–1997 period [Maneekarn and Ushijima, 2000]. However, in an epidemiological surveillance carried out during 2000–2001 [Khamrin et al., 2006], G2 was detected at a low prevalence, and in this study it was undetectable in 2002. Unexpectedly, G2 was found to be the most predominant genotype in 2003, with a prevalence rate as high as 83.3% of the rotaviruses detected in this study. High nucleotide sequence identities (99.3–100%) between G2 strains isolated in this study in Chiang Mai

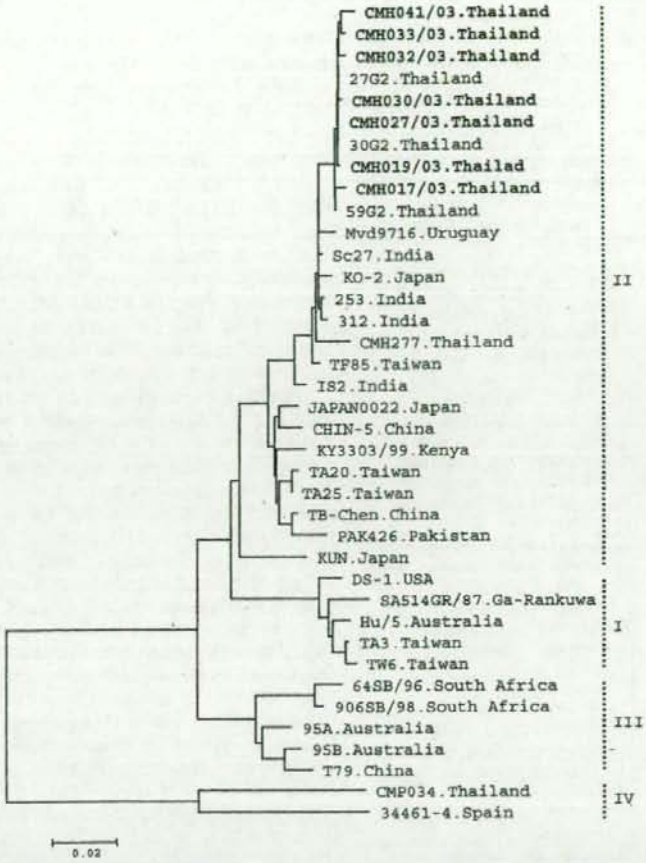

Fig. 3. Phylogenetic analyses of VP7 gene nucleotide sequences of rotavirus G2 strains. The tree was generated based on the neighbor-joining method by using the Mega 3.1 program. The G2 strains used in the analysis were obtained from the GenBank database. The accession numbers of the VP7 reference sequences are listed as follows: 27G2 (AY653298), 30G2 (AY653299), 59G2 (AY653300), Mvd9716 (AF480275), Sc27 (AJ293722), KO-2 (AF401754), 253 (X95370), 312 (X95371), CMH277 (AY707784), TF85 (AF106299), IS2 (X95273), JAPAN0022 (D50117), CHIN-5 (D50114), KY3303/99 (AY261350), TA20 (AF106281), TA25 (AF106283), TB-Chen (AY787646), PAK426 (D50125), KUN (D50124), DS-1 (AB118023), SA514GR/87 (AY261338), Hu/5 (A01028), TA3 (AF106280), TW6 (AF044338), 64SB/96 (AY261341), 906SB/98 (AY261347), 95A (U73955), 95B (U73956), T79 (AF450292), CMP034 (DQ534015), 34461-4 (AY766085).

and those in Bangkok during the same epidemic season (2002–2004) suggest that all of them might have been derived from the same evolutionary ancestor. It should be noted that G2 strains isolated in Chiang Mai, as described in this study and our previous study [Khamrin et al., 2006], were found to be associated with P[4], just like the G2 strains from other parts of the world.

Over the last decade, the G1 rotavirus has been recognized as one of the most common genotypes responsible for acute gastroenteritis in children worldwide [Gentsch et al., 2005; Santos and Hoshino, 2005]. In Thailand, G1 was the most predominant genotype from 1985 to 1989 and co-predominant with G2 from 1987 to 1988 [Maneekarn and Ushijima, 2000]. However, surveillance studies of rotaviruses in children hospitalized with diarrhea in Chiang Mai revealed that G1 was disappeared for four consecutive years (2000–2001 [Khamrin et al., 2006] and in 2002–2003), as shown in Figure 1 of this study. Surprisingly, G1 reemerged as the most predominant genotype in 2004, with a frequency of detection rate as high as about 60%.

Recently, Arista et al. [2006] proposed classifying G1 into seven major lineages based on the phylogenetic analysis of G1 strains reported from several countries worldwide. In addition, lineage I could be split further into three sub-lineages (Ia, Ib, and Ic). The G1 strains detected in this study were formed exclusively in lineage I and appeared to be closely related to G1 strains reported previously from other Asian countries, and they fell into sub-lineage Ic. Nevertheless, based on phylogenetic analysis, G1 strains detected in this study

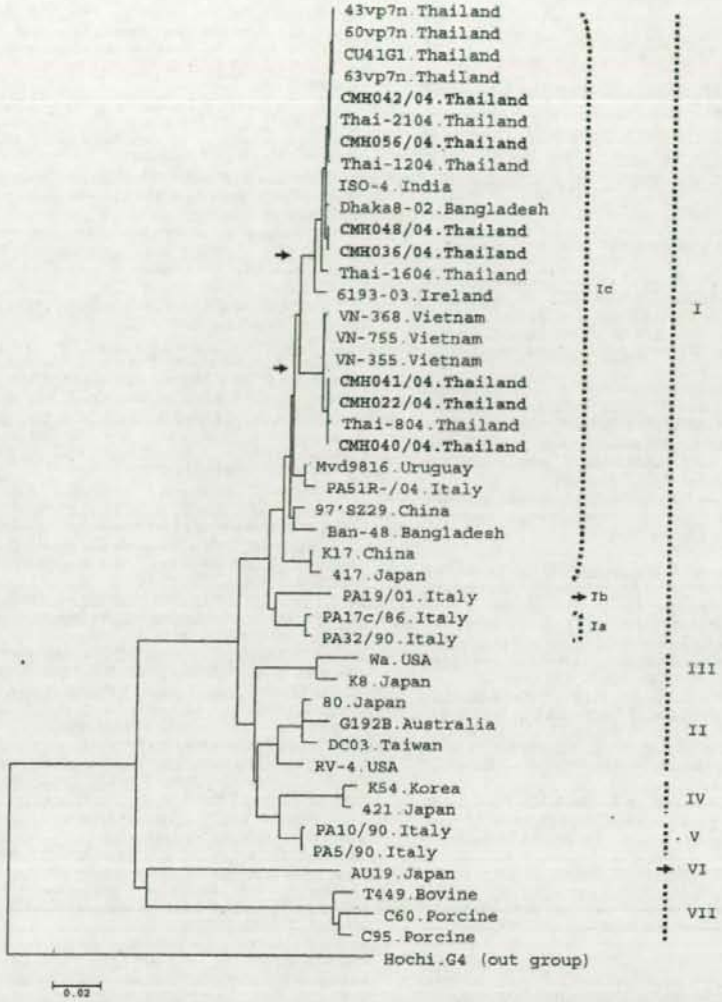

Fig. 4. Phylogenetic analyses of VP7 gene nucleotide sequences of rotavirus G1 strains. The tree was generated based on the neighbor-joining method by using the Mega 3.1 program. The G1 strains used in the analysis were obtained from the GenBank database. The accession numbers of the VP7 reference sequences are listed as follows: 43vp7n (DQ674873), 60vp7n (DQ674881), CU41G1 (DQ236055), 63vp7n (DQ674883), Thai-2104 (DQ512982), Thai-1204 (DQ512980), ISO-4 (AY098670), Dhaka8-02 (AY631049), Thai-1604 (DQ512981), 6193-03 (DQ207389), VN-368 (DQ512969), VN-755 (DQ512974), VN-355 (DQ512968), Thai-804 (DQ512979), Mvd9816 (AF480293), PA51R-/04 (DQ377599), 97'SZ29 (AF260952), Ban-48 (U26364), K17 (D16320), 417 (D16328), PA19/01 (DQ377593), PA17c/86 (DQ377567), PA32/90 (DQ377574), Wa (K02033), K8 (D16344), 80 (D16325), G192B (AF043678), DC03 (AF183859), RV-4 (M64666), K54 (U26377), 421 (D16326), PA10/90 (DQ377587), PA5/90 (DQ377573), AU19 (AB018697), T449 (M92651), C60 (L24164), C95 (L24165), Hochi (AB039035).

were grouped into two discrete clusters. One cluster showed a close genetic relationship with Thai G1 strains isolated in Chiang Mai and Bangkok, while the other one displayed a close genetic relationship with Thai and Vietnamese strains. It is possible that the G1 strains circulating in Thailand might have originated from two different ancestors. Unfortunately, no sequence data of G1 strains isolated before 2000 were available in the databank. Therefore, a comparison of nucleotide sequences between recent Thai G1 strains and older strains was not possible.

In summary, this study presents the changing distribution of rotaviruses in children hospitalized with acute gastroenteritis. The results demonstrate the decline of G9 and reemergence of G1 and G2 rotaviruses in Chiang Mai, Thailand from 2002 to 2004.

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Molecular and epidemiological trend of norovirus associated gastroenteritis in Dhaka City, Bangladesh

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Abstract

Background: Diarrhea, over the years, has killed millions of people and continues to be a major threat in Bangladesh.
Objectives: To determine the incidence of norovirus infection in infants and young children with acute gastroenteritis in Dhaka City, Bangladesh and to determine the genogroup and genotype in norovirus-positive stool specimens.
Study design: Fecal specimens were collected from infants and children with acute gastroenteritis in Dhaka City, Bangladesh from October 2004 to September 2005, and examined for norovirus by reverse transcription-polymerase chain reaction.
Results: Noroviruses were detected in 41 of 917 fecal specimens. Molecular analysis of norovirus was carried out by sequencing methods. Only norovirus GII/4 strains were detected during this study. The dominant genotype throughout the study period was GII/4. Norovirus infections were most commonly observed in winter and rainy seasons in Dhaka City. The common clinical symptoms in norovirus-infected patients were diarrhea (90%), vomiting (75%) and abdominal pain (46%).
Conclusions: This is the first epidemiological research of norovirus in Bangladesh. Norovirus is an important enteropathogen responsible for viral gastroenteritis among infants and children in Bangladesh.

Keywords: Norovirus; RT-PCR; Dhaka; Genogroup; Genotype

1. Introduction

Many viral strains cause acute gastroenteritis in humans. Norovirus (NoV), rotavirus (RV) and astrovirus (ASV) are the most common causes of sporadic cases of this disease and have been responsible numerous outbreaks of nonbacterial gastroenteritis in a variety of settings, including hospitals, day care centers, nursing homes and schools (Atmar and Estes, 2001; Gallimore et al., 2004; Hale et al., 2000; Milazzo et al., 2002). The mortality of acute gastroenteritis among children is greater in developing than developed countries. Annual mortality associated with acute gastroenteritis was estimated to be 2.1 million in 2000 (Mulholland, 2004; Parashar et al., 2003). Group A rotaviruses (GARVs), family Reoviridae, are a major cause of acute diarrhea worldwide in infants (Parashar et al., 2003), while NoVs are recognized as the most important cause of nonbacterial gastroenteritis outbreaks in school-age children and adults worldwide (Koopmans et al., 2003; Parashar and Glass, 2003).

NoV, a member of the calicivirus family together with vesivirus, lagovirus and sapovirus (Farkas et al., 2004), contains single-stranded positive-sense RNA genomes of 7.6 kb excluding the poly-A tail that encodes three open reading frames (ORFs) (Jiang et al., 1993; Lambden et al., 1993). ORF1 encodes a nonstructural polyprotein, which is cleaved

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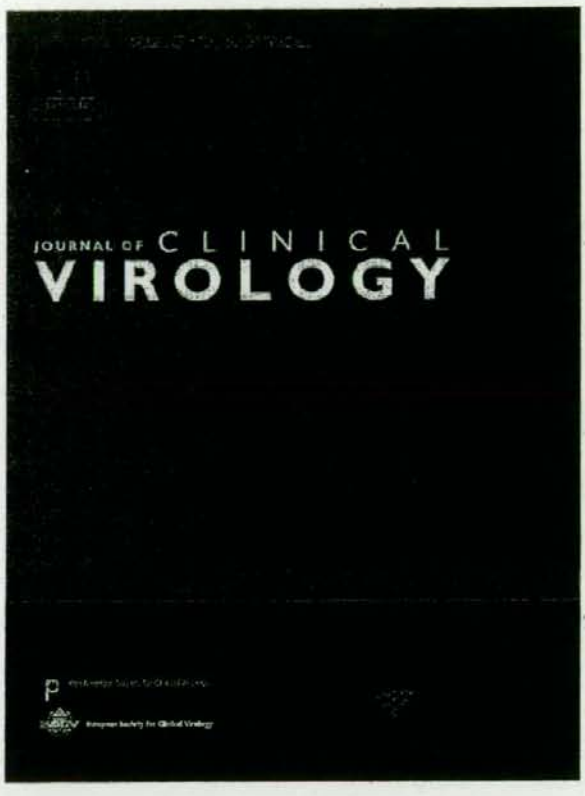

into functional proteins by a virus-encoded 3C-like protease. ORF2 and ORF3 encode the major capsid protein VP1 and minor capsid protein VP2, respectively (Glass et al., 2000; Jiang et al., 1992).

The detection of NoV infections has been limited by the inability to grow it in culture, but molecular detection methods are now available for use during epidemiological studies (Isakbaeva et al., 2005; Kageyama et al., 2004; Matsui et al., 1991). NoV can be divided into five distinct genogroups. NoVGI and NoVGII are mainly infectious to humans. A recent study indicated that NoV GI and GII strains consist of at least 15 and 18 genotypes, respectively (Okada et al., 2005; Seah et al., 1999).

2. Materials and methods

2.1. Study population and fecal specimens

One stool was collected from 917 infants and children with acute gastroenteritis in Dhaka City, Bangladesh from October 2004 to September 2005. Acute gastroenteritis was defined by three or more loose stools in a 24 h period. Stool samples were collected from those patients when viral gastroenteritis was clinically suspected and stored at −20 °C.

2.2. Viral RNA extraction

Fecal specimens were thawed, diluted with distilled water to 10% suspensions, and centrifuged at 10,000 × *g* for 10 min. The supernatant was removed and heated at 65 °C for 10 min. Viral RNA was extracted from 140 μl of the supernatant using a spin-column technique (QIAamp Viral RNA kit; Qiagen®, Hilden, Germany) according to the manufacturer's instructions.

2.3. Reverse transcription

For reverse transcription (RT), 4 μl extracted viral RNA as mixed with a reaction mixture consisting of 5× First strand buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Roche, Mannnheim, Germany), 10 mM DTT (Invitrogen), superscript reverse transcriptase III (200 U/μl) (Invitrogen, Carlsbad, CA, USA), random primer (1 μg/μl) (hexadeoxyribonucleotide mixture) (Takara, Shiga, Japan), RNase inhibitor (33 U/μl) (Toyobo, Osaka, Japan) and MilliQ water. The total of reaction mixture is 8 μl. The RT step was carried out at 50 °C for 1 h, followed by heating at 99 °C for 5 min and then held at 4 °C (Phan et al., 2005).

2.4. Polymerase chain reaction (PCR)

NoV was detected by PCR analysis of cDNA with specific primers previously published (Yan et al., 2003) that target the junction between ORF1 and ORF2. We used G1-SKF (CTGCCCGAATTYGTAAATGA), G1-SKR (CCAACCCARCCATTRTACA) for NoVGI and COG2F (CARGARBCNATGTTYAGRTGGATGAG), G2-SKR (CCRCCNGCATR-HCCRTTRTACAT) for NoVGII. The PCR was performed at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s and a final extension at 72 °C for 7 min and then held at 4 °C.

2.5. Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide (0.5 μg/ml) for 20 min and then visualized under ultraviolet (UV) light. The bands were recorded by photography (Ushijima et al., 1992).

2.6. Nucleotide sequence analysis

The nucleotide sequences of NoV-specific PCR products were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA). Sequence analysis was performed using CLUSTAL X software (version 1.6). A phylogenetic tree was constructed with 100 bootstrap resamples of the nucleotide alignment data sets using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (Phylip).

2.7. Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 3.2 software package (Kumar et al., 2001). The following reference strains and accession numbers were used in the phylogenetic analysis: Hawaii Calcivirus (U07611), Mexicovirus (U22498), Torontovirus (U02030), Lordsdale (X86557), Bristol (X76716), NLV/VA98387/1998 (AY038600), Hu/NV/Hokkaido/194/2004/JP (AB240180), Hu/NV/Hokkaido/231/2004/JP (AB240183), Hu/NV/Hokkaido/306/2005/JP (AB240187), Hu/G-II/Nagano/2004/H/JP (DQ095875) and Stockholm/97 (AF194182).

2.8. Nucleotide sequence submission

The nucleotide sequence data reported in this paper have been deposited in GenBank under the following accession numbers: DQ889461–DQ889501.

3. Results

3.1. Detection of noroviruses

NoVs were detected in 41/917 of the fecal specimens collected from infants and children with acute gastroenteritis. All fecal specimens were also tested for the presence of sapovirus, astrovirus by RT-multiplex PCR. Among diarrheal

Table 1
Clinical features of norovirus-positive and norovirus-negative patients

Sign and symptoms	Norovirus-positive (N=41 (%))	Norovirus-negative (N=876 (%))
Diarrhea	37 (90%)	621 (72%)
Vomiting (≥3 times per day)	31 (75%)	309 (35%)
Abdominal pain	19 (46%)	248 (28%)
Fever (>100 °F)	11 (27%)	217 (24%)
Three to five times loose stools within 24 h	29 (70%)	578 (65%)
Six or more loose stools within 24 h	12 (30%)	298 (35%)
Age at hospitalization (≤24 months)	34 (82%)	824 (94%)

viruses detected, norovirus was the most prevalent (4.5%), followed by 2.7% of sapovirus and 0.3% of astrovirus (Dey et al., 2007). The youngest subject was 2-month-old and the oldest 38 months; the average age was 13 months. All fecal specimens were tested for the detection of NoV by RT-multiplex PCR method.

3.2. Clinical features of norovirus-positive and norovirus-negative patients

Clinical data of the 41 patients infected with NoV are listed in Table 1. The most common clinical symptoms were vomiting (75%), diarrhea (90%), abdominal pain (46%) and fever (27%). The most common clinical features of norovirus-negative patients were diarrhea (72%), vomiting (35%), abdominal pain (28%) and fever (24%). The percentage of vomiting, diarrhea and abdominal pain in norovitus-positive patients were higher than norovirus-negative patients.

3.3. Nucleotide sequence and phylogenetic analyses of norovirus

All the NoV amplicons were characterized for genogroup, genotypes and genetic relationship with the reference strains based on their capsid regions (Okada et al., 2005) classification scheme. Their partial nucleotide sequences were compared to each other as well as to reference NoV strains available in the DDBJ DNA/GenBank database by BLAST. The nucleotide sequence of the 5′ ends of the NoV capsid gene was determined by direct sequencing with the amplified fragments. All of the NoV sequences were classified into genogroup II (Fig. 1) and the NoV genogroup II clustered into one genotype (GII/4).

CLUSTAL X indicated that these NoVs were very similar at the amino acid level and were most closely related to the NLV/VA98387/1998, Hu/NV/Hokkaido/194/2004/JP, Hu/NV/Hokkaido/231/2004/JP, Hu/NV/Hokkaido/306/2005/JP and Hu/GII/Nagano/2004/H/JP strain.

3.4. Seasonal distribution of norovirus

The seasonal pattern of NoV infection in this study is shown in Fig. 2. Two peaks of NoV infection were observed. One peak occurred from November 2004 through March 2005 and another one occurred from August through September in 2005. Ninety percent (37 of 41) positive samples were collected during this peak time. It was found that the nucleotides as well as the amino acid sequences of capsid regions among norovirus isolates were of high identity (above 96%).

4. Discussion

NoV is the most common nonbacterial etiological agent for gastroenteritis outbreaks and a common cause of acute gastroenteritis in children. These outbreaks have a significant public health impact worldwide (Fankhauser et al., 2002; Pang et al., 1999). The NoV genogroup II viruses are dominant in all parts of the world. Several genotypes co-circulate, but the majority of infections are caused by a few genotypes (Koopmans et al., 2003). Predominance of GII strains over GI have been widely observed with sporadic cases of acute diarrhea and during outbreaks (Bon et al., 1999; Buesa et al., 2002; Farkas et al., 2000; Kirkwood and Bishop, 2001; Pang et al., 1999; Roman et al., 2002). This study showed that all Bangladeshi NoV sequences belonged only to genogroup II, which clustered into one genotype (GII/4). NoV detected in this study was closely homologous at the nucleotide and amino acid level. Nucleotide sequence analysis indicated that the common GII/4 strains were circulating among Bangladeshi children and infants.

The present study demonstrated two peaks of NoV infection. This result clearly indicated that norovirus infection was most commonly observed in late autumn to winter seasons (November–March) in Dhaka City.

Most of the norovirus-positive samples collected during this period. In this study, most of the patients (92%) were belonged to 1–24 months age group. The lowest age was 2 months, the highest was 3.2 years (38 months) and the average age was 1.1 years (13 months the NoV has been associated with gastroenteritis outbreaks in all age groups (Green et al., 2001). However, less is known of the age distribution of norovirus infection. In this study, infections were most commonly detected in children less than 3 years of age.

The most common signs and symptoms of infants and children infected with NoV were diarrhea (90%), vomiting (75%) and abdominal pain (46%). These results were consistent with other research. We found more patients with severe diarrhea (90%) than with vomiting (75%), which is in agreement with (Wyatt et al., 1974). Fever (body temperature more than 100 °F) was found in 27% of, which was similar to patients with acute gastroenteritis without. Number of loose stool per day was rather extended with most of the individuals having six to eight times (43%) stool per day.

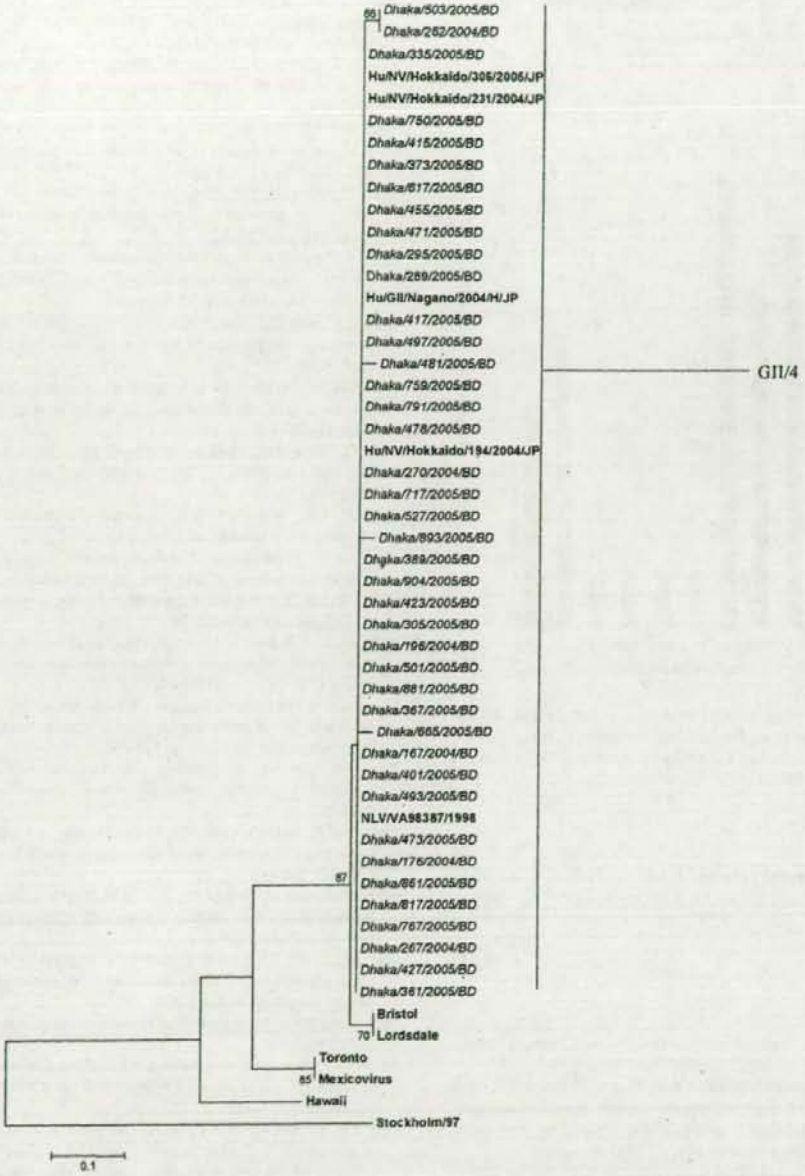

Fig. 1. Phylogenetic tree of nucleotide sequences of Bangladeshi norovirus. The tree was constructed from partial amino acid sequences of capsid region of norovirus. Reference strains of norovirus were selected from DDBJ/GenBank under the accession number indicated in bold. Bangladeshi norovirus was highlighted in italic. Stockholm/93 strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. The GenBank accession numbers of this study are: DQ889461–DQ889501.

We found that NoV-infected persons developed symptoms of severe vomiting and watery diarrhea and typically remain symptomatic for 2–3 days.

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

In conclusion, this is the first epidemiological research of norovirus in Bangladesh.

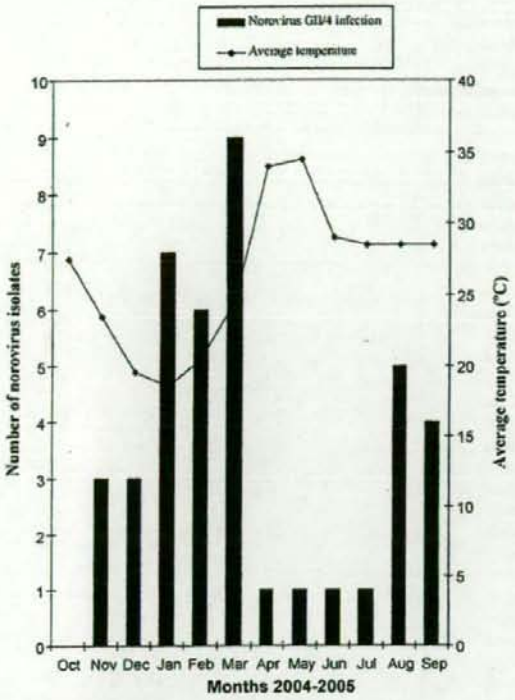

Fig. 2. Seasonal pattern of norovirus infection in infants and children with acute gastroenteritis in Dhaka City, Bangladesh during October 2004–September 2005. Average temperature data were obtained from the Metrological Institute, Dhaka.

Acknowledgements

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Genetic Diversity of Noroviruses and Sapoviruses in Children Hospitalized With Acute Gastroenteritis in Chiang Mai, Thailand

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Human caliciviruses, including norovirus (NoV) and sapovirus (SaV), are recognized as common pathogens that cause acute viral gastroenteritis in children and adults throughout the world. To gain an overview of molecular epidemiology of human caliciviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand, from 2002 to 2004, NoV and SaV were detected and characterized molecularly for identification of their genotypes. From a total of 248 fecal specimens collected, 35 (14.1%) were positive for NoV GII genogroup. Among the 35 NoV GII, GII/4 was the most predominant genotype (22 strains), followed by GII/3 (7 strains), GII/1 (2 strains), GII/7 (2 strains), GII/2 (1 strain), and GII/16 (1 strain). In addition, only three specimens (1.2%) were positive for SaV, each of which was classified into two different genogroups. One isolate was clustered with GIV genogroup, while the other two belonged to two distinct genotypes of the SaV GI cluster, GI/1 and GI/2 genotypes. This study demonstrated that human caliciviruses are important enteric viruses that caused acute gastroenteritis in the hospitalized children in Chiang Mai, Thailand from 2002 to 2004. Moreover, a great genetic diversities of NoV and SaV were observed. ***J. Med. Virol. 79:1921–1926, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: noroviruses; sapoviruses; acute gastroenteritis; Chiang Mai; Thailand

INTRODUCTION

Viral gastroenteritis is one of the most common illnesses in humans worldwide, and different agents such as rotavirus, astrovirus, adenovirus, and calicivirus have been associated with the disease [Clark and McKendrick, 2004]. Among different types of viruses that cause diarrhea, rotavirus is the most common, and is a major cause of severe gastroenteritis in infants and young children worldwide [Parashar et al., 2006]. Recently, however, human caliciviruses have emerged as significant etiologic agents of diarrheal disease across all age groups. Norovirus (NoV) and sapovirus (SaV) are two of the four genera of the family *Caliciviridae*, which is a nonenveloped, positive-sense, single-stranded RNA [Green et al., 2001]. These viruses are a leading cause of gastroenteritis worldwide and are responsible for outbreaks in various epidemiological settings, including restaurants, schools, day-care centers, hospitals, nursing homes, and cruise ships [McEvoy et al., 1996; Russo et al., 1997; McIntyre et al., 2002; Akihara et al., 2005].

Extensive molecular epidemiological studies of calicivirus infection in humans have been conducted. Application of RT-PCR and DNA sequencing techniques for the detection and characterization of NoV and SaV became the standard tests for detecting these pathogens [Yan et al., 2004]. These detection techniques have enhanced markedly our understanding of the epidemiology of NoV and SaV infections. Thousands of NoV and SaV strains have been identified, named, and classified into genogroups and genetic clusters.

Currently, based on the diversity of capsid sequences, NoVs are grouped into five genogroups (GI–GV), of which three have been found in humans; GI, GII, and GIV [Kageyama et al., 2004; Zheng et al., 2006]. Human NoV genogroups are subdivided further into at least

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15 genotypes in GI, 18 genotypes in GII, and only 1 genotype in GIV [Kageyama et al., 2004; Vinje et al., 2004; Okada et al., 2005]. Several epidemiological studies clearly indicated that NoV GII is the main causative agent among NoV, which causes acute diarrhea in humans [Hansman et al., 2004; Phan et al., 2006a,b; Tseng et al., 2007]. SaVs can be divided into five genogroups (GI–GV), of which GI, GII, GIV, and GV are known to infect humans, whereas SaV GIII infects porcine species [Farkas et al., 2004; Wang et al., 2006; Hansman et al., 2007].

In Thailand, very few molecular epidemiological studies of NoV and SaV have been conducted and various genotypes were circulated in different epidemiological settings [Guntapong et al., 2004; Hansman et al., 2004; Veeravigrom et al., 2004]. The frequency of NoV and SaV detection rates ranged from 8.6%–17.5% and 4.8%–15.0% of diarrheal disease in hospitalized cases. However, only two studies have been characterized further for their genotypes by sequence and phylogenetic analyses. The study carried out in Chiang Mai during 2000 to 2001 demonstrated that out of 105 specimens collected, 8 and 4 were found to be a single infection with NoV and SaV, while 1 was a mixed infection [Hansman et al., 2004]. The other study was conducted during 2002–2003. The stool specimens were collected from five different geographical areas in Thailand (Sa Kaeo, Chanthaburi, Songkhla, Nong Khai, and Tak). Of the 80 specimens examined, 11 and 9 of NoV and SaV single infections were identified, respectively, and three were found to be mixed infections [Guntapong et al., 2004].

With the aim of having an overview of molecular epidemiology of human caliciviruses in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand, the prevalence of NoV and SaV infections were examined from 2002 to 2004. The genotypes of NoV and SaV were identified by sequence and phylogenetic analyses.

MATERIALS AND METHODS

Specimens Collection

A total of 248 fecal specimens were collected from pediatric patients aged less than 5-years-old, who were hospitalized with acute gastroenteritis at McCormick Hospital, Chiang Mai, Thailand. The study period was from March 2002 to December 2004.

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

The RNA genome of NoV and SaV were first extracted from 10% fecal suspension supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). The presence of the NoV (GI and GII) and SaV in fecal specimens was detected by RT-PCR using the protocol described previously [Yan et al., 2004]. A forward primer, G1-SKF (nt 5342–5361) 5′-CTGCCCGAATTYGTAAATGA-3′, was used in combination with the reverse primer G1-SKR (nt 5653–5671) 5′-CCAACCCARCCATTRTACA-3′, for the amplification of NoV GI, which specifically generated a PCR amplicon of 330 bp. For NoV GII identification, a forward primer, COG2F (nt 5003–5028) 5′-CARGARBCNATGTTYAGRTGGATGAG-3′, was used in combination with the reverse primer G2-SKR (nt 5367–5389) 5′-CCRCCNGCATRHCCRTTRTACAT-3′, which showed a PCR product size of 387 bp. For SaV detection, a 434 bp fragment was generated using forward primer SLV5317 (nt 5317–5339) 5′-CTCGCCACCTACRAWGCTTGGTT-3′ and reverse primer SLV5749 (nt 5727–5749) 5′-CGGRCYTCAAAVSTACCBCCCCA-3′. All of the virus positive samples were analyzed further for their genotypes by nucleotide sequence and phylogenetic analyses.

Sequence and Phylogenetic Analyses

The PCR amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced by using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated sequencer (ABI 3100; Perkin Elmer-Applied Biosystems, Inc.). The primers used for amplification of the partial capsid genes were also used as sequencing primers. The sequences obtained were compared to those of NoV and SaV strains deposited in the GenBank using the BLAST program. The genotypes of NoV and SaV were classified by using the clustering methods determined previously by Kageyama et al. [2004] and Phan et al. [2007b].

Nucleotide Sequence Accession Numbers

The partial nucleotide sequences of the capsid gene were deposited in GenBank under the accession number EF600759–EF600793 for NoV strains and EF600794–EF600796 for SaV strains.

RESULTS

Detection and Genotype Distribution of Noroviruses

Between March 2002 and December 2004, a total of 248 fecal specimens were collected from pediatric patients hospitalized with diarrhea, and 35 (14.1%) were found positive for NoV by using the RT-PCR screening method (Table I). Of these, all of the positive samples were identified as NoV GII genogroup, and none was a mixed infection between NoV and SaV. The age of the patients, who were infected with NoV, ranged from 4 months up to 3-years-old. All of the NoV positive samples were characterized further for their genotypes by sequencing of the partial capsid regions. The genotypes were classified according to the phylogenetic clustering method described by Kageyama et al. [2004]. A total of 35 NoV GII isolates were clustered exclusively with other GII reference strains and these GII strains were classified further into six distinct genotypes, including GII/1, GII/2, GII/3, GII/4, GII/7,

TABLE I. Prevalence of NoV and SaV Infections in Pediatric Patients Hospitalized With Acute Gastroenteritis in Chiang Mai, Thailand From 2002 to 2004

Year	No. of samples collected	No. of samples positive for virus (%)	
		NoV (GII)	SaV
2002	43	6 (14.0)	0 (0.0)
2003	45	5 (11.1)	2 (4.4)
2004	160	24 (15.0)	1 (0.6)
Total	248	35 (14.1)	3 (1.2)

and GII/16 (Fig. 1). It was observed that GII/4 was the most predominant genotype and accounted for the majority (22 of 35; 62.8%) of NoV isolates detected in the present study. In addition to the GII/4, NoV GII/3 was detected as the second most common genotype and accounted for 20% (7 of 35) of the NoV isolates. The prevalence rates of GII/1 and GII/7 genotypes were equal at 5.7% (2 of 35 isolates). Moreover, a relatively low frequency of GII/2 and GII/16 genotypes at 2.9% (1 of 35) was observed.

Detection and Genotype Distribution of Sapoviruses

Of 248 samples obtained from infants and young children hospitalized with diarrhea, 3 (1.2%) were found positive for SaV by the RT-PCR screening method (Table I). The detection rate of SaV was definitely much lower (1.2%; 3 of 248) than that of NoV (14.1%; 35 of 248). All of the SaV positive samples were identified as a single SaV infection, with none being a mixed infection between NoV and SaV. The age of patients ranged from 8 months to 1-year-old. Of these, all three SaV isolates were directly sequenced and phylogenetically analyzed based on partial nucleotide capsid regions. The phylogenetic tree shown in Figure 2 was generated based on the clustering methods described by Phan et al. [2007b]. From the phylogenetic tree, great genetic diversity of SaV genotypes was observed. All three SaV isolates were clustered exclusively with other human SaV reference strains and classified further into two distinct genogroups. One of the three SaV isolates was clustered with GIV genogroup, while the other two SaV isolates belonged to two distinct genotypes of the GI cluster, GI/1 and GI/2 genotypes, respectively.

DISCUSSION

Based on early antigenic analyses, and more recently extensive sequence analysis, the circulations of both NoV and SaV in nature have been shown to be highly variable. Genetic analysis of NoV in Chiba, Japan from 1999 to 2004 demonstrated that 31.8% of samples collected from sporadic or outbreak cases were positive for NoV. Phylogenetic analysis of these NoV isolates showed a great genetic diversity and at least 13 and 16 genotypes were identified in GI and GII genogroups, respectively [Okada et al., 2005]. Recently, molecular epidemiological studies of SaV have been conducted in several countries, including Australia, Japan, Thailand, UK, USA, and Vietnam, and it was found that the rates of detection and overall prevalence of SaV infections varied from one country to another, with usually much less frequency than NoV infections [Hansman et al., 2004, 2006; Blanton et al., 2006; Gallimore et al., 2006; Nguyen et al., 2007; Phan et al., 2007a].

In Thailand, the first NoV and SaV epidemiological study was conducted in Chiang Mai, during 2000 and 2001 [Hansman et al., 2004]. This surveillance study indicated that NoV and SaV detection rates were 8.6% and 4.8%, respectively. Based on the clustering methods determined previously by Kageyama et al. [2004], NoV GII/4 genotypes was the most predominant genotype. The other co-circulating strains belonged to GI/3, GI/7, GI/8, GII/7, GII/8, and GII/10. For SaV detection, the most prevalent genotype was GI/1, followed by GII/1. Interestingly, one isolate of SaV was found to be an intragenogroup recombinant strain [Hansman et al., 2004; Katayama et al., 2004]. However, the study conducted by Guntapong et al. [2004] from 2002 to 2003 reported a higher prevalence of NoV and SaV infection at 17.5% and 15.9%, respectively. The most prevalent genotype of NoV was GII/4, while the other co-circulating strains were GII/1, GII/3, GII/6, and GII/16. However, the NoV GI genogroup was undetectable from this surveillance study. The majority of SaV strains were GI/1, while one was GV and another represented a novel genotype in the GII cluster. In this study, the prevalence and distribution of NoV and SaV genotypes were investigated in children hospitalized with diarrhea in Chiang Mai, Thailand in three consecutive years from 2002 to 2004. It was found that NoV and SaV circulated in this area with the prevalence rates of 14.1% and 1.2%, respectively. Of these, over 60% of NoV detection was the GII/4 genotype. These results are consistent with the previous findings in Thailand, in which the prevalence rates of NoV were higher than SaV infections, and NoV GII/4 was the most predominant genotype circulating in this country. In 2000 and 2001, GII/4 was accounted for 33.3% of all the NoV genotypes detected [Hansman et al., 2004]. Interestingly, the following years of 2002 and 2003 NoV GII/4 detection rate was increased dramatically to 64.3% [Guntapong et al., 2004]. This data also correlate with our result that during 2002–2004, 62.8% of NoV detection was identified as GII/4 genotype. The accumulated data from previous studies and this study indicate that NoV GI was first detected in Thailand during 2000 and 2001. However, in the three consecutive years of 2002–2004, NoV GI disappeared completely and has remained undetectable. For SaV detection, the prevalence rate is much lower (1.2%) than those of previous reports (4.8% and 15.9%). In addition, it should be noted that although the surveillance data between this study and the study reported by Guntapong et al. [2004] were conducted in the same periods, the vast genetic diversity between the two studies has been observed. For SaV, two different genotypes of GI/1 and GI/2 together with one of the

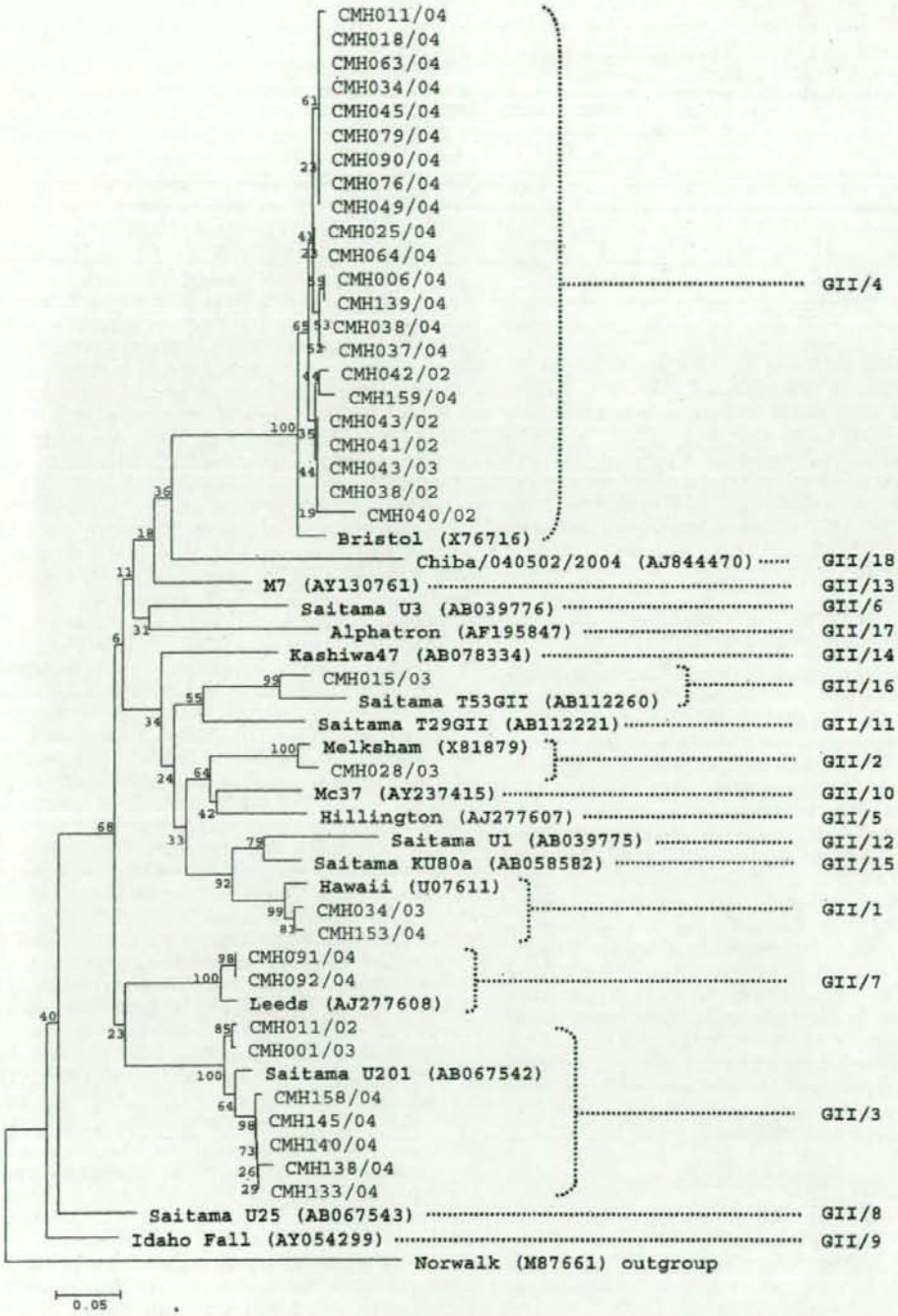

Fig. 1. Phylogenetic analysis of partial capsid sequences of NoV detected in pediatric patients hospitalized with acute gastroenteritis in Chiang Mai, Thailand from 2002 to 2004. The tree was constructed on the basis of the neighbor-joining method and the numbers on each branch indicate the bootstrap values. The NoV outgroup and reference strains of GII/1–GII/18 are presented in boldface and GenBank accession numbers are given in parentheses.

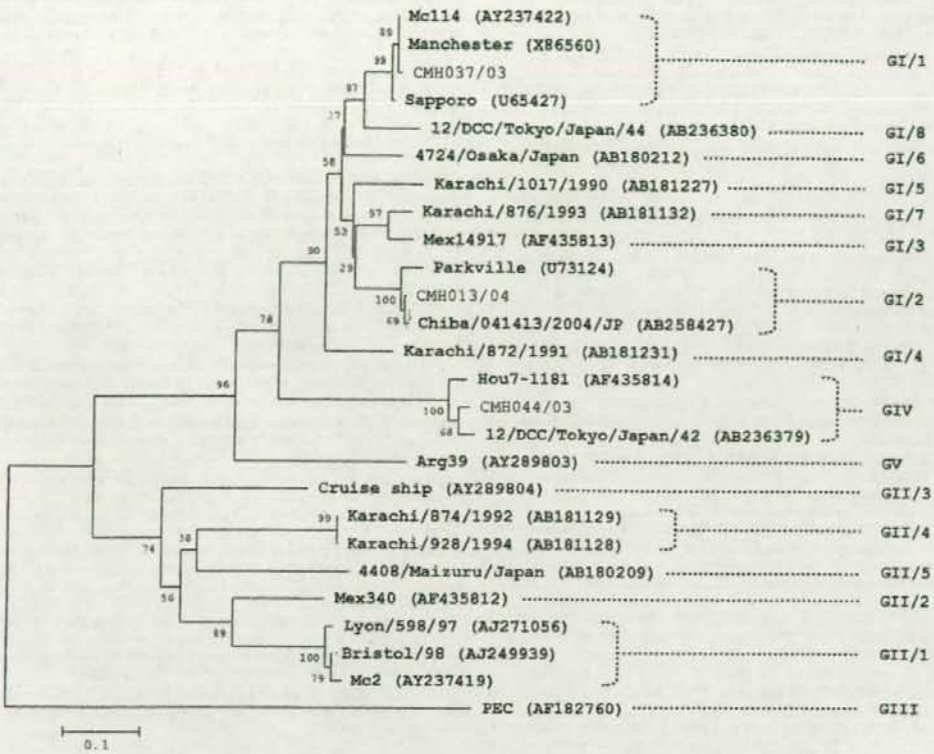

Fig. 2. Phylogenetic analysis of partial capsid sequences of SaV detected in pediatric patients hospitalized with acute gastroenteritis in Chiang Mai, Thailand from 2002 to 2004. The tree was constructed on the basis of the neighbor-joining method and the numbers on each branch indicate the bootstrap values. The SaV reference strains of GI to GV are presented in boldface and GenBank accession numbers are given in parentheses.

SaV GIV genogroup were isolated in this study, while previous studies identified the circulation of SaV GI, GII, and GV strains. The discrepancy of SaV strains detected by the two studies might be due to the study sites, that is, Guntapong et al. [2004] study was done in Sa Kaeo, Chanthaburi, Songkhla, Nong Khai, and Tak while our study was conducted in Chiang Mai city.

In summary, this study demonstrated that human caliciviruses are important enteric viruses causing acute gastroenteritis in hospitalized children in Chiang Mai, Thailand from 2002 to 2004. The great genetic diversities of NoV and SaV have also been observed.

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Sequence Analysis of Vietnamese P[6] Rotavirus Strains Suggests Evidence of Interspecies Transmission

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Nucleotide and amino acid sequences of the VP8* gene of five Vietnamese P[6] rotavirus strains detected from hospitalized patients with acute gastroenteritis were analyzed and compared with other human and porcine P[6] rotaviruses. It is of interest that these strains had greatest identity with two Italian porcine rotavirus strains, 134/04-10 and 134/04-11. To our knowledge, these five Vietnamese rotaviruses are the rare P[6] rotavirus strains belonging to lineage I that cluster into sublineage Ic with porcine rotaviruses, and not into sublineage Ia, as other human P[6] rotaviruses have done so far. Sequence analysis of the VP7 gene of these P[6] rotavirus strains was also performed. The results showed that the Vietnamese G9P[6] strain had high similarity with other human G9 rotaviruses, confirming a human–animal reassortant virus, whereas other three G4P[6] strains had best identity with porcine G4 rotavirus strains, suggesting interspecies transmission of rotavirus between porcine and humans. This result provides the important data on molecular characteristics of Vietnamese rotaviruses, and highlights interspecies transmission events of rotaviruses in Vietnam as well as in Asia. ***J. Med. Virol. 79:1959–1965, 2007.***

KEY WORDS: rotavirus; P[6]; interspecies transmission

INTRODUCTION

Group A rotaviruses are members of the family *Reoviridae*. They are the major cause of acute gastroenteritis in infants and young children. In estimation, rotavirus causes approximately 22% of childhood diarrhea hospitalizations, and is responsible for about 611,000 childhood deaths per year over the world [Parashar et al., 2006]. The rotavirus genome consists of 11 segments of double stranded RNA (dsRNA) enclosed in a triple layered capsid. The outer capsid layer is composed of two proteins, VP7 that defines the G types (derived from glycoprotein), and VP4, that defines P types (derived from protease-sensitive protein) [Estes, 2001]. At least 15 different G genotypes and 27 P genotypes have been established, based on the sequence analysis of VP7 and VP4 genes, respectively [Rao et al., 2000; Martella et al., 2007; Steyer et al., 2007]. Epidemiological studies around the world have demonstrated that rotaviruses of types G1–G4, P[4], and P[8] are responsible for most infections, and four G-P combinations, G1P[8], G2P[4], G3P[8], and G4P[8] have been linked to 88.5% of the rotavirus diarrhea cases among children worldwide [Santos and Hoshino, 2005]. Besides humans, rotavirus also causes gastroenteritis in a wide variety of domestic animals, including porcine, bovine, cattle, etc. Recently, the unusual G and P genotypes which were predominant in animals, such as G5, G8, or P[6], have been increasingly found in humans [Linhares et al., 2002; Kebaabetswe et al., 2005; Nielsen et al., 2005]. Many reports have pointed out the possibility of interspecies transmission of rotavirus between humans and animals [Rahman et al., 2003; Khamrin et al., 2006; Martella et al., 2007].

P[6] human rotavirus strains were first detected exclusively from asymptomatic newborns, raising hopes that such strains may serve as components for future vaccine candidates [Flores et al., 1986; Vesikari et al.,

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1991]. However, recent studies could detect P[6] rotaviruses not only from asymptomatic, but also from symptomatic children [Jagannath et al., 2000; Linhares et al., 2002; Rahman et al., 2003]. Although P[6] rotaviruses were detected from various countries on different continents, most of them cluster in one distinct lineage called the M37-like lineage, except two strains; AU19, which is a unique human G1 rotavirus displaying a supershort RNA pattern [Nakagomi et al., 1999], and Gottfried, originally isolated from the intestinal content of a suckling pig with diarrhea [Bohl et al., 1984]. M37, Gottfried, and AU19 viruses are considered as prototype strains of lineage I, II, and III P[6] rotaviruses, respectively. Banyai et al. [2004] described the identification of six unusual human P[6] strains which do not cluster into lineage I as other human P[6] rotaviruses have done so far. These six P[6] strains may be classified into two novel lineages, IV and V. Then, Martella et al. [2006] introduced porcine P[6] rotaviruses which were more related to human strains, either P[6]-I or P[6]-V, than to porcine reference strain Gottfried (lineage II). However, the porcine P[6] strains within lineage I displayed a certain heterogeneity, and they could be clustered into three sub-lineage, Ib to Id, while the human P[6]-I strains were highly homogenous and clustered into a unique lineage Ia. Recently, the reports from China and Vietnam first introduced the identification of human P[6] strains (LL36755, KH210, and KH228) belonging to lineage I that cluster into sublineage Ic with porcine rotaviruses, and not into sublineage Ia, showing the diversity of human P[6] rotavirus strains as well as the possibility of interspecies transmission of rotaviruses between porcine and humans [Ahmed et al., 2007; Duan et al., 2007].

Human P[6] rotaviruses have been identified in several epidemiological studies in Vietnam [Doan et al., 2003; Nguyen et al., 2005], and sequence analysis of VP4 and VP7 genes of Vietnamese G5P[6] strains suggests evidence of animal-like rotavirus infection in humans [Ahmed et al., 2007]. In this study, we investigate the nucleotide sequences of VP4 and VP7 gene among five Vietnamese rotavirus strains bearing the P[6] genotype detected in a 1-year surveillance in Ho Chi Minh City, and compare with other human and animal P[6] rotaviruses.

MATERIALS AND METHODS

P[6] Rotavirus Strains

A 1-year surveillance of common viral agents was conducted in the Children's Hospital 1, Ho Chi Minh City, Vietnam between October 2002 and September 2003 [Nguyen et al., 2007]. Briefly, fecal specimens from 1,010 hospitalized patients with acute gastroenteritis were analyzed by RT—multiplex PCR, and group A rotavirus could be determined in 681 of 1,010 (67.4%) samples. Six hundred seventy-four out of 681 rotavirus-positive fecal specimens had enough material to be analyzed further by P genotyping according to the method described by Gentsch et al. [1992]. The results showed that P[8] was the most predominant genotype (362/674, 53.2%), followed by P[4] (202/674, 30.0%), P[6] (5/674, 0.7%), and P[19] (1/674, 0.15%). Five detected P[6] rotavirus strains were used in this study. Vesikari's numerical score was used to evaluate the severity of patients [Ruuska and Vesikari, 1990].

RT-PCR and Nucleotide Sequencing

For VP4 gene, an 887bp amplicon including the entire VP8* fragment and part of the VP5* fragment was amplified by Con3 and Con2 primers [Gentsch et al., 1992]. Regarding VP7 gene, two primers Beg9 and End9 were used to get a 1,062 bp amplicon [Gouvea et al., 1990]. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were sequenced by using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA) and Big Dye Terminator Cycle Sequencing Kit version 3.1, according to the manufacturer's instruction. The primer pairs Con3–Con2, and Beg9–End9, were used as sequencing primers.

Phylogenetic Analysis

Multiple sequence alignments were calculated using the CLUSTALW program, and phylogenetic analyses were conducted with the MEGA2 software package [Kumar et al., 2001], based on different P[6] rotavirus sequences available in GenBank.

Accession Numbers

The nucleotide sequences of Vietnamese rotavirus strains described in this study have been deposited in GenBank under accession numbers: DQ471353, EF179115–EF179118, for VP4 gene; and EF544998–EF545001, for VP7 gene.

RESULTS

Characterization of Patients

Five hospitalized patients had fecal specimens that were identified as having P[6] rotavirus infection. Table I shows the combined G genotype, patient profile, clinical symptoms, and Vesikari's numerical scores of Vietnamese P[6] strains.

Sequence and Phylogenetic Analysis of the VP8*

Partial gene encoding for the VP4 of five P[6] rotavirus strains was successfully sequenced including the hypervariable region VP8* cleavage and a small part of the conserved region VP5*. A BLAST search demonstrated that all five strains shared the greatest sequence identity with P[6] rotavirus strains, confirming the result of RT-PCR genotyping. Since the shortest VP4 gene of P[6] rotaviruses available from GenBank was 500 nucleotides in length, a comparison was performed with a shorter region spanning nucleotide 200–700 and amino acid 65–230. Nucleotide and amino acid comparison showed that Vietnamese P[6] strains had higher

TABLE I. Clinical Data of Hospitalized Patients Infected by P[6] Rotavirus in Children's Hospital 1, Ho Chi Minh City, 2002–2003

Strain	G-type	Age (month)	Date of collection	Duration of diarrhea (days)	Max no. of diarrheal stools/24 hr	Duration of vomiting (days)	Max no. of vomiting episodes/24 hr	Fever (°C)	Dehydration	Treatment	Vesikari's score
VN584/2003	Mixed[a]	12	April 2003	4	7	3	5	Mild	None	Hospitalization	13
VN592/2003	G4	11	April 2003	5	7	0	0	None	None	Hospitalization	7
VN602/2003	G4	23	April 2003	10	7	2	2	Moderate	None	Hospitalization	14
VN846/2003	G4	15	August 2003	4	5	0	0	None	None	Hospitalization	5
VN904/2003	G9	8	August 2003	5	4	1	3	None	None	Hospitalization	9

[a]Mixed infection between G4 and G9.

homology with lineage I strains (85.8–94.0% for nucleotide and 82.5–96.3% for amino acid) than other lineages (79.2–86.2% for nucleotide and 79.5–92.1% for amino acid). Furthermore, the similarity between Vietnamese strains and Italian porcine P[6] strains, which belonged to sublineage Ic, was much higher than that of human rotaviruses in sublineage Ia, normally called M37-like lineage (Table II). These results and the phylogenetic tree of P[6] human and porcine rotaviruses clearly indicated that Vietnamese P[6] rotaviruses clustered into sublineage Ic together with two porcine P[6] rotaviruses detected from Italy and other three human P[6] strains that likely originated from porcine (Fig. 1).

A deduced amino acid comparison of the VP8* was performed between Vietnamese P[6] rotavirus strains and other human and porcine rotaviruses among 5 lineages from residues 65 to 230. The highly conserved prolines at residues 68, 71, 224, and 225 were seen in all studied strains. Five Vietnamese P[6] strains shared the conserved amino acids with other lineage I P[6] rotavirus strains, within a submitted region (aa 105-L, aa 134-T). However, Vietnamese P[6] rotavirus strains did not bear several conserved residues with other human P[6] rotaviruses within sublineage Ia (aa 91, I instead of V; aa 101, V instead of I; aa 147, A instead of V; triple GGR instead of YNS at positions 170–172; and aa 202, T instead of V). Interestingly, Vietnamese strains shared the conserved residue with the porcine reference strain Gottfried and other porcine rotaviruses within lineage I at position 216 (asparagine). The comparison results also clearly showed that sublineage Ic, which included Vietnamese strains, was found to be a single conserved amino acid substitution (aa 140-Y) that is phenylalanine in other P[6] rotaviruses.

Sequence and Phylogenetic Analysis of the VP7 Gene

Nearly full-length of the gene encoding for VP7 protein of four studied strains was successfully determined the nucleotide sequence (the strain VN584/2003 was not enough material for further analysis). A BLAST search confirmed the results of G genotyping by nested PCR. Nucleotide and amino acid comparison showed that the Vietnamese G4 strains had higher homology with other porcine strains, including the Gottfried strain (85.8–94.0% for nucleotide and 82.5–96.3% for amino acid) than other human G4 rotavirus strains (79.2–86.2% for nucleotide and 79.5–92.1% for amino acid). In contrast, the Vietnamese G9 strain had the greatest identity with human rotavirus strains (Table III). The phylogenetic tree of VP7 gene clearly showed that all Vietnamese G4 strains clustered together with porcine rotaviruses, whereas the G9 strain, VN904/2003, was in the same cluster with recent human G9 rotavirus strains (Fig. 2).

DISCUSSION

The prototype M37 strain was first recovered from an asymptomatic infant in a newborn nursery, followed by a wide detection of similar strains among children with