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Genetic Diversity of Norovirus and Sapovirus in Hospitalized Infants with Sporadic Cases of Acute Gastroenteritis in Chiang Mai, Thailand

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Stool specimens from hospitalized infants with sporadic gastroenteritis in Chiang Mai, Thailand, between July 2000 and July 2001 were examined for norovirus and sapovirus by reverse transcription-PCR and sequence analysis. These viruses were identified in 13 of 105 (12%) specimens. One strain was found to be a recombinant norovirus.

Norovirus and sapovirus are two of the four genera of the family *Caliciviridae* and are well-characterized agents of human acute gastroenteritis (6–9). These viruses can be transmitted by a variety of routes, including food (15) and water (16). Three genogroups (GI, GII, and GIII) for norovirus and four genogroups (GI, GII, GIII, and GIV) for sapovirus are thought to exist, though only norovirus GI and GII and sapovirus GI, GII, and GIV are known to infect humans (11, 18). Numerous molecular epidemiological studies have revealed a global distribution of these viruses (2–4, 17, 19). However, very few molecular epidemiological studies have been conducted in Asian countries other than Japan. In this study, we detected norovirus and sapovirus in stool specimens from hospitalized infants with gastroenteritis in Thailand and partially sequenced the capsid gene to determine genogroups and genotypes.

One hundred five stool specimens collected from hospitalized infants (ranging from 1 month to 5 years of age) with acute sporadic gastroenteritis in Chiang Mai, Thailand, between July 2000 and July 2001 were examined for norovirus and sapovirus by reverse transcription-PCR. This included 52 specimens from McCormic Hospital, 21 specimens from Chiang Mai University Hospital, 23 specimens from Nakornping Hospital, and nine specimens from Sanpatong Hospital. RNA was extracted with the QIAamp viral RNA minivacuum protocol (Qiagen) according to the manufacturer's instructions. Reverse transcription was carried out in a final volume of 20 μl with 10 μl of RNA in 50 pmol of random hexamer (Takara), 1× Superscript II reverse transcription buffer (Invitrogen), 10 mM dithiothreitol (Invitrogen), 0.4 mM each of the four deoxynucleoside triphosphates (Roche), 1 U of RNase inhibitor (Toyobo), and 10 U of Superscript II reverse transcriptase (Invitrogen). Reverse transcription was performed at 42°C for 1 h, followed by deactivation of reverse transcriptase at 72°C for 15 min.

The norovirus PCR primers were selected from three reports that described detection of a broad range of strains (10, 13, 14). For norovirus GI we used primers COG1F (sense) and G1SKR (antisense). For norovirus GII we used primers G2F3 (sense) and G2SKR (antisense). For sapovirus, we used novel capsid gene region primers (corresponding to nucleotides 5083 to 5516 of Manchester virus; GenBank accession number X86560), the SV5317 primer (sense; 5'-CTC GCC ACC TAC RAW GCB TGG TT-3'), and the SV5749 primer (antisense; 5'-CGG RCY TCA AAV STA CCB CCC CA-3' [where R is A or G; W is A or T; S is C or G; Y is C or T; V is A, C, or G; and B is C, G, or T]). PCR was carried out with 5 μl of cDNA in a PCR mixture containing 33 pmol of each primer, 1× *Taq* DNA polymerase buffer B (Promega), 0.2 mM each of the four deoxynucleoside triphosphates, 2.5 U of *Taq* polymerase (Promega), and up to 50 μl of distilled water. PCR was performed at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 5 min. Reverse transcription-PCR products were sequenced and used for genetic classification. Partial and complete nucleotide sequencing and phylogenetic analysis were performed as previously described (11). The nucleotide sequences determined in this study have been deposited in GenBank under accession numbers AY237410 to AY237423.

Norovirus and sapovirus were detected in 12% (13 of 105) of stool specimens from infants admitted to three of the four hospitals in the Chiang Mai region. The age at infection ranged from 4 months to 5 years. All but one of the infants infected with sapovirus were 12 months of age or younger, the youngest infant being 4 months of age. Norovirus also mostly infected infants 12 months of age or younger. One infant was infected with both norovirus and sapovirus strains. Figure 1a shows the phylogenetic tree of the nine norovirus capsid sequences isolated together with reference sequences. The Thai sequences belonged to three distinct norovirus GI genotypes and three norovirus GII genotypes. One norovirus GII sequence (isolate Mc37) that did not cluster with any of the published genotypes was characterized further by complete genome sequencing.

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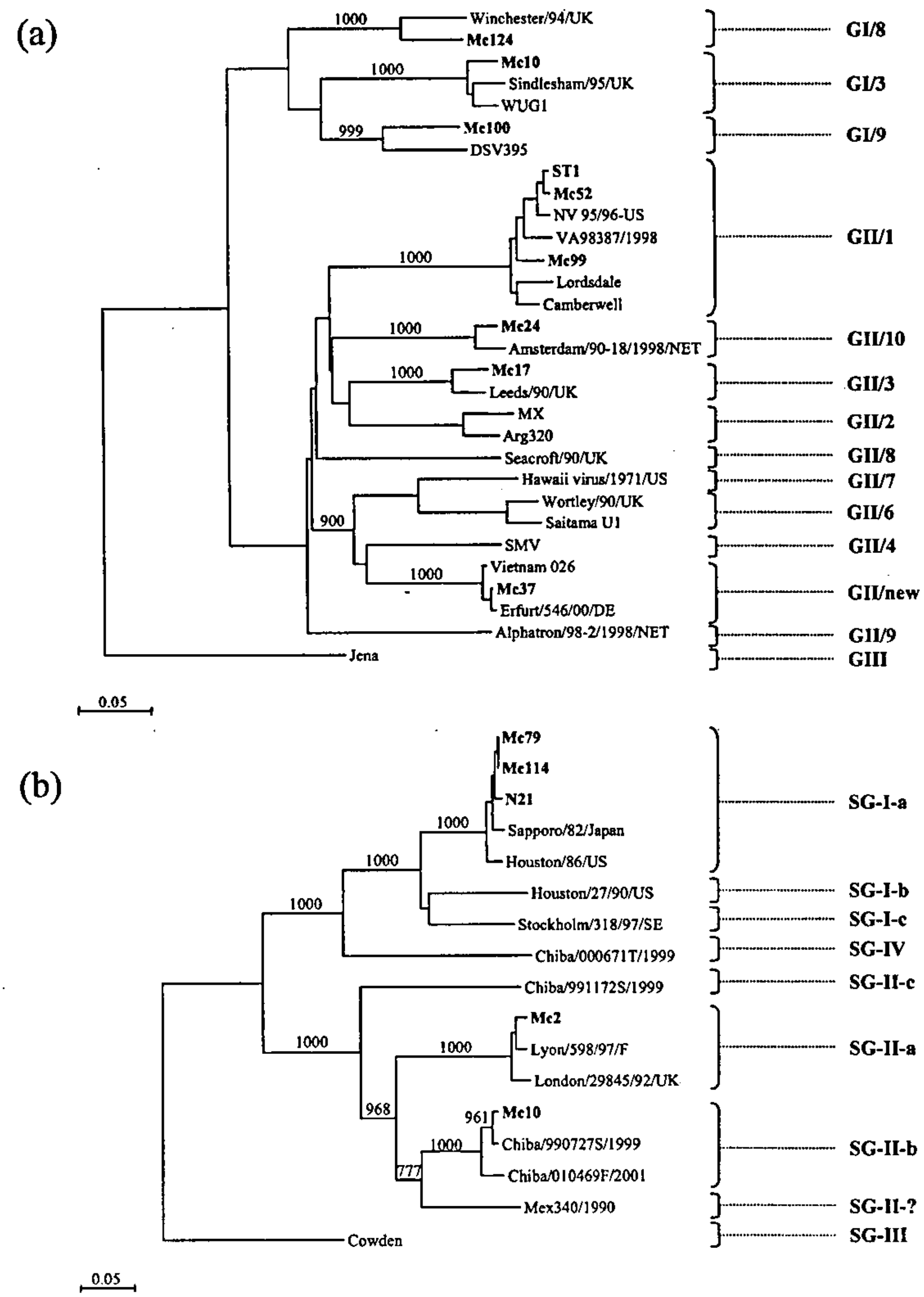

FIG. 1. Phylogenetic analysis of sequences isolated in Thailand. (a) Norovirus capsid sequences (264 bp). (b) Sapovirus capsid sequences (376 bp). The numbers on each branch indicate the bootstrap values for the genotype. Thai sequences are represented in bold. For example, Mc2 is the strain isolated at McCormic Hospital from patient 2. Mc, McCormic Hospital; St, Sanpatong Hospital; N, Nakornping Hospital. Norovirus sequences were classified according to the scheme of Katayama et al. (11), and sapovirus sequences were classified based on the scheme of Okada et al. (18). GenBank accession numbers for the reference strains are as follows: Vietnam 026, AF504671; Alphatron/98-2/1998/NET, AF195847; Amsterdam/98-18/1998/NET, AF195848; Arg320, AF190817; Camberwell, U46500; Chiba/000671T/1999, AJ412805; Chiba/990727S/1999, AJ412795; Chiba/991172S/1999, AJ412797; Chiba/010469F/2001, AJ412820; Cowden, AF182760; DSV395, U04469; Erfurt/546/00/DE, AF427118; Hawaii virus/1971/US, U07611; Houston/86/US, U95643; Houston/27/90/US, U95644; Jena, AJ011099; Leeds/90/UK, AJ277608; London/29845/92/UK, U95645; Lordsdale, X86557; Lyon/598/97/F, AJ271056; Mex340/1990, AF435812; MX, U22498; 408/97003012/1996/FL (NV 95/96-US), AF080558; Saitama U1, AB039775; Sapporo/82/Japan, U65427; Seacroft/90/UK, AJ277620; Sindlesham/95/UK, AJ277615; SMV, AY134748; Stockholm/318/97/SE, AF194182; VA98387/1998, AY038600; Winchester/94/UK, AJ277609; Wortley/90/UK, AJ277618; and WUG1, AB081723.

The genome of Mc37 comprised 7,541 nucleotides, excluding the poly(A) tail, and contained three open reading frames (ORFs). The ORF1 sequence showed 97.2% nucleotide identity to that of Saitama U1 virus (AB039775) but only 71.3 and 67.9% nucleotide identity in ORF2 and ORF3, respectively. Consequently, strain Mc37 likely represents a novel recombinant norovirus.

Figure 1b shows the phylogenetic tree of the five sapovirus sequences isolated together with reference sequences. The sapovirus primers detected both GI and GII sapovirus sequences. Three of the five sapovirus sequences belonged to one sapovirus GI cluster, SG-I-a. The two other sapovirus sequences, sapovirus isolates Mc2 and Mc10, belonged to two distinct sapovirus GII clusters, SG-II-a and SG-II-b, respectively. The sapovirus Mc2 sequence showed 78.5% nucleotide identity to the sapovirus Mc10 sequence. The sequences with the closest matches to the sapovirus Mc10 sequence were from two strains isolated in Japan, Chiba/010469F/2001 virus (AJ412820) and Chiba/990727S/1999 virus (AJ412795), showing 95 and 97% nucleotide identity, respectively. The next closest sequence in the GenBank database (Mex340/1990, AF435812) showed only 82% nucleotide identity.

Our results are consistent with those from similar studies. In a report from Spain, 14.19% of stool specimens were positive for norovirus and sapovirus (1), and the majority of strains belonged to norovirus GII (10.65%), followed by norovirus GI (2.26%) and sapovirus (1.29%). Also, in an Australian report, the overall annual minimum incidence rate in hospitalized children was 8.5% for norovirus and 0.6% for sapovirus infection (12). The majority of norovirus strains detected in this Australian report and another from Ireland (5) were of the Lordsdale virus cluster (GII/1). Our study identified several norovirus sequences in this cluster that closely matched a norovirus 95/96-US strain sequence. Recently, several reports have highlighted the importance of the 95/96-US strain's having global distribution and causing a significant number of outbreaks of gastroenteritis (2, 5, 17, 19). In conclusion, these data have described great genetic diversity among both norovirus and sapovirus strains cocirculating in the Chiang Mai region of Thailand and increased the evidence for the worldwide distribution of these viruses.

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Detection of norovirus and sapovirus infection among children with gastroenteritis in Ho Chi Minh City, Vietnam

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Summary. This report describes norovirus (NoV) and sapovirus (SaV) infections in hospitalized children with acute sporadic gastroenteritis in Ho Chi Minh City, Vietnam. Stool specimens collected between December 1999 and November 2000 were examined for NoV and SaV using reverse transcription-PCR and phylogenetic analysis. NoVs were detected in 72 of 448 rotavirus-negative specimens, counted as part of an overall annual detection rate of 5.4% (72 of 1,339 children). This included four NoV genogroup I (GI) strains and 68 NoV GII strains. Only one SaV GI strain was detected in the rotavirus-negative specimens. Over 73% of the NoV sequences belonged to GII/4 (Lordsdale cluster) and were detected in all months except March. We also detected GII/3 strains (Saitama U201 cluster), a naturally occurring recombinant NoV, between January 2000 and March 2000 but not after this period. Other NoV strains belonging to GI/4, GI/8, GII/1, and GII/7 were also detected but were infrequent. In addition, two almost identical NoV GII strains (strains 026 and 0703) collected six months apart were classified into a new genotype that includes the Mc37 strain, which was previously shown to be a recombinant NoV. During this one-year study, the NoV prevailed at the end of the rainy season and the beginning of the dry season. Further epidemiological studies may be necessary to determine whether the GII/4 strains continue to dominant in this region.

Introduction

To date, two types of caliciviruses that cause acute gastroenteritis in humans have been identified, those in genus *Norovirus* (NoV, formerly known as "Norwalk-like viruses") and those in genus *Sapovirus* (SaV, formerly known as "Sapporo-like viruses"). NoVs are a leading cause of gastroenteritis worldwide and cause outbreaks in various epidemiological settings including hospitals [43], cruise ships [34], schools [13], and restaurants [35]. The prototype strain of NoVs is the Norwalk virus (Hu/NV/Norwalk virus/1968/US), which was first discovered from an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio, U.S.A. in 1968 [22]. NoVs have been divided into five genogroups, among which only genogroup I (GI) and GII are known to infect humans [23]. A recent study indicated that NoV GI and GII strains consist of at least 14 and 17 genotypes, respectively [21]. The NoV genome contains three open reading frames (ORFs). The first ORF (ORF1) encodes non-structural proteins, including the RNA-dependent RNA polymerase (RdRp), ORF2 encodes the major capsid protein (VP1), and ORF3 encodes a small capsid protein (VP2) [2]. NoVs are uncultivable by standard culture methods. However, expression of either VP1 alone or a region encoding both VP1 and VP2 using recombinant baculoviruses resulted in the formation of virus-like particles (VLPs) that are morphologically and antigenically similar to the native virion [15, 16, 19, 26].

SaVs are thought to mostly infect infants, occasionally causing outbreaks [38, 41]. The prototype strain of SaVs is the Sapporo virus (Hu/SV/Sapporo virus/1977/JP), which was originally discovered from an outbreak in a home for infants in Sapporo, Japan, in 1977 [4]. Although many fewer SaV strains than NoV strains have been identified, SaVs are divided into four genogroups, among which only SaV GI, GII, and GIV are known to infect humans. The SaV genogroups are tentatively comprised of four GI clusters, three GII clusters, and one GIV cluster [41]. The SaV genome is organized in a slightly different way than is the NoV, since it contains only two ORFs. ORF1 encodes all the non-structural proteins and the major capsid protein, while ORF2 encodes a small protein, believed to be similar to VP2 of NoV [1].

In both NoV and SaV, the genotypes are generally maintained across the three ORFs. However, several strains failed to maintain their sequence identities for ORF1 and ORF2, and they were shown to be recombinant NoVs [24, 30, 45].

Immunological and seroepidemiological studies have shown that the prevalence of serum antibody levels to NoV and SaV in infants was lowest in the first year of life, rising after two years of age [17, 32, 37]. One study conducted in the UK found that the prevalence of NoV antibody differed regionally [10]. Additionally, children in developing countries may be exposed to these viruses more frequently than are children from developed countries due to lower hygiene standards. Numerous molecular epidemiological studies have revealed a global distribution of these viruses [37, 39, 46]; however, little is known about their infections in children in Asian countries other than Japan.

The objectives of this study were to describe the NoV and SaV detection rates in rotavirus-negative stool specimens from children with acute sporadic gastroenteritis in Ho Chi Minh City, Vietnam. We characterized the genotypes of NoV and SaV strains detected in stool specimens and the NoV seasonal trend. In addition, we genetically and antigenically analyzed a recombinant NoV strain detected in this city.

Materials and methods

Specimens

Stool specimens were collected from children one month to 15 years of age presenting with acute sporadic gastroenteritis at the General Children's Hospital No. 1 in Ho Chi Minh City, Vietnam, between December 1999 and November 2000 [6]. Four hundred and forty-eight rotavirus-negative stool specimens from an available 1,339 specimens were selected for NoV and SaV analysis. Between 16 and 56 of the rotavirus-negative specimens (mean, 37 specimens) were screened for each month of the study period.

One additional NoV strain, 9912-02F (AB044366), isolated from an adult male in an outbreak of gastroenteritis in November 1999 in Hiroshima, Japan, was used as a reference strain for genetic and antigenic analysis of the recombinant NoVs.

Statistical analysis

We used the chi-squared test (χ^2) to determine the significance of each age group and the seasonality of NoV detection and the Fisher Exact test to determine the significance of the NoV and SaV detection rates in Vietnam and Thailand [11]. Mixed infections were excluded from the analysis. A P-value < 0.05 was considered statistically significant.

RNA extraction

A 10% (w/v) stool suspension was prepared with sterilized MilliQ water and centrifuged at $10{,}000 \times g$ for 10 min. The QIAamp Viral RNA Mini Vacuum Protocol (Qiagen, Hilden, Germany) was used to extract RNA from 140 µl of the clarified supernatant according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

cDNA synthesis was carried out with 10 µl of the RNA in 20 µl of the reaction mixture containing 50 pmol random hexamer (Takara, Tokyo, Japan), 1× Superscript II RT buffer (Invitrogen, Carlsbad, Calif.), 10 mM DTT (Invitrogen), 0.4 mM of each dNTP (Roche, Mannheim, Germany), 1 U RNase inhibitor (Toyobo, Tokyo, Japan), and 10 U Superscript RT II (Invitrogen). RT was performed at 42 °C for 1 h, followed by inactivation of RT at 72 °C for 15 min. For detection of NoV, we selected PCR primers from three reports that described the performance in detecting a broad range of strains [20, 26, 28]. For NoV GI we used sense COG1F and antisense G1SKR primers. For NoV GII sense G2F3 and antisense G2SKR primers were used. For detection of SaV, we used sense SV5317 and antisense SV5749 primers that amplify the capsid gene region [11]. NoV GII RdRp region was also amplified to identify recombinant NoVs using primers previously described [46]. PCR was carried out with 5 µl of cDNA in 50 µl of the mixture containing 33 pmol of each primer, 1× *Taq* DNA polymerase buffer B (Promega, U.S.A.), 0.2 mM of each dNTP, and 2.5 U *Taq* polymerase (Promega, U.S.A.). After an initial denaturation at 94 °C for 5 min, 35 cycles of amplification were performed using the GeneAmp PCR System 9600 (PE Biosystems, Foster City, CA). Each cycle consisted of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and extension reaction at 72 °C for 1 min followed by final extension at

72 °C for 5 min. Water samples were used in each RT-PCR as negative controls to monitor false-positives and contamination. Products were visualized under UV light in a 1% agarose gel stained with ethidium bromide. The nucleotide sequences of the amplified fragments were directly determined as described [11]. Complete genome sequencing was performed as described [24].

Phylogenetic analysis

Sequence analysis was performed using Clustal X (version 1.82), and the columns containing gaps were removed. We calculated the genetic distance using Kimura's two-parameter method. Phylogenetic trees with 1,000 bootstrap replicates were generated using the neighbor-joining method with Clustal X. We used SimPlot software to compare recombinant NoV sequences [31]. GenBank accession numbers for the reference strains are as follows: Norwalk/68/US, M87661; BS5/98/GE, AF093797; WUG1, AB081723; Sindlesham/95/UK, AJ277615; Southampton/91/UK, L07418; SaitamaSzUG1/99/JP, AB039774; Musgrove/89/UK, AJ277614; Chiba 407/87/JP, AB042808; Winchester/94/UK, AJ277609; Desert Shield DSV395/US, U04469; SaitamaKU8GI/99/JP, AB058547; V4/00/SW, AF407000; Lordsdale/93/UK, X86557; Camberwell/94/AU, AF145896; 416/97003156/1996/LA/US, AF080559; SaitamaU3/97/JP, AB039776; Saitama U201/98/JP, AB067542; Mexico/89/MX, U22498; Arg320/95/AR, AF190817; Hillington/90/UK, AJ277607; Erfurt/546/00/GE, AF427118; Mc37/99/Thai, AY237415; SaitamaKU5GII/00/JP, AB058575; Snow Mountain/76/US, U70059; Saitama U1, AB039775; 9912-02F, AB044366; Wortley/90/UK, AJ277618; Hawaii/71/US, U07611; Girlington/93/UK, AJ277606; Kashiwa47/00/JP, AB078334; SaitamaU25/98/JP, Amsterdam/98/NE; Leeds/90/UK, AJ277608; Gwynedd/273/1994/US, AF414409; and Alphatron/98/NE, AF195847.

Expression of NoV capsid protein

To characterize antigenicity, we expressed the VP1 of the 026 and 9912-02F strains in insect cells. Briefly, PCR-generated fragments were cloned according to Gateway Technology and Baculovirus Expression protocol (Invitrogen). For 026 strain, we used a sense primer attB1Viet026 (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC C<u>AT GAA GAT GGC GTC GAA TGA CGC CGC TCC A</u>-3′). For 9912-02F strain, we used a sense primer attB1Hiro (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA AGG AGA TAG AAC C<u>AT GAA GAT GGC GTC GAG TGA CGC CGC TCC</u>-3′). The underlined sequences represented the VP1 start sequences. For the antisense primer we used attB2Tx30SXN (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTA GAC TAG TTC TAG ATC GCG AGC GGC CGC CCT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT-3′).

Recombinant bacmids were transfected into Sf9 cells using Effectene according to the manufacturer's instructions (Qiagen, Hilden, Germany). Sf9 cells were incubated for five days at 26 °C, after which the culture medium was clarified by low-speed centrifugation, and the supernatant was stored as the seed baculovirus. Tn5 cells were infected with the seed baculovirus at 26 °C and harvested six days postinfection. The VLPs secreted into the cell medium were separated from the cells by low-speed centrifugation, concentrated by ultracentrifugation at 30,000 rpm at 4 °C for 2 h (Beckman SW-32 rotor), and resuspended in 100 μl of Grace's medium. The VLPs were purified by CsCl equilibrium gradient ultracentrifugation at 45,000 rpm at 15 °C for 18 h (Beckman SW-55 rotor). Rabbits were immunized with 10 μg of VLPs in Freund's complete adjuvant. After one month, the animal received a booster. Hyperimmune serum was collected one week after the last injection.

SDS-PAGE

We used SDS-PAGE to examine the VP1 expression using a 5–20% gradient polyacrylamide gel (ATTO, Tokyo, Japan). The samples were mixed with a 1/10 volume of buffer solution containing 62.5 mM Tris-HCl (pH 6.8), 25% (w/v) glycerol, 2% (w/v) SDS, and 0.01% Bromophenol Blue with 5% (v/v) 2-mercaptoethanol and then boiled for 5 min. Electrophoresis was performed in 25 mM Tris/192 mM glycine/0.1% SDS buffer at 20 mA for 1.5 h. The proteins were stained with Coomassie Brilliant Blue R-250.

Electron microscopy

The cell medium was examined for VLPs by negative-stain electron microscopy (EM). The cell medium samples were diluted in distilled water (1:10) and applied to a collodion-coated 400-mesh electron microscopy grid and stained with 4% uranyl acetate (pH 4).

Nucleotide sequences accession numbers

The nucleotide sequence data determined in this study have been deposited in GenBank under accession numbers: AF504649–AF504652, AF504655–AF504657, AF504660–AF504665, AF504667–AF504686, AY237424, AY237429, AY237431–AY237471, AY237473–AY237483, AY242861, AY579403–AY579409, and AY581294.

Results

NoV and SaV epidemiology

Four hundred and forty-eight rotavirus-negative stool specimens from children one month to 15 years of age presenting with acute sporadic gastroenteritis were selected for NoV and SaV analysis [6]. NoVs were detected in 72 of 448 specimens, with an overall annual detection rate of 5.4% (72 of 1,339 children). SaV was detected in only one of 448 rotavirus-negative specimens. No mixed infections of NoV and SaV were found. The age distribution was grouped by 0–5, 6–11, 12–23, 24–35, and >35 months of age. The NoV infection rates between the age groups or the male and females were not significantly different (data not shown).

NoV seasonal distribution

The NoV detection rate was analyzed between December 1999 and November 2000 (Fig. 1). NoV was detected throughout the 12-month period. The NoV detection rates were high in September 2000 (6.3%), October 2000 (6.7%), November 2000 (12.6%), and January 1999 (7.9%). From February to August, the detection rates remained relatively low, between 1.9 and 5.7 percent. The climate in Ho Chi Minh City is distinctively seasonal. The dry season typically lasts from November to April, and the hottest months are from February to May. The rainy season, characterized by sudden heavy rains, begins in May and ends in October. The wet months are from June to September. NoV was detected more frequently between the months of September and January (8%, 49/610) than between February and August (3.2%, 23/729), and this difference was statistically significantly

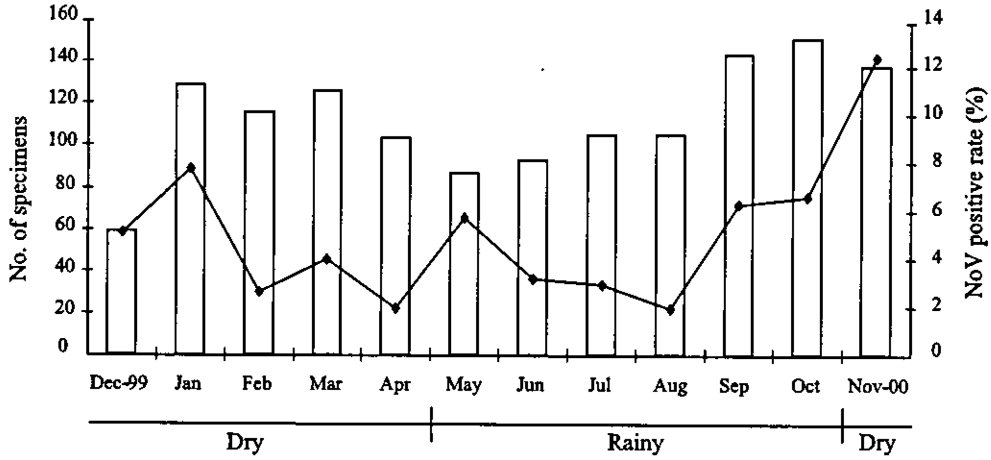


Fig. 1. Monthly distribution of NoV-positive specimens from children with acute sporadic gastroenteritis in Ho Chi Minh City, Vietnam. The two different climatic seasons (dry and rainy) in Ho Chi Minh City are shown. No. of specimens, □; positive rate (%), -◆-

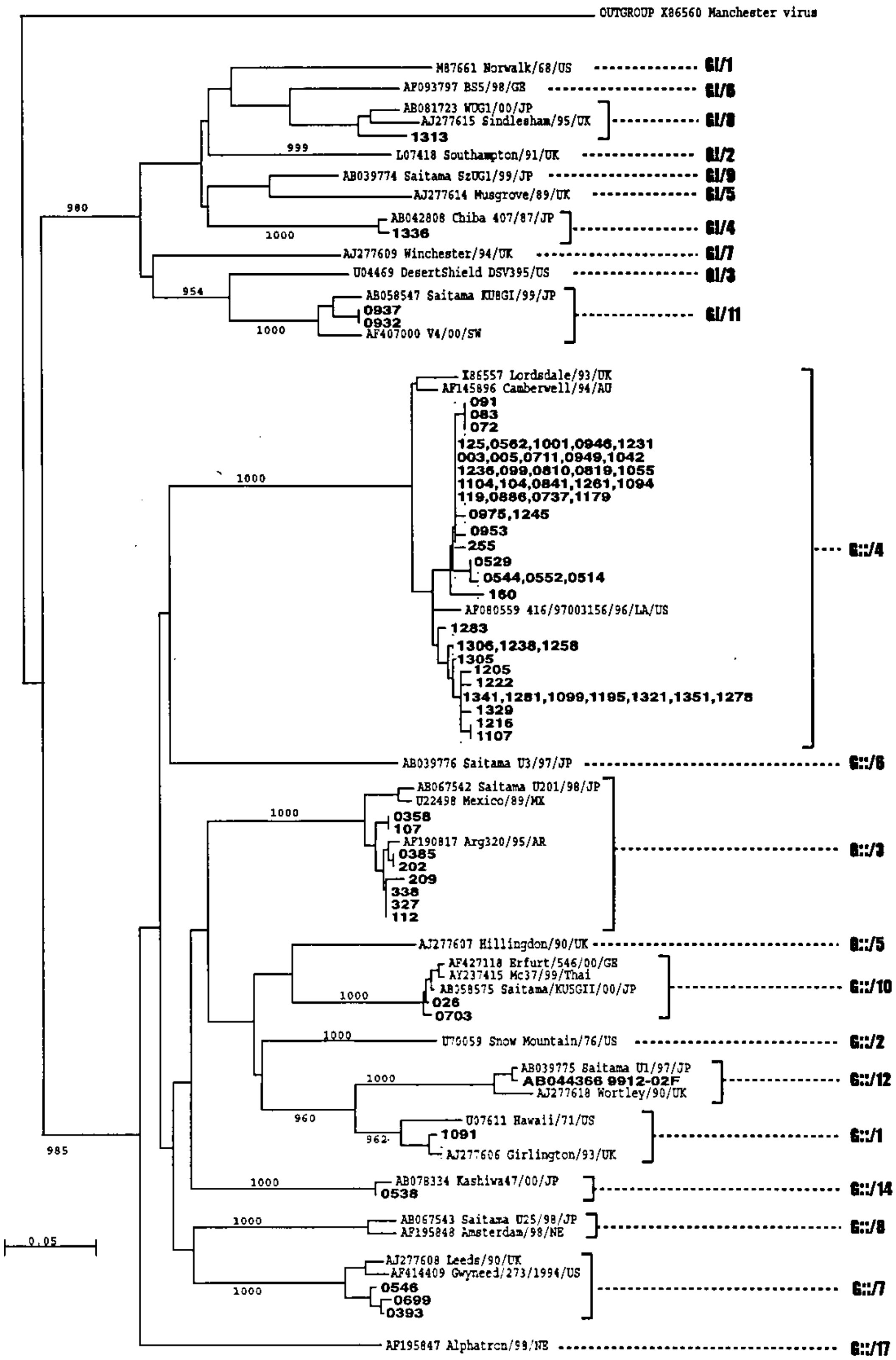
($\chi^2 = 15.5$, $P < 0.005$). This trend suggested that NoV prevailed at the end of the rainy season and the first half of the dry season.

Sequence analysis

The nucleotide sequence of the 5′ ends of the NoV capsid gene was determined by direct sequencing with the amplified fragments. This region has been shown to be suitable for genotyping [24]. The numbering of genotypes were based on the recently published list [21]. In total, 72 NoV strains including four GI and 68 GII strains were phylogenetically analyzed and classified. As shown in Fig. 2, two of the four GI strains clustered into two GI genotypes, GI/8 (WUG1 cluster) and GI/4 (Chiba 407 cluster), respectively. The remaining two GI strains, sharing over 98% nucleotide identity, clustered into a recently published genotype (GI/11 genotype). The majority of GII strains (78%, 53/68) belonged to the GII/4 genotype (Lordsdale cluster). We found strains from this genotype in all months except March. Strains belonging to GII/3 (Saitama U201 cluster), GII/7 (Leeds cluster), and GII/1 (Hawaii cluster) were relatively uncommon, with eight, three, and one strain(s) found, respectively. The remaining three GII strains were grouped into two recently published genotypes (GII/10 and GII/14 genotypes).

To verify the sequence identities of the GII strains, we did additional amplification of the RdRp region and sequenced it using 23 out of 68 strains (Fig. 3).

Fig. 2. Phylogenetic tree of NoVs based on the partial capsid region (255 bp) using Manchester virus as the outgroup. The strains detected in Ho Chi Minh City were indicated by strain number (bold letter). The numbers on the branches indicate the bootstrap values for the clusters. Bootstrap values of 950 or higher were considered statistically significant for the grouping [24]. The scale represents nucleotide substitutions per site


OUTGROUP X86560 Manchester virus
M87661 Norwalk/68/US GI/1
AF093797 BS5/98/GE GI/6
AB081723 WUG1/00/JP
AJ277615 Sindlesham/95/UK GI/8
1313
999
L07418 Southampton/91/UK GI/2
AB039774 Saitama SzUG1/99/JP GI/9
AJ277614 Musgrove/89/UK GI/5
980
AB042808 Chiba 407/87/JP
1336 GI/4
1000
AJ277609 Winchester/94/UK GI/7
U04469 DesertShield DSV395/US GI/3
954
AB058547 Saitama KU8GI/99/JP
0937
0932 GI/11
AF407000 V4/00/SW
1000
X86557 Lordsdale/93/UK
AF145896 Camberwell/94/AU
091
083
072
125,0562,1001,0946,1231
003,005,0711,0949,1042
1236,099,0810,0819,1055
1104,104,0841,1261,1094
119,0886,0737,1179
0975,1245
0953
255
0529
0544,0552,0514
160
AF080559 416/97003156/96/LA/US
1283
1306,1238,1258
1305
1205
1222
1341,1281,1099,1195,1321,1351,1278
1329
1216
1107
1000
GII/4
AB039776 Saitama U3/97/JP GII/6
AB067542 Saitama U201/98/JP
U22498 Mexico/89/MX
0358
107
AF190817 Arg320/95/AR
0385
202
209
338
327
112
1000
GII/3
AJ277607 Hillingdon/90/UK GII/5
AF427118 Erfurt/546/00/GE
AY237415 Mc37/99/Thai
AB058575 Saitama/KU5GII/00/JP
026
0703
1000
GII/10
U70059 Snow Mountain/76/US GII/2
1000
AB039775 Saitama U1/97/JP
AB044366 9912-02F
AJ277618 Wortley/90/UK
1000
GII/12
U07611 Hawaii/71/US
1091
AJ277606 Girlington/93/UK
960
962
GII/1
985
AB078334 Kashiwa47/00/JP
0538
1000
GII/14
0.05
AB067543 Saitama U25/98/JP
AF195848 Amsterdam/98/NE
1000
GII/8
AJ277608 Leeds/90/UK
AF414409 Gwynedd/273/1994/US
0546
0699
0393
1000
GII/7
AF195847 Alphatron/98/NE GII/17

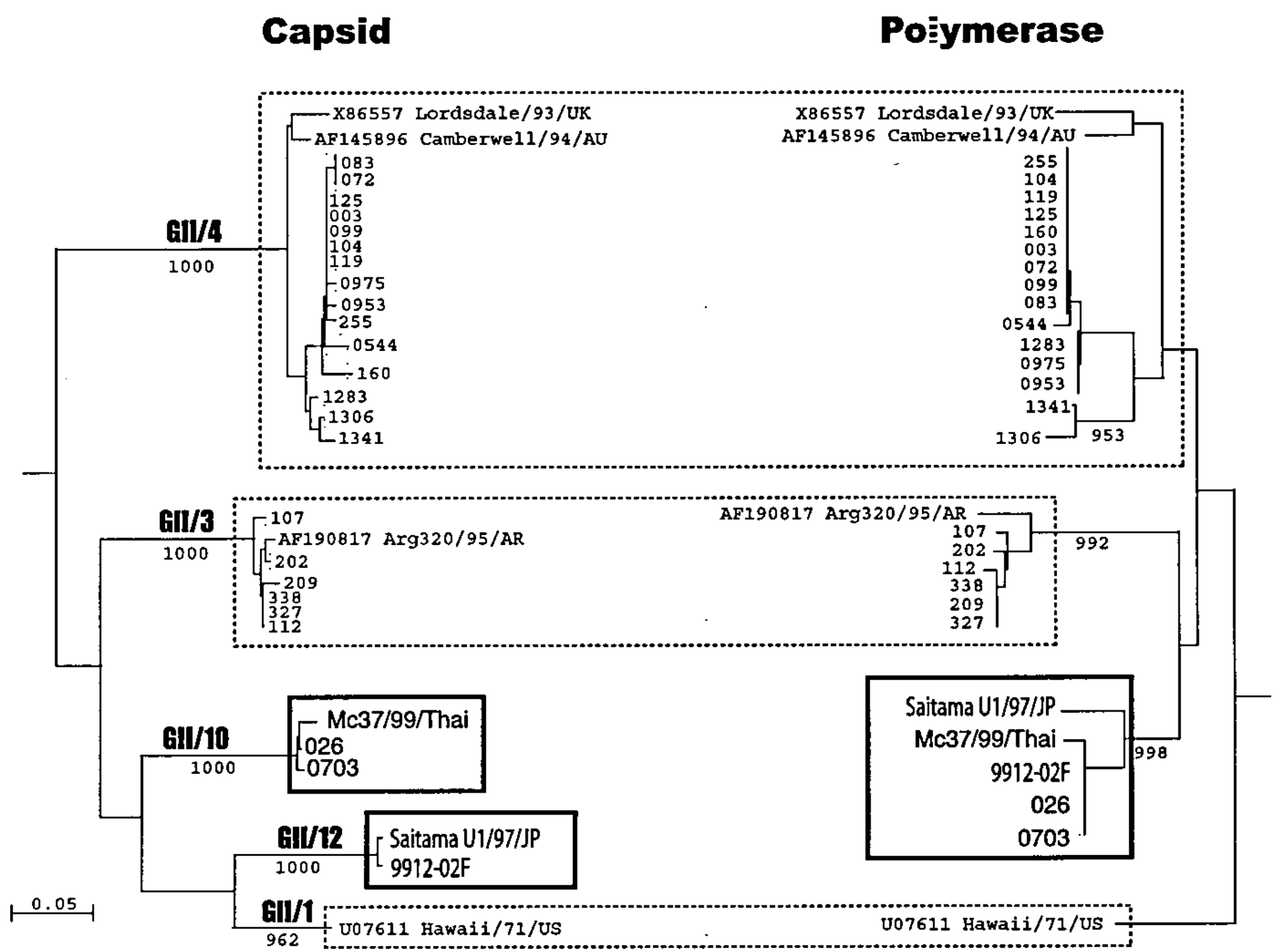


Fig. 3. Phylogenetic trees of 23 NoV GII strains. The left panel shows a tree based on the partially sequenced capsid (264 bp) regions, and the right panel shows that of RdRp (298 bp). The scale represents nucleotide substitutions per site. The strains boxed by broken lines are ones whose genotypes are maintained in both RdRp and capsid. The strains boxed by bold lines are ones whose genotypes are not maintained

Unfortunately, NoV strains from GII/1, GII/7, and GII/14 could not be amplified with our RdRp primers. Therefore the true identities of these strains have not yet been determined.

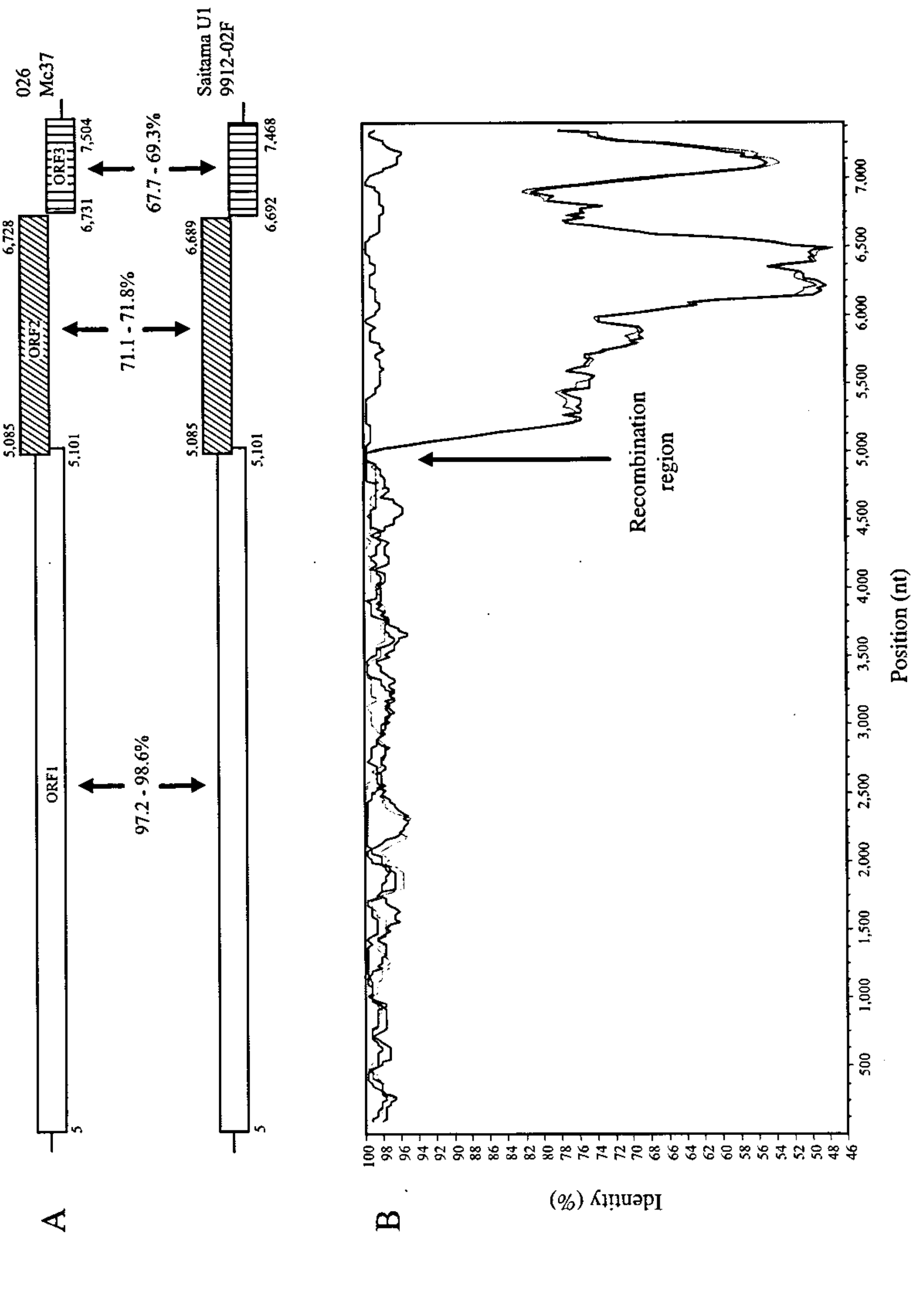
In 15 strains belonging to GII/4 genotype (Lordsdale cluster) and six strains belonging to the GII/3 genotype (the Saitama U201 cluster in which the Arg320 strain is included), the genotype was maintained in both RdRp and capsid regions (Fig. 3). Two other strains from GII/10, the 026 and 0703 strains, shared almost 100% nucleotide identity in both the RdRp and capsid regions, which indicates that they are the same strain. These two strains also shared almost 100% nucleotide identity with Mc37 strain, a recombinant NoV [11], in both the RdRp and capsid regions, demonstrating that 026 and 0703 strains were also recombinant viruses. As given in Fig. 3, these three strains shared over 95% and 98% nucleotide identities in the RdRp with the Saitama U1 and 9912-02F strains, respectively. Therefore, these five strains are included in the same cluster as far as their RdRp is considered. A previous phylogenetic analysis using 18 complete genome sequences demonstrated that the Saitama U1 strain was also a recombinant NoV [24].

In contrast to RdRp-based grouping, the 9912-02F and Saitama U1 strains were grouped into the GII/12 genotype, not into the GII/10 genotype, when capsid-based grouping was performed (Fig. 3). Therefore, these five strains are all recombinant viruses sharing the similar RdRp nucleotide sequence, but forming two distinct genotypes by a capsid-based grouping (see below).

In our study we identified only one SaV belonging to the Manchester cluster (SV GI). Although only one set of primers was used to detect SaV sequences, our primers were shown to be capable of amplifying SaV GI and GII genogroups [11], suggesting that SaV were relatively uncommon in this population.

Genetic and antigenic analyses of the recombinant strains

As mentioned above, two strains (026 and 0703) from GII/10 genotype were suspected to be recombinant NoV strains based on their partial RdRp and capsid sequences. To additionally analyze this finding, we determined the complete genome sequence of 026 and 9912-02F. The closest matching genome sequence to 026 was Mc37, sharing over 98.5% nucleotide identity. Therefore, 026 and Mc37 represented the same strain, though isolated from different countries and at different times. However, another closely matching partial RdRp sequence on the database was 9912-02F, which was isolated in Hiroshima, Japan, and had over 95% nucleotide identity with the 026 strain (Fig. 3). After complete genome sequencing of 9912-02F, we found that the ORF1 sequences of 026 and 9912-02F shared over 99% amino acid similarity, while the ORF2 and ORF3 sequences had only 78% and 67% amino acid similarity, respectively (Fig. 4A). Interestingly, ORF1, ORF2, and ORF3 sequences of 9912-02F shared 98.4%, 98.7%, and 94.4% amino acid similarity with Saitama U1, respectively. Therefore, 9912-02F and Saitama U1 likely represented the same strain, though they were obtained from different regions in Japan.


A
026
Mc37
Saitama U1
9912-02F
ORF1
ORF2
ORF3
5
5,085
5,101
6,728
6,731
7,504
6,689
6,692
7,468
97.2 - 98.6%
71.1 - 71.8%
67.7 - 69.3%
B
Identity (%)
Position (nt)
Recombination
region

vice versa. There is no direct evidence to support either scenario at the moment. Further extensive studies by sequence analysis of ORF1 and ORF2 using other strains is needed.

SaV infection causes gastroenteritis in all age groups, though it occurs predominantly in infants and young children [5]. Our study detected SaV in only one of the 448 children hospitalized with non-rotavirus gastroenteritis in Ho Chi Minh City. Several reports have noted that SaV detection was usually much lower than NoV detection [3, 25, 42, 47]. In one of these studies, Pang et al. found NoV in 10% of hospitalized children with gastroenteritis and 3% with SaV, while Kirkwood et al. found only 0.6% with SaV. In addition, several reports found SaV gastroenteritis is milder in symptoms as compared with NoV, therefore often not requiring hospitalization [25, 42, 44]. On the other hand, we performed a similar epidemiological study among hospitalized infants with gastroenteritis in Thailand and found SaV in 3.8% (4/105 of single infection) of the stool specimens [11]. Comparisons of the Thailand and Vietnam studies showed that this dissimilarity of the SaV detection rates was significant (Fisher Exact $P < 0.005$), whereas the dissimilarity of the NoV detection rates was not significant (8/105 were NoV positive in Thailand of single infection; Fisher Exact $P = 0.1$). The same primers and conditions were used in both studies, which suggested that SaV was an uncommon etiological agent of gastroenteritis in Ho Chi Minh City. Climatic and environmental conditions as well as cultural differences, including eating habits and hygiene practices, may be important factors that accounted for these differences in the SaV detection between these two countries [33]. Further epidemiological investigations of SaV in these two countries may help determine why SaV detection was significantly different and help ascertain the possible routes of SaV infections.

In many countries, NoV infection is prevalent in the winter months [12, 29, 36], though several studies showed no seasonal distribution [37, 40]. In our study, NoV infections prevailed at the end of the rainy season and the first half of the dry season, which was statistically significant. During this period the average temperature is cooler than the rainy season, which suggests a winter-like prevalence.

In conclusion, this study has shown that NoV was an important cause of sporadic gastroenteritis in Ho Chi Minh City. NoV strains belonging to the GII/4 genotype represented the dominant NoV strain, though several other NoV strains were also found to be co-circulating. SaV was detected in only one specimen, suggesting that SaV infection was an uncommon cause of gastroenteritis in Ho Chi Minh City.

Acknowledgments

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[7, 9, 46]. We found 17 (25%) out of 68 strains that closely matched the 95/96-US strain (Fig. 2). These results suggested that the 95/96-US-like strain was also an important cause of sporadic cases of gastroenteritis. Further epidemiological studies may help determine whether strains from GII/4 including 95/96-US-like strains continue to be dominant in this region.

We showed that six strains from GII/3 were in fact Arg320-like strains, based on the partial RdRp and capsid sequences (Figs. 2 and 3). These six Arg320-like strains were detected between January 2000 and March 2000 but not after this period. Interestingly, a similar study of sporadic gastroenteritis conducted in Japan between April 1996 and March 2000 reported that Arg320-like strains suddenly appeared and spread between October 1999 and February 2000 [14]. Likewise, we found that 95/96-US-like strains suddenly appeared in October and November 2000 in Ho Chi Minh City. This sudden appearance and disappearance of strains may indicate that the population developed immunity. However, several studies have also found that dominant strains can persist in one region over a number of years, which may suggest that some strains, such as the 95/96-US strain, could be more virulent [39, 46].

Other NoV strains belonging to GI/4, GI/8, GII/1, and GII/7 were co-circulating, but these were infrequent. We also identified three recently published genotypes (Fig. 2), one in the GI (GI/11 genotype) and two in the GII (GII/10 and GII/14 genotypes). Recently, NoV GI and GII strains were predicted to consist of at least 14 and 17 genetic genotypes, respectively [21], but this number is expected to increase with improved detection techniques and increased surveillance [20].

Two NoV GII strains (026 and 0703) were shown to be almost the same virus as Mc37 strain, a recombinant NoV (Figs. 2 and 3). In 1999, Jiang et al. [18] first reported a naturally occurring recombinant NoV, and later several other strains were described as recombinants [24, 30, 45]. They discovered a region of genetic recombination between the RdRp and capsid genes. Our sequence analysis was comparable to these recombinant NoV studies. Genomic sequence analysis of 026 with other recombinant NoVs showed the region of genetic recombination was between 5,033 and 5,100 nucleotides (with reference to the 026 sequence) (Fig. 4B). We further analyzed 026 by expressing the VP1 of 026 and 9912-02F in a baculovirus expression system. Hyperimmune sera against the VLPs indicated that GII/12 and GII/10 are distinct antigenic types, though a considerable level of cross-reactivity was found between them. A similar cross-reactivity was also reported when the antigenicity was examined by antibody ELISA [27].

Co-circulation of two potential parental strains may allow a recombination event when their nucleic acid sequences come within physical contact in infected cells, e.g., during copy choice recombination. If 026, 0703, and Mc37 represented NoV "strain A", and 9912-02F and Saitama U1 represented NoV "strain B", at least two possible scenarios of genetic recombination are suggested. Scenario one: both "strain A" and "strain B" are recombinant NoVs and the parent strain(s) have not yet being identified. Scenario two: "strain A" was a parent of "strain B", or

We next used four complete nucleotide sequences to further analyze the 026 sequence using the SimPlot software [31]. When we compared the nucleotide sequence of 026 with those of Mc37, Saitama U1, and 9912-02F, we found an apparent region of genetic recombination between 5,033 and 5,100 nt (Fig. 4B). We found 100% sequence homology over this 68-nt region. After this region the homology was notably different, and the SimPlot analysis showed a sudden drop in nucleotide identity for 9912-02F and Saitama U1 but not for Mc37. These results demonstrated that the ORF1 sequence among these four strains was almost identical, but the ORF2 and ORF3 sequences of Saitama U1 and 9912-02F strains were distinctly different from those of 026 and Mc37 strains.

In addition to the genetic analysis described above, we performed antigenic analysis by using virus-like particles (VLPs) and immune sera to them. VP1 of 026 and 9912-02F were expressed in insect cells as described in Materials and methods, and hyperimmune sera against these VLPs were used to determine their cross-reactivity. An ELISA OD value of 0.15 was set for the cutoff point, and the reactivity was measured at 492 nm. The titers of 026 serum against 026 and 9912-02F VLPs were 2,058,000 and 512,000, respectively, a fourfold difference. The titers of 9912-02F serum against 9912-02F and 026 VLPs were 1,024,000 and 128,000, respectively, an eightfold difference. These results demonstrated that 026 and 9912-02F likely represented distinct antigenic types, which correlates with the genetic analysis described earlier [27].

Discussion

In this study, we reported the NoV and SaV detection in stool specimens from hospitalized children with acute sporadic gastroenteritis in Ho Chi Minh City, Vietnam, between December 1999 and November 2000. The results have shown an overall annual NoV and SaV detection rate of 5.5% (73 of 1,339 children). A similar study conducted in Australia detected NoV and SaV in 9% (32/353) of stool specimens from children with acute gastroenteritis [25], while another study conducted in Ireland detected NoV in 8% (29/360) of stool specimens from children with sporadic gastroenteritis [8]. In our study, the majority of NoV strains (over 73%) belonged to GII/4, and these were isolated in all months except March. A similar finding was previously reported by a phylogenetic analysis using RdRp [14]. Also belonging to GII/4 is the 95/96-US strain, which caused the majority of outbreaks of gastroenteritis in the United States (60/152) during the 1995–1996 season [39], and which has been found in more than seven different countries

Fig. 4. **A** The genomic organization of 026, Mc37, Saitama U1, and 9912-02F viruses and **B** the SimPlot analysis of 026, Mc37, Saitama U1, and 9912-02F genomes. The reference 026 genome sequence was compared to Mc37 (blue), Saitama U1 (green), and 9912-02F (red) sequences. A window size of 200 bp with an increment of 20 bp was used. All gaps were removed. The recombination region is suspected to be located between ORF1 and ORF2, as shown by the arrow

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