specimens from children with acute gastroenteritis in Karachi, Pakistan; to determine the age-related distribution, geographical distribution, and seasonal patterns of AstV, NV (GI, GII), and SV infections; and to characterize the detected viruses according to type.

MATERIALS AND METHODS

Fecal Specimens

Fecal specimens were collected from 517 infants and young children admitted to the Civil Karachi Hospital, Dow Medical College, Karachi city, Pakistan with acute gastroenteritis from 1990 to 1994. All fecal specimens were determined previously to be negative for rotavirus and adenovirus [Nishio et al., 2000]. These specimens were diluted with Eagle's minimum essential medium to 10% suspensions, and clarified by centrifugation at 5,000*g* for 20 min. Supernatants were stored at −30°C until use.

Extraction of Viral RNA

Viral RNA was extracted from 140 μl of a 10% fecal suspension by the use of a spin column technique according to the instructions in the QIAamp® Viral RNA Mini Kit Handbook.

Viral Detection and RT-PCR

AstV, NV (GI, GII), and SV were detected by RT-PCR analysis of extracted viral RNA. Four pairs of specific primers published were mixed [Yan et al., 2003] for the multiplex PCR.

Nucleotide Sequencing and Phylogenetic Analysis

All PCR products (DNA) positive for NV and SV were purified and then sequenced. Sequence analysis was performed using E-CLUSTAL W and the neighbor-joining method was used for the construction of the phylogenetic tree [Lui et al., 1995; Katayama et al., 2002]. The nucleotide sequence data for the capsid region from strains Karachi/1001/1990 and Karachi/730/1992 has been submitted to the DDBJ DNA database and has been assigned accession numbers AB126940 and AB126249, respectively. Reference strains and accession numbers used in this study are as follows: Birmingham (AJ277612), Southampton (L07418), Musgrove (AJ277614), Chiba (AB022679), NV68 (M87661), WUG1 (AB081723), SzUG1 (AB039775), Stav (AF145709), Hawaii (U07611), Girllington (AJ277606), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Alphatron (AF195847), Toronto (U02030), Seacroft (AJ277620), Leeds (AJ277608), Lordsdale (X86557), Manchester (X86560), London/92 (U95645), Stockholm (AF194182), Sapporo/82/JP (U65427), Plymouth (X86559), Houston/86 (U95643), Potsdam (AF294739), Lyon/30388/98/F (AJ251991), Lyon/598/97/F (AJ271056), Chiba/00067T/ 1999 (AJ412805), Chiba/010604F/2001 (AJ412826).

Human AstV Serotyping

Human AstV serotyping was carried out using RT-PCR analysis of extracted viral RNA with serotype specific primers (1–8) described by Sakamoto et al. [2000].

RESULTS

Epidemiology of Viral Infections

Of 517 fecal specimens negative for rotavirus and adenovirus, 168 were collected in 1990; 86 in 1991; 76 in 1992; 99 in 1993; and 88 in 1994. In each of the 5 years, the number of male was higher than that of female.

Among all children with acute gastroenteritis, 95.5% were aged less than 35 months. AstV, NV (GI, GII), and SV were detected mostly in the under 35 month age range. The viral infectious rate was highest in the 6–11 months old group (29.3%) and lowest in children over 35 months of age (13.0%). Surprisingly, infants under 6 months of age had a rather high rate of viral infections (24.1%) (Table I).

Viral enteropathogens were detected in 122 (23.6%) children with acute gastroenteritis, of these, 69 (56.6%) were boys. AstV was identified in 58 (47.5%) fecal specimens. NV was detected in 51 specimens (41.8%) of which NV GII was responsible for 39 cases (76.5%) and NV GI for 12 cases (23.5%). SV was found in 17 specimens (13.9%).

The percentages of monoinfections were 44.3% (54 specimens), 40.2% (49), and 12.3% (15) for AstV, NV, and SV, respectively. Two (1.6%) of all the viral positives were mixed infections between AstV and NV GII, and two more (1.6%) were mixed infections between AstV and SV.

In this study, the majority of AstV (81.1%) was detected in September and October 1990 (Fig. 1). This type of seasonality is indicative of an outbreak of AstV. All the Pakistani children in this presumed outbreak were under 3 years old. The highest incidence group was the 6–11 month old group (40%), and the second highest group was the 12–23 month old group (30%). We detected only one AstV in 2-year-old children (3.3%), and no case was found in children over 2 years old. Infants aged less than 6 months had a rather high rate of AstV infection (26.7%). Unlike the yearly distribution of AstV, there was no large difference between NV and SV infection rates from 1990 to 1994. The NV incidence was higher than those of AstV and SV in each of the 5 years except for 1990 (Table II).

TABLE I. Age-Related Gastroenteritis and Viral Infections Among Children in Karachi City, Pakistan

Age (month)	Number of tested specimens (%)	Number of positive specimens (%)
<6	87 (16.8)	21 (24.1)
6–11	164 (31.7)	48 (29.3)
12–23	179 (34.6)	38 (21.2)
24–35	64 (12.4)	12 (18.8)
>35	23 (4.5)	3 (13.0)
Total	517	122

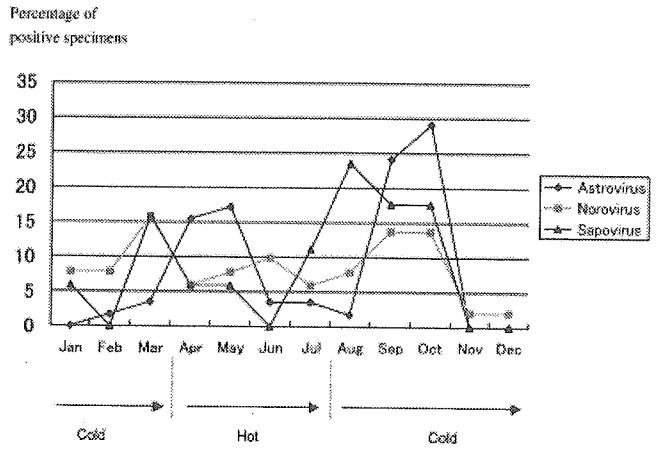

Fig. 1. Monthly distributions of astrovirus (AstV), norovirus (NV) (GI, GII), and sapovirus (SV) infections identified in children during the 5-year period, showing the cold season and hot season in Karachi, Pakistan.

Human AstV Serotypes

In our study, all 58 AstV (100%) detected were serotypes 1. Interestingly, we identified one possible infection with mixed serotypes 1 and 3. More remarkably, this same sample, Karachi/831/1991, contained AstV and NV GII. Apparently, this sample represented a triple mixed infection. Other AstV serotypes could not be found.

NV Nucleotide Sequencing and Phylogenetic Analysis

The total of 51 NV nucleotide sequences including 12 NV GI and 39 NV GII were analyzed by phylogenetics and grouped using the recent NV capsid region classification scheme of Katayama et al. [2002]. The NV GI sequences clustered into three distinct GI genotypes. Most of the NV sequences (66.7%) belonged to NV GI genotype 7 (NV GI/7) (typified by the Chiba virus cluster). Three NV sequences (25%) clustered into NV GI/1 (known as the NOR89JB virus cluster). Only one NV sequence (8.3%) was classified into NV GI/6 (known as the Musgrove virus cluster) (Fig. 2).

All 39 NV GII sequences were classified into five distinct GII genotypes. Sixteen (41.0%) of these NV sequences were similar to the genotype 1 (known as the Lordsdale virus). Twelve (30.8%) NV sequences belonged to the Hawaii virus cluster (the genotype 7), six (15.4%) to the genotype 2 (typified by the Toronto), two to the genotype 4 (known as the Melksham), and two more to the genotype 8 (known as the Seacroft). In addition to the five distinct GII genotypes, we also identified one sequence, isolate Karachi/1001/1990, representing one new NV GII genotype (Fig. 3).

SV Nucleotide Sequencing and Phylogenetic Analysis

The phylogenetic analysis indicated that SV group I (typified by the Manchester) was a more common genogroup (70.6%) than SV group II (23.5%) (London virus). Interestingly, one strain named Karachi/730/1992 was found and one strain, Karachi/730/1992, was shown to be different from the other SV strains; this was classified into the group Chiba/000671T/1999 and tentatively called genogroup IV by Okada et al. [2002] (Fig. 4). The nucleotide identity between Karachi/730/1992 and Chiba/000671T/1999 was 95%, and the amino acid identity was 96%.

DISCUSSION

AstV is a leading cause of infantile viral gastroenteritis worldwide [Koci et al., 2003]. Outbreaks of gastroenteritis resulting from AstV infection in Brazil [Silva et al., 2001], and in France [Belliot et al., 1997] have been reported, but this is the first similar report from Karachi, Pakistan. In this outbreak, the highest incidence of cases fell into the 6–11 month old age group, and the rate of incidence decreased with increasing age over 12 months. Quite possibly, children aged from 6 to 11 months might lack antibody protection to AstV, whereas by the time children have reached 11 months they have begun to acquire viral immunity.

Our findings showed that in infants less than 6 months old the prevalence of viral infections was high. Perhaps because of limited breastfeeding or for other reasons, these infants might not have enough maternally acquired passive antibody against viral enteropathogens. Studies conducted in Pakistan show a decline in breastfeeding since 1966, especially in urban areas [Lambert, 1988]. Many women perceive childbirth as being a major physical stress, and consider early initiation of breastfeeding as being just an added stress. In several urban squatter settlements, human breastmilk was regarded as a potential menace [Mull, 1992].

TABLE II. Incidence of Norovirus (NV) (GI, GII) Compared to That of Astrovirus (AstV), and Sapovirus (SV) in Children With Gastroenteritis in Karachi, Pakistan

Year	Number of specimens tested	Number (%) of AstV positive	Number (%) of NV positive	Number (%) of SV positive
1990	168	37 (22.0)	18 (10.7)	4 (2.4)
1991	86	7 (8.1)	8 (9.3)	5 (5.8)
1992	76	4 (5.3)	8 (10.5)	4 (5.3)
1993	99	6 (6.1)	7 (7.1)	1 (1.0)
1994	88	4 (4.5)	10 (11.4)	3 (3.4)

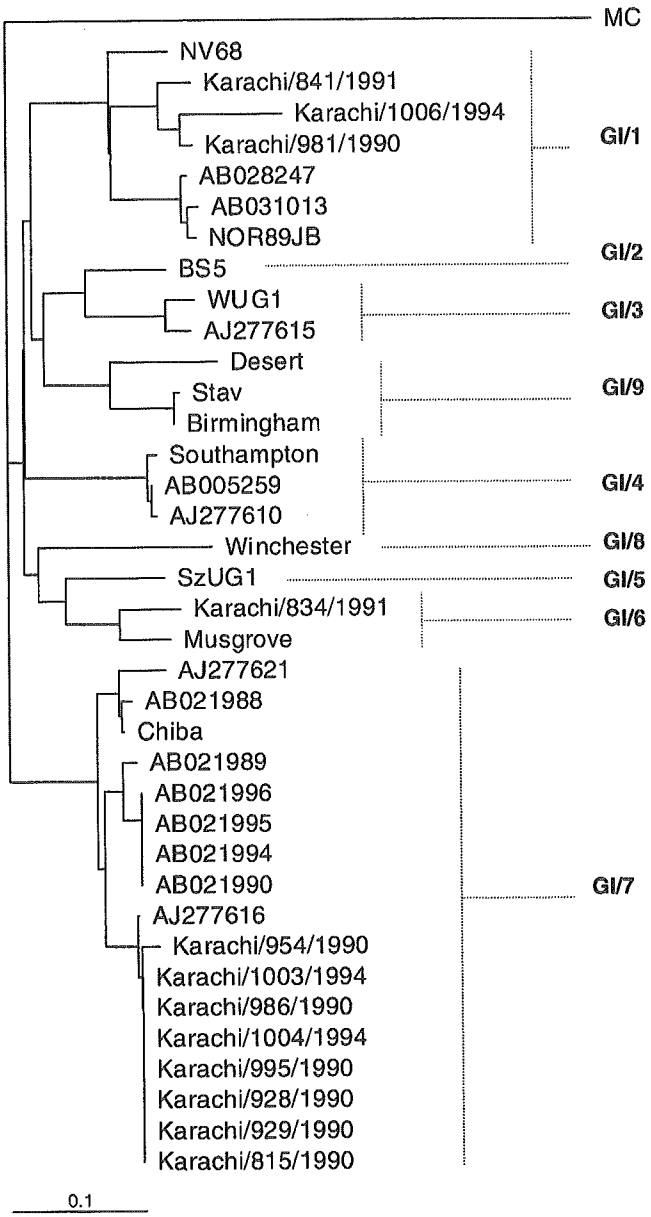

Fig. 2. Phylogenetic tree of NV GI (NV GI). The tree was reconstructed for partial sequences of the capsid region of NV GI found in Karachi city, Pakistan from 1990 to 1994. Reference strains of NV GI were registered in DDBJ/GenBank under the accession number indicated in the text. In the phylogenetic tree, NV GI was classified into nine distinct serotypes from 1 to 9. Manchester (MC) strain was used as an out-group for phylogenetic analysis.

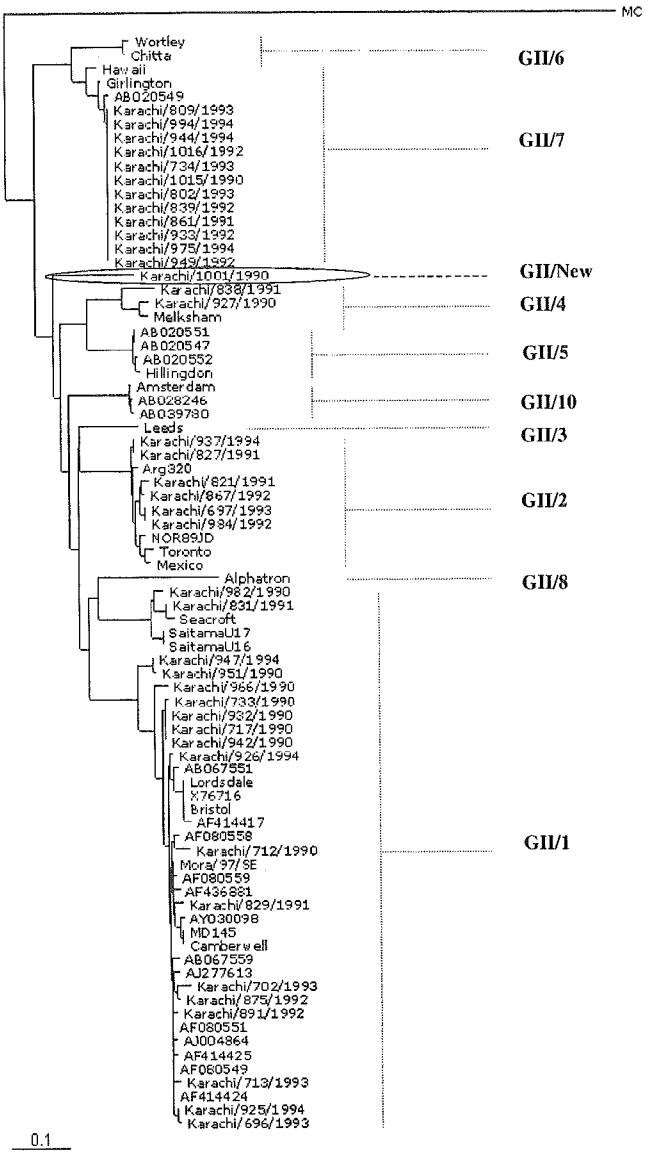

Fig. 3. Phylogenetic tree of NV GII (NV GII). The tree was reconstructed for partial sequences of the capsid region of NV GII identified in Karachi city, Pakistan during the 5-year period (1990–1994). Reference strains of NV GII were registered in DDBJ/GenBank under the accession number indicated in the text. In the phylogenetic tree, NV GII was classified into ten distinct serotypes from 1 to 10. The new serotype was signified in the oval circle. Manchester virus (MC) was used as an out-group strain for phylogenetic analysis.

Some hospitals in Pakistan separated newborns from their mothers, so that infants would not have a chance to initiate early breastfeeding. In practice, health practitioners did not inform the mothers of the importance of breastfeeding, and did not instruct them on how to establish and sustain breastfeeding. Further, many mothers preferred to give their babies formula instead of breastfeeding because of fear of insufficient milk supply due to their workload [Lambert, 1988]. Infants commonly drink unsafe water during the first 6 months after birth in Pakistan, and this may increase the risk of gastroenteritis [Shah et al., 2003]. Moreover, as substitutes for breastfeeding, the majority of infants were given honey as part of a quasi-religious ritual along with other foods intended for "cleansing of the stomach" [Badruddin et al., 1997].

The climate in Karachi city, Pakistan is distinctively seasonal. The summer lasts from April to July, and the hot temperature may reach over 37°C. The cold season characterized by less rain begins in August and ends in March. The coldest month is January when the temperature may dip as low as 5°C. In this study, AstV

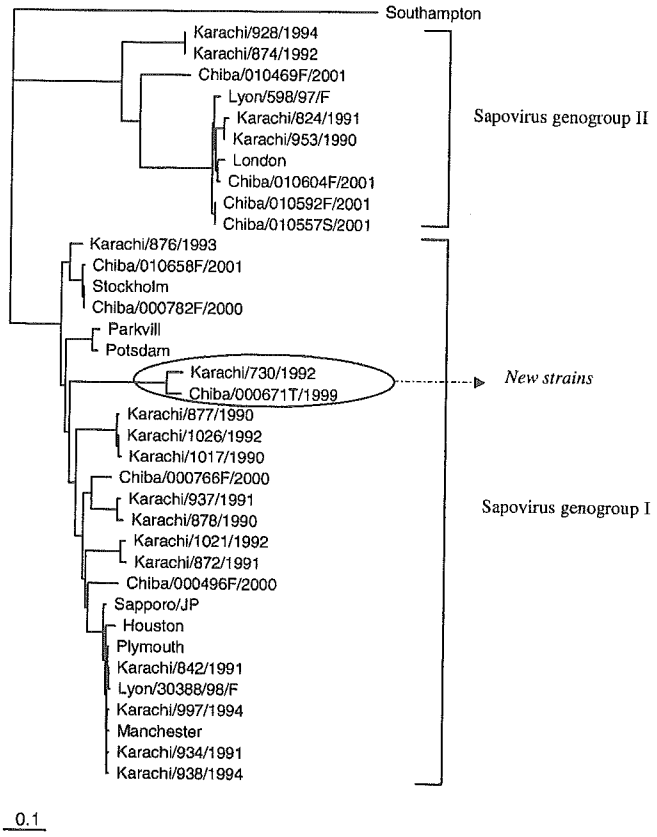

Fig. 4. Phylogenetic tree of SV. The tree was reconstructed for partial sequences of the capsid region of SV detected in Karachi city, Pakistan from 1990 to 1994. Reference strains of SV were registered in DDBJ/GenBank under the accession number indicated in the text. In the phylogenetic tree, SV was classified into genogroup I and II. The new strains were highlighted in the oval circle. Southampton strain was used as an out-group for phylogenetic analysis.

was observed to prevail in the hot summer months except for the outbreak in September and October. In many countries, AstV is prevalent in the rainy and cold season, and several studies did not find a seasonal correlation.

The NV detection rate was highest in March and secondly high in September as well as October. Moreover, almost all SV were found in August, September and October. These results suggest that these two major groups of human caliciviruses are prevailing during the cold season.

AstV was divided into eight serotypes in which serotype 1 was the most frequently detected. The mixed infections among these serotypes are uncommon [Palombo and Bishop, 1996; Sakamoto et al., 2000; Silva et al., 2001]. An outbreak of gastroenteritis associated with AstV serotype 1 occurred during September and October 1990 in Karachi, Pakistan. The outbreak of gastroenteritis attributable to AstV serotype 1 was also reported in Brazil [Silva et al., 2001]. Interestingly, we found one sample with a triple mixed infection between AstV (serotypes 1 and 3) and NV GII. Another less likely possibility, however, was that this AstV had undergone genetic recombination between serotypes 1 and 3. Although interesting, this mixed infection caused by different serotypes, in different AstV, and the extent of infection with different serotypes of the same virus were not addressed in this study.

NV belonging to the Lordsdale cluster represent the highest detection rate in sporadic gastroenteritis among children in Karachi, Pakistan. Other strains including one new NV GII genotype were also identified as being co-circulating.

The results indicated that SV group I (typified by the Manchester virus, MC) was a dominant genogroup. Interestingly, one strain named Karachi/730/1992 was similar to the group of Chiba/000671T/1999 tentatively called as genogroup IV by Okada et al. [2002]. These two viruses had a high identity on the nucleotide as well as the amino acid. However, the phylogenetic tree of SV showed that Karachi/730/1992 was a part of a genogroup I.

This is the first report from Karachi city, Pakistan of acute gastroenteritis associated with human AstV, NV (GI, GII), and SV, and the first indication of an outbreak attributable to AstV serotype 1. Our findings confirm the presence of these viruses in acute gastroenteritis among infants and young children in Karachi city, Pakistan.

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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 × 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 CAT GAA GAT GGC GTC GAA TGA CGC CGC TCC A-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 CAT GAA GAT GGC GTC GAG TGA CGC CGC TCC-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

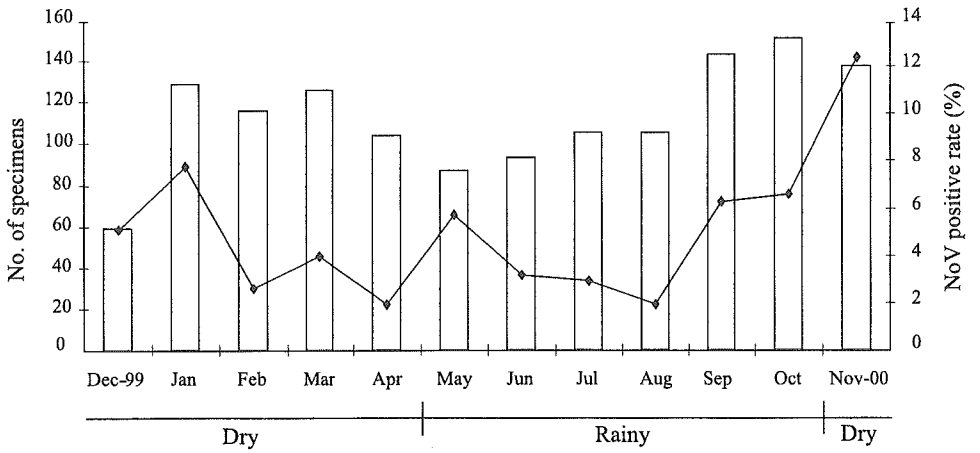


Fig. 1. Monthly distribution of NoV-positive specimens from children with acute sporadic gastroenteritis in Ho Chi Minh City, Vietnam. The two different climatic seasons (dry and rainy) in Ho Chi Minh City are shown. No. of specimens, □; positive rate (%), -◆-

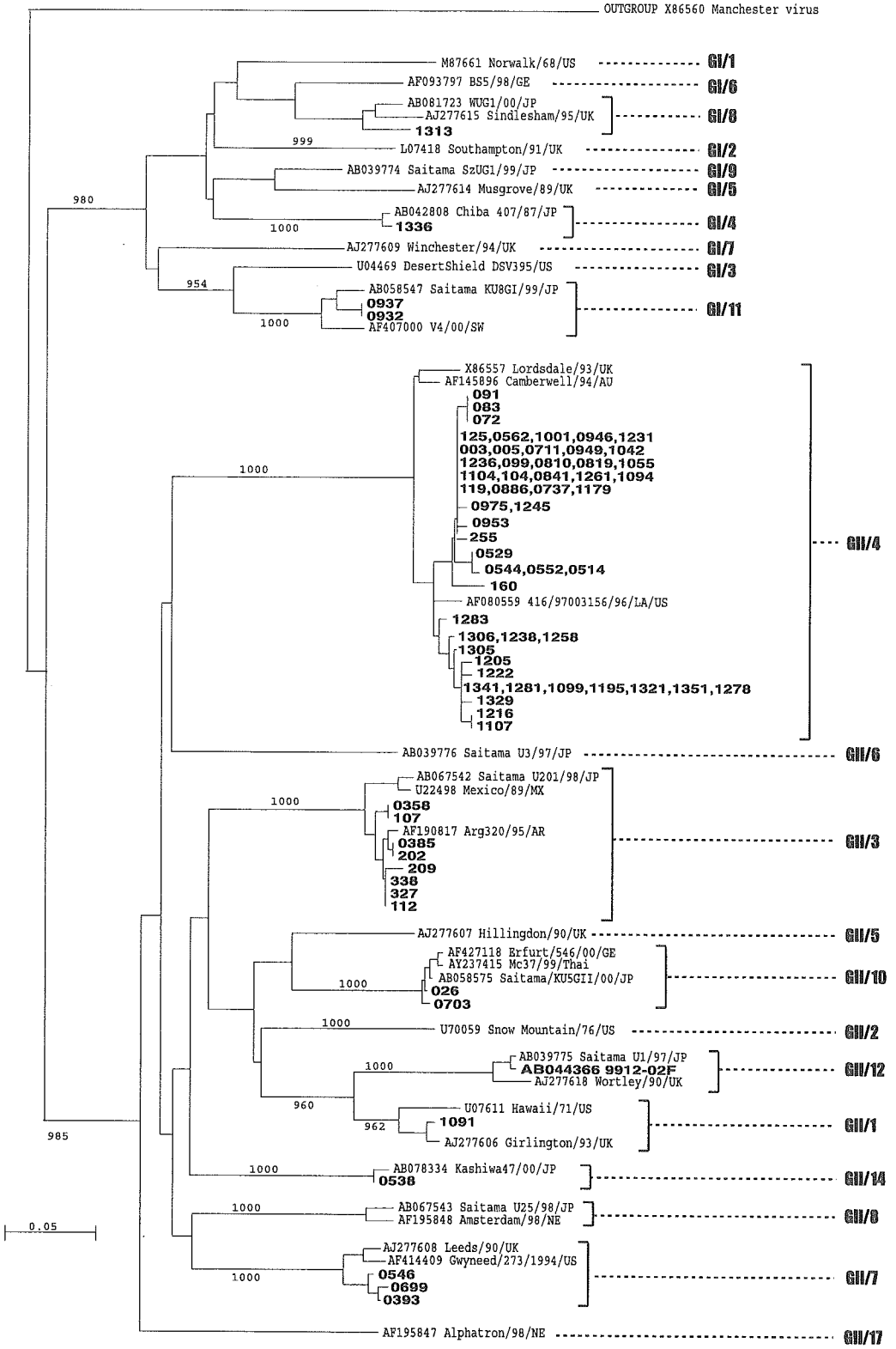
($\chi^2 = 15.5$, $P < 0.005$). This trend suggested that NoV prevailed at the end of the rainy season and the first half of the dry season.

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
1313
GI/8
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
AF407000 V4/00/SW
GI/11
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
1000
0358
107
AF190817 Arg320/95/AR
0385
202
209
338
327
112
GII/3
AJ277607 Hillingdon/90/UK
GII/5
AF427118 Erfurt/546/00/GE
AY237415 Mc37/99/Thai
AB058575 Saitama/KU5GII/00/JP
1000
026
0703
GII/10
1000
U70059 Snow Mountain/76/US
GII/2
AB039775 Saitama U1/97/JP
1000
AB044366 9912-02F
AJ277618 Wortley/90/UK
GII/12
960
U07611 Hawaii/71/US
962
1091
AJ277606 Girlington/93/UK
GII/1
985
1000
AB078334 Kashiwa47/00/JP
0538
GII/14
1000
AB067543 Saitama U25/98/JP
AF195848 Amsterdam/98/NE
GII/8
0.05
AJ277608 Leeds/90/UK
AF414409 Gwyneed/273/1994/US
1000
0546
0699
0393
GII/7
AF195847 Alphatron/98/NE
GII/17

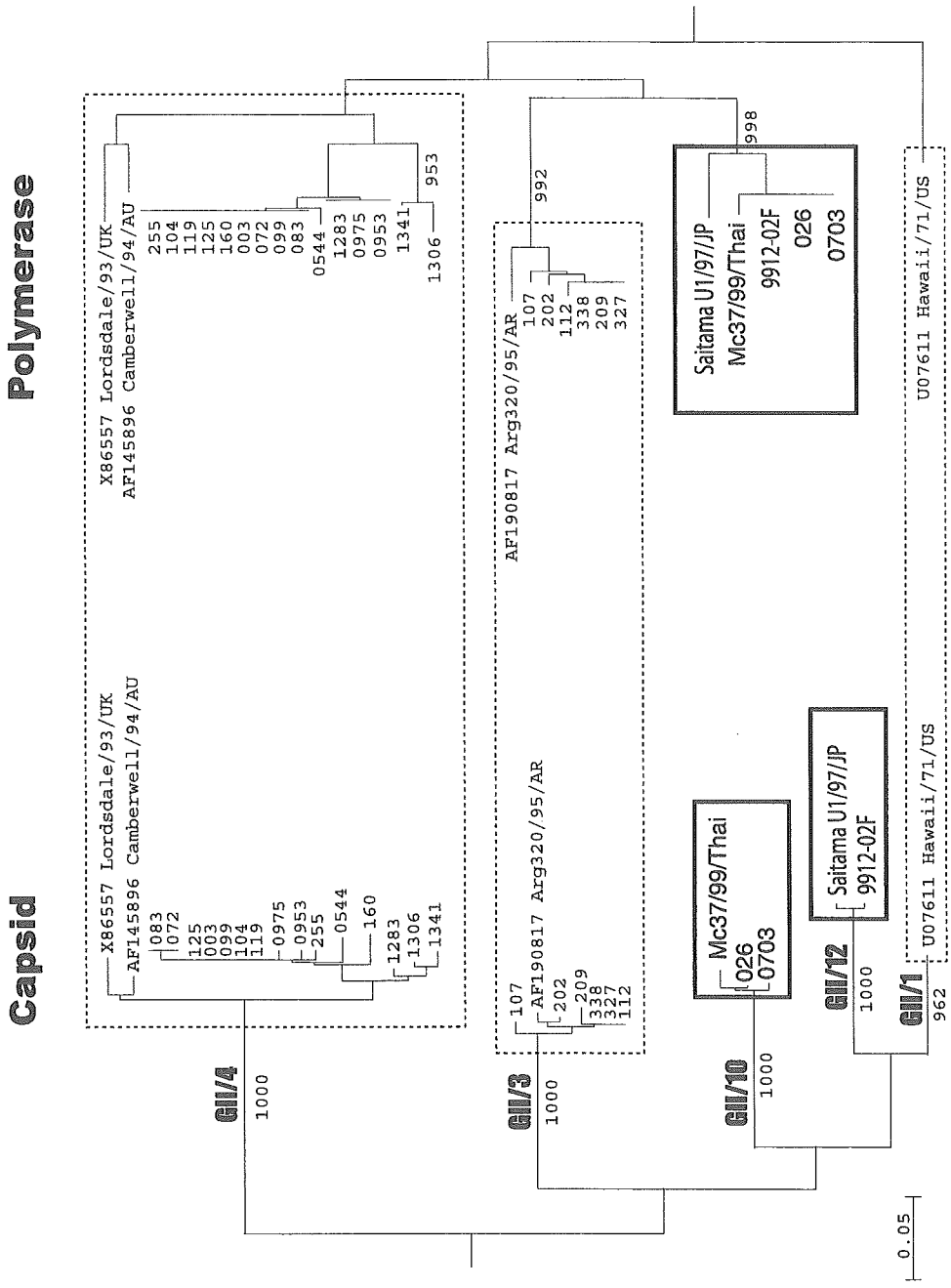


Fig. 3. Phylogenetic trees of 23 NoV GII strains. The left panel shows a tree based on the partially sequenced capsid (264 bp) regions, and the right panel shows that of RdRp (298 bp). The scale represents nucleotide substitutions per site. The strains boxed by broken lines are ones whose genotypes are 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.

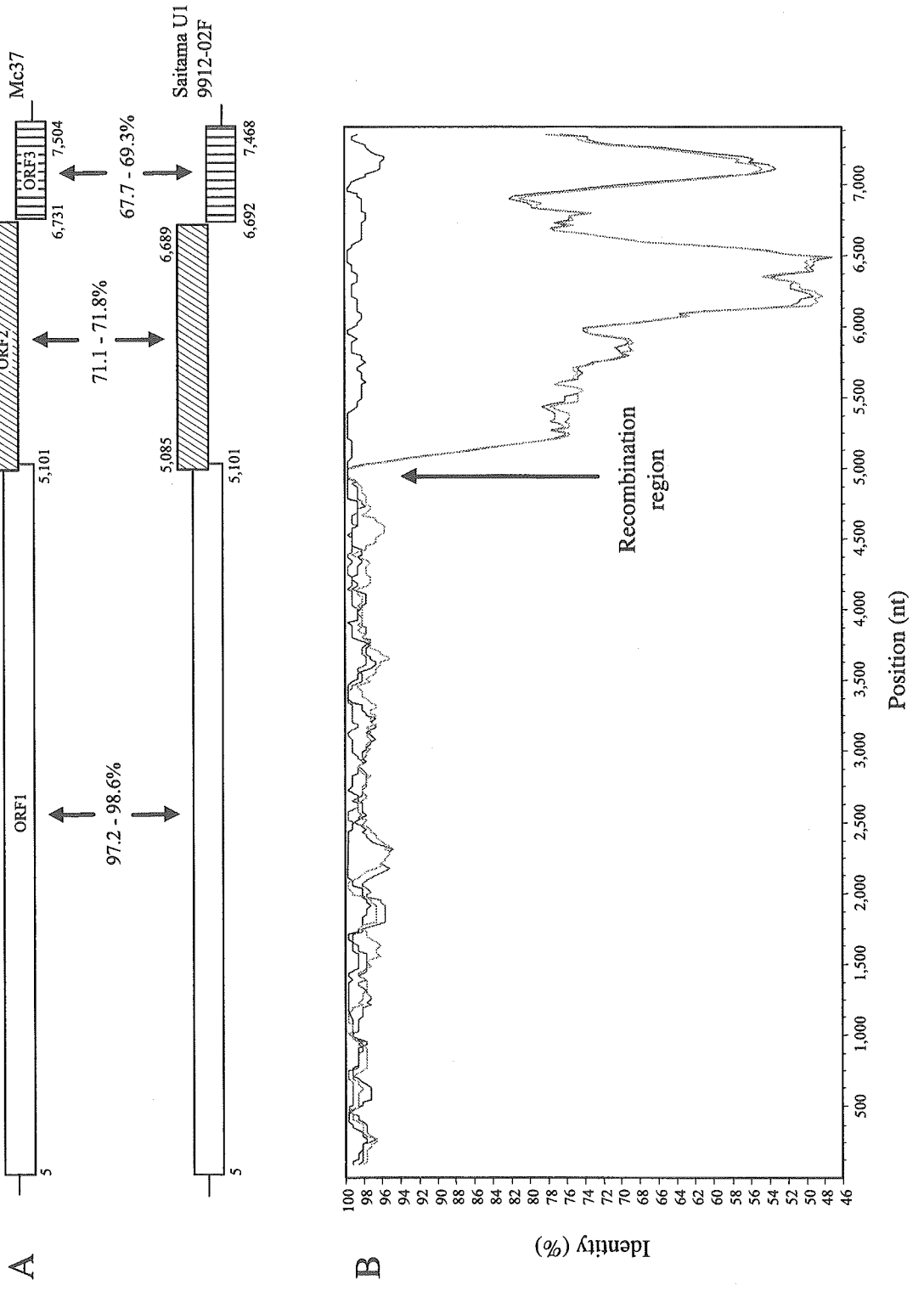
In 15 strains belonging to GII/4 genotype (Lordsdale cluster) and six strains belonging to the GII/3 genotype (the Saitama U201 cluster in which the Arg320 strain is included), the genotype was maintained in both RdRp and capsid regions (Fig. 3). Two other strains from GII/10, the 026 and 0703 strains, 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

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

[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

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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