Table 1 Specific primers used in multiplex PCR

Viruses and primers	Target region	Polarity [a]	Sequence (5' to 3')[b]	Position
Adenovirus				
Ad1	Hexon	+	TTCCCCATGGCICAYAACAC	1834–1853
Ad2	Hexon	−	CCCTGGTAKCCRATRTTGTA	2315–2296
Group A rotavirus				
Beg9	VP7	+	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1–28
VP7-1'	VP7	−	ACTGATCCTGTTGGCCATCCTTT	373–395
Group C rotavirus				
G8NS1	VP7	+	ATTATGCTCAGACTATCGCCAC	353–374
G8NA2	VP7	−	GTTTCTGTACTAGCTGGTGAAC	683–704

[a] : +, Forward primer ; −, reverse primer.

[b] : Within nucleotide sequence of primers, I = inosine ; K = G or T ; R = A or G ; Y = C or T.

method. Group B rotavirus (RVB, CAL-1), norovirus genogroup I (NV/GI, Osaka/3626), norovirus genogroup II (NV/GII, Osaka/3581), sapovirus (SV, Osaka/3668) and cultured human astrovirus serotype 1 (AstV-1, H1 (Oxf), accession no. L23513) were used as negative controls. Among these, group B rotavirus detected by polyacrylamide gel electrophoresis (PAGE) was kindly provided by Dr. Kobayashi (Sapporo Medical University, Sapporo, Japan), and NV/GI Osaka/3626, NV/GII Osaka/3581 and SV Osaka/3668 were sequenced in both directions using specific primers made previously (data not shown).

Nucleic acid extraction and reverse transcription (RT)

Viral nucleic acid was extracted from stool supernatants with a QIAamp Viral RNA kit (QIAGEN, Germany) according to the manufacturer's protocol. After heating the nucleic acid extract, RT was carried out as previously described[27]. Briefly, for each sample, 7.5μl of the nucleic acid extract were heated at 94℃ for 2 min followed by mixing with a 7.5μl RT reaction mixture consisting of 0.375μl (1μg/μl) of random primer (Takara, Shiga, Japan), 0.75μl (200U/μl) of SuperScript Reverse Transcriptase II (SS RTII ; Invitrogen) and other components[15], so as to obtain a total reaction volume of 15.0μl. The RT was carried out at 42℃ for 1 hr, followed by heating at 99℃ for 5 min to inactivate the enzyme, and immediate cooling at 4℃.

Specific-primer pairs and multiplex PCR assay for adenovirus and group A and C rotaviruses

Three primer pairs were used in multiplex PCR. Primers Ad1 and Ad2 were used for amplifying the hexon gene of all subgenera A to F adenoviruses[26], generating a 482bp PCR product. Primers Beg9 and VP7-1' were used for amplifying the VP7 gene of group A rotavirus[24], generating a 395bp PCR product. Primers G8NS1 and G8NA2 were used for amplifying the VP7 gene of group C rotavirus[12], generating a 352bp PCR product. The sequence and relative locations of three primer pairs are shown in Table 1.

In the multiplex PCR assay, 2.5μl of cDNA were added to a 500μl microcentrifuge tube with 2.5μl of 10X Taq DNA polymerase buffer (Promega, Madison, WI), 0.5μl of each of the three specific-primer pairs (33μM each), 2.0μl (2.5mM/μl) of dNTPs (Roche, Mannheim, Germany) and 0.125μl (5U/μl) of Taq DNA polymerase (Promega), to achieve a total volume of 25.0μl with the additional of MilliQ water. PCR was performed at 94℃ for 3 min followed by 35 cycles of 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 60 sec, and a

平成16年 8 月20日

final extension at 72℃ for 7 min, followed by cooling at 4℃. All PCR products were electrophoresed in 1.5% agarose gels, followed by staining with ethidium bromide (EtBr, 0.5μg/ml) for 20 min then visualized under ultraviolet (UV) light.

Specificity testing of the mixture *of three* specific-primer pairs

Specificity testing was performed for the positive controls of adenovirus 41, group A rotavirus serotype 1 and group C rotavirus in order to determine the specificity of the mixture of three primer pairs. Two combinations including the PCR mixture containing three primer pairs and each single template, and three primer pairs and three templates were tested with the same thermal cycler program as described above. Specificity testing was also performed for negative controls of RVB, NV/GI, NV/GII, SV and AstV-1.

The final concentrations of each mixture were the same as noted above, and MilliQ water was added to achieve a total volume of 25.0μl.

Sensitivity testing of RT-multiplex PCR and screening-EIA method for adenovirus and *group* A rotavirus

To compare the sensitivity level of RT-multiplex PCR and the screening-EIA method for the presence of adenovirus and group A rotavirus, ten positive controls including four adenovirus-positive fecal supernatants (JP3018, JP3022, JP3026, JP3135), one adenovirus culture (AdF40), four rotavirus-positive fecal supernatants (JP2998, JP3000, JP3011, JP3209) and one rotavirus culture (Wa) were 10-fold serially diluted (10^{-1} to 10^{-5}) in MilliQ water, and subjected to nucleic acid extraction for RT-multiplex PCR and the EIA test.

Confirming the positive results of multiplex PCR for detection of target viruses *in* fecal specimens by monoplex PCR

Adenovirus and group A and C rotavirus positives by multiplex PCR in fecal specimens were further confirmed by monoplex PCR with single specific-primer pairs (0.5μl of 33μM each) Ad1/Ad2, Beg9/VP7-1' and G8NS1/G8NA2, respectively, with the same thermal cycler program as described above for multiplex PCR.

Serotyping of group A rotavirus

Group A rotavirus serotyping was performed using RT-PCR analysis of extracted viral RNA with previously published serotype specific primers (1 to 4 and 9) presented by Das et al. (1994). All group A rotavirus-positives by RT-multiplex PCR were serotyped according to the revised protocols of Gouvea's and Das' working group, as described elsewhere[6]. The expected molecular sizes of the RT-PCR products for group A rotavirus serotypes 1 to 4 and 9 of the primer pair consisting of 9con1 and 9T1-1, 9T1-2, 9T-3P, 9T-4 and 9T-9B were 158, 244, 466, 403 and 110bp, respectively.

Sequencing the multiplex PCR products of untypable group A rotavirus- and group C rotavirus-positives

The PCR products of untypable group A rotavirus positives 395bp in size by multiplex PCR and the

amplicon of the VP7 gene of group C rotavirus were purified and sequenced. Sequence data analyses were then performed.

Assessment of sensitivity of the RT-multiplex PCR assay and screening-EIA for detection of adenovirus and group A rotavirus in fecal specimens

In order to assess and compare the sensitivity of the RT-multiplex PCR with EIA, a total of 207 fecal specimens were further tested by EIA with polyclonal antibodies specific for adenovirus and group A rotavirus, as previously reported[15)25)28)]. Briefly, 96 wells polyvinyl microplates were coated with 100μl of anti-adenovirus or group A rotavirus rabbit serum diluted 1 : 8,000 in carbonate buffer (pH 9.6) and kept overnight at 4℃. The plate was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), and 50μl of PBS and 50μl of specimen supernatants were then added. After shaking for 1 hr at room temperature, the plate was kept overnight at 4℃. The plate was washed three times with PBS-T, and 100μl of guinea pig antiserum against adenovirus or group A rotavirus, diluted 1 : 8,000 with 1% bovine serum albumin (BSA) in PBS-T, were added to wells, followed by shaking for 1hr at room temperature. After washing three times, 100μl horseradish peroxidase (HRP) conjugated rabbit anti-guinea pig IgG diluted 1 : 3,000 with 1% BSA in PBS-T were added, followed by shaking of the plate for 1hr at room temperature. The plate was washed four times and 100μl of O-phenylenediamine (OPD)-H_2O_2 were added. The plate was kept in the dark for 30 min and the reaction was stopped by 50μl of 20% sulfuric acid. A result was considered positive only when the OD value was >0.2 and greater than two times that of the background.

RT-multiplex PCR assay for norovirus (genogroups I and II), sapovirus and astrovirus

Fecal specimens negative for adenovirus and rotavirus were further tested for astrovirus, norovirus (GI, GII), and sapovirus by RT-PCR. The identification of viral group was performed with RT-multiplex PCR with specific primers, as previously reported[27)].

Results

Specificity testing of the mixture of three specific-primer pairs

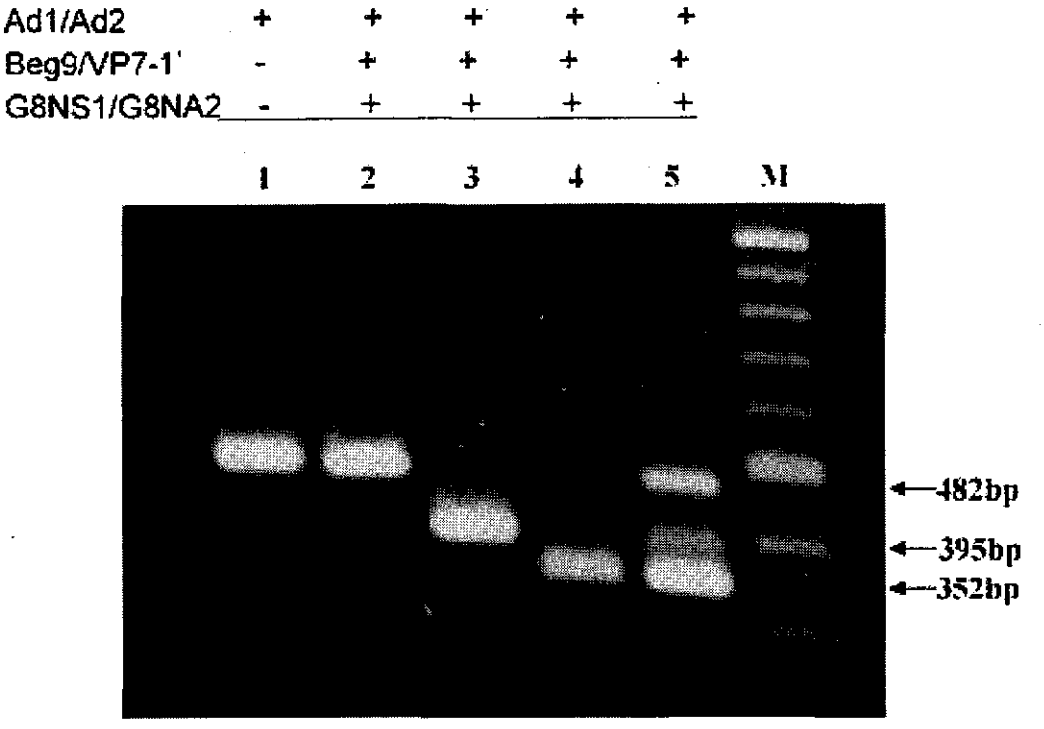
Specificities of the mixture of three specific-primer pairs used in multiplex PCR for adenovirus- and group A and C rotavirus-positive controls are shown in Fig. 1. Each primer pair amplified the expected product independently and specifically, with sizes of 482bp, 395bp and 352bp generated by Ad1/Ad2, Beg9/VP7-1' and G8NS1/G8NA2, respectively. No cross-reaction with non-targets was identified. For the negative controls RVB, NV/GI, NV/GII, SV and AstV-1, no amplicon was generated (data not shown).

Sensitivity testing of RT-multiplex PCR assay and screening-EIA

For the 10-fold dilution series of adenovirus- and group A rotavirus-positive controls, it is noteworthy that the multiplex PCR assay remained positive up to a dilution of 10^{-2} to 10^{-5} for adenovirus and 10^{-5} for rotavirus. The positive results of the highest dilution in the screening-EIA were 10^{-1} and 10^{-2} for adenovirus and group A rotavirus, respectively. Overall, the sensitivity level of the multiplex PCR was apporximately 10- to 10,000-times that of the screening-EIA (Table 2).

平成16年 8 月20日

Fig. 1 Specificity test of the mixture of three specific primer pairs for positive controls Adenovirus 41 (Ad41) and group A and C rotaviruses. Lanes : (1) amplicon of Ad41 by monoplex PCR with single adenovirus-specific primer pair Ad1/Ad2 ; (2–4) amplicon of Ad41, group A and C rotaviruses, respectively, by multiplex PCR with the mixture of three primer pairs ; (5) amplicons of the mixture of three positive controls by multiplex PCR with the mixture of three primer pairs ; M, DNA molecular size markers ; +, primer pair used in PCR ; −, primer pair not used in PCR.



Detection of target viruses in fecal specimens by RT-multiplex PCR

Out of 207 fecal specimens, 114 (55.1%) were positive for the target viruses. Of these, 11 were adenoviruses, 101 were group A rotaviruses and co-infection with adenovirus and group A rotavirus was found in one case. Group C rotavirus was detected in one 18-month-old child (Fig. 2). The highest prevalence of group A rotavirus responsible for diarrhea was 88.6% (101/114 viral positives) and was observed mainly in October and November. In contrast, the seasonal pattern of adenovirus was unclear due to the limited number of positives.

Confirming adenovirus- and group A and C rotavirus-positives by multiplex PCR with monoplex PCR

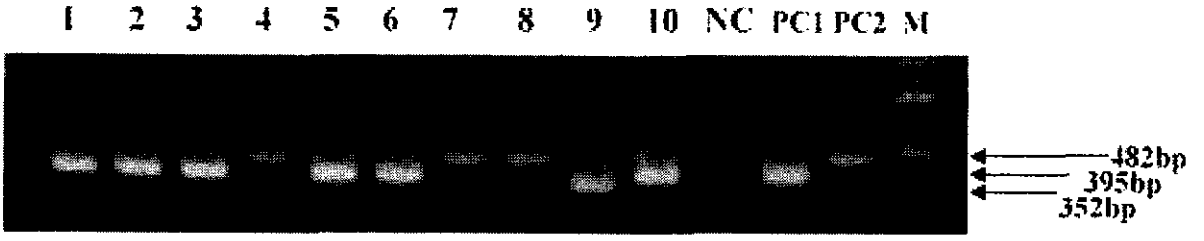
All positives for adenovirus and group A and C rotaviruses by RT-multiplex PCR were further tested to confirm the findings with monoplex PCR. No false positives were identified (data not shown). Moreover, out of 102 (including one co-infected case) group A rotavirus-positives, 96 were serotyped successfully by RT-PCR. Among these, serotypes 1, 2, 3, and 9 accounted for 65.6% (63/96), 7.3% (7/96), 5.2% (5/96) and 17.7% (17/96), respectively. Interestingly, four cases were identified with co-infection, i.e. 2

Table 2 Sensitivity testing of RT-multiplex PCR and screening-EIA for detection of adenovirus and group A rotavirus in 10-fold dilution series of positive controls

Sample No. *	The screening-EIA **				The (RT) multiplex PCR **					
	10^0	10^{-1}	10^{-2}	10^{-3} to 10^{-5}	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Ad40	+	+	+	−	+	+	+	+	+	+
JP3018	+	−	−	−	+	+	+	+	+	−
JP3022	+	+	+	−	+	+	+	+	+	+
JP3026	+	−	−	−	+	+	+	−	−	−
JP3135	+	−	−	−	+	+	+	+	+	+
Wa	+	−	−	−	+	+	+	+	+	+
JP2998	+	−	−	−	+	+	+	+	+	+
JP3000	+	−	−	−	+	+	+	+	+	+
JP3011	+	−	−	−	+	+	+	+	+	+
JP3209	+	+	−	−	+	+	+	+	+	+

* : Ad40, cultured adenovirus subgenus F serotype 40 strain ; Wa, cultured group A rotavirus serotype 1 strain ; JP, positive fecal specimen collected in Japan, as described in text.
** : +, positive ; −, negative.

Fig. 2 Examples of agarose gels containing multiplex PCR products of adenovirus and group A and C rotaviruses in fecal specimens. Lanes : (1, 2, 3, 5, 6 and 10) group A rotavirus positives ; (4, 7 and 8) adenovirus positives ; (9) group C rotavirus positive ; (NC) negative control ; (PC1) positive control RVA with 395bp amplicon ; (PC2) positive control Ad41 with 482bp amplicon ; M, DNA molecular size marker.



(serotypes 1 and 2), 1 (serotypes 1 and 3) and 1 (serotypes 3 and 9). However, six specimens still could not be serotyped (data not shown).

Sequencing of VP7 gene amplicons for untypable group A- and C rotavirus-positives

Amplicons of the VP7 gene of six untypable group A rotavirus positives were further sequenced to characterize their serotypes. Based on the sequence analysis, 2 specimens were similar to group A rotavirus serotype 1 and 4 specimens were serotype 2. To clarify these observations, nucleotide alignments between sequence data of these positives and their specific primers were conducted. Interestingly, the findings showed the nucleotide alignments at primer regions of group A rotavirus positives to be rather different from those of the primers, which explained the failure in serotyping six specimens (data not shown). Furthermore, one group C rotavirus detected by multiplex PCR was also confirmed by sequencing.

平成16年 8 月20日

Table 3 Results of RT-multiplex PCR assay and screening-EIA for detection of adenovirus and group A rotavirus in fecal specimens

(A) Adenovirus detection					(B) Group A rotavirus detection				
		EIA					EIA		
		+	−	Total			+	−	Total
multiplex PCR	+	6	6	12 *	multiplex PCR	+	80	22	102 *
	−	3	192	195		−	2	103	105
	Total	9 *	198	207		Total	82 *	125	207

* : One co-infection with adenovirus and group A rotavirus was included.
The correspondence rates between the two methods were calculated : for adenovirus detection, (6+192) /207 = 95.7% ; for group A rotavirus detection, (80+103)/207 = 88.4%.

Assessment of RT-multiplex PCR assay and screening-EIA sensitivities for detection of adenovirus and group A rotavirus in fecal specimens

Detection rates for adenovirus and group A rotavirus, including the Ad and RVA co-infection specimen, by the RT-multiplex PCR assay were 5.8% (12/207) and 49.3% (102/207), respectively. With the EIA method, however, adenoviruses were responsible for 4.3% (9/207) and group A rotavirus for 39.6% (82/207) of positives (Table 3). Correspondence rates between the two assays were 95.7% (198/207) and 88.4% (183/207) for adenovirus and group A rotavirus, respectively. In total, 28 positives for adenovirus and group A rotavirus by the RT-multiplex PCR assay were negative by EIA, whereas 5 negatives by multiplex PCR were positive by EIA including 3 adenoviruses and 2 group A rotaviruses (Table 3).

RT-multiplex PCR assay for norovirus (*GI and GII*), sapovirus and astrovirus

The fecal specimens negative for adenovirus and rotavirus were further tested for the presence of norovirus (GI, GII), astrovirus, and sapovirus by RT-multiplex PCR. Out of 93 fecal specimens negative for adenovirus and rotavirus, 4.8% (10) were identified as positive for all four viruses. Norovirus (GI, GII), sapovirus and astrovirus were detected in 1, 4, 2 and 3 specimens, respectively.

Discussion

The current study assessed a novel RT-multiplex PCR assay which permitted simultaneous detection of all subgenera of adenoviruses, and group A and C rotaviruses in fecal specimens. Primers used in multiplex PCR were selected for their specificity and broad detection range for these viruses, and three size-specific amplicons could be distinguished by agarose gel electrophoresis. The identity of each multiplex PCR product was confirmed by using specific-monoplex PCR with single specific-primer pairs. A total of 207 fecal specimens were further screened for adenovirus and group A rotavirus by EIA in order to characterize the sensitivity of multiplex PCR. Twenty-eight fecal specimens found to be positive by multiplex PCR assay were negative on screening-EIA and the opposite was also found in a few specimens. Taken together, the results clearly indicate RT-multiplex PCR to be a more sensitive and reliable method than EIA for the detection of adenovirus and rotavirus in clinical specimens and to be a potentially useful tool for diagnostic purposes and molecular epidemiological studies of the diversity of diarrheal viruses.

Viral nucleic acids (non-viral particles) in fecal specimens can be detected by a sensitive method like

PCR assay but not by EIA. This might explain the inconsistency between PCR and EIA in some specimens, which were positive by PCR assay but negative by EIA. Although the PCR assay had been considered much more sensitive than EIA, some investigators reported that several fecal specimens positive by EIA were negative by PCR. This phenomenon might be due to the volumes used in PCR, which were much smaller than those used for EIA, or virus without a genome possibly exist in fecal specimens. In our experiments, five cases including 3 adenoviruses and 2 group A rotaviruses positive by EIA but negative by multiplex PCR were further tested by IC Kit (Orion Diagnostica, Espoo, Finland) according to the manufacturer's protocol. Interestingly, none of the 5 were positive for adenovirus or rotavirus by IC. This confirmed the consistency of results between the multiplex PCR assay and IC. These findings might increase our understanding of the possibility of nonspecific responses by polyclonal antibodies specific for adenovirus and group A rotavirus used in EIA.

During December 2001 and April 2003, 48.8% (101/207) of patients with diarrhea seeking hospital treatment were infected with group A rotavirus (except for the one case with adenovirus- and group A rotavirus-coinfection) which accounted for 89.4% (101/114) of all targets detected. This clearly indicates group A rotavirus to play an important role and to have become a leading cause of viral gastroenteritis in children in Yunnan Province, China. Consistent with previous studies carried out in Beijing and other regions of China, rotavirus infection in the present study occurred mainly in November and December[17]. Furthermore, only a single group C rotavirus case was detected. To date, the epidemiology of adenovirus- and group C rotavirus-infections in Yunnan children has not been known.

The objective of the present study was to develop a sensitive and specific multiplex PCR assay in order to detect all subgenera of adenovirus and group A and C rotaviruses in clinical specimens. During assay development, the RT step, which is necessary for the synthesis of cDNA from rotavirus ds-RNA, but not for detecting adenovirus ds-DNA target, does not alter the ability of multiplex PCR to detect adenovirus. Our data demonstrated three types of diarrheal viruses to be detectable in fecal specimens using the developed assay. The current development has extended our previous study, in which we achieved rapid diagnosis of norovirus (genogroups I and II), sapovirus and astrovirus in clinical cases of acute gastroenteritis. Our present assay has major potential advantages for simultaneous routine screening for multiple target viruses in fecal specimens. The combination of these two multiplex assays raises the possibility of rapid detection of most diarrheal viruses in the laboratory diagnosis of clinical cases. Our next goal is the development of a rapid and efficient PCR detection assay for group B rotavirus, which can be combined with our RT-multiplex PCR. This advancement is anticipated to be available in the near future.

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平成16年 8 月20日

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糞便検体中のアデノウイルスおよびA群，C群ロタウイルスのRT-multiplex PCR法の開発（中国小児下痢症患者の検体を用いて）

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ロタウイルス，アデノウイルス，ノロウイルス，サポウイルス，アストロウイルスは世界中の散発的及び集団的急性胃腸炎の発生に関わると考えられている．そのため，下痢症患者の糞便検体からこれらのウイルスをスクリーニングする迅速かつ感度のよい検出法が求められている．以前の研究で，我々は糞便検体からノロウイルス（グループI，グループII），サポウイルスとアストロウイルスを同時に検出するRT-sm PCRを開発した．最近，我々は新たにアデノウイルス，A群とC群ロタウイルスを同時に検出するRT-multiplex PCRを開発した．本研究ではこの方法で2001年12月から2003年4月までに中国雲南省小児下痢症患者から採取した糞便検体を用いてアデノウイルス，A群とC群ロタウイルスを検査した．207検体のうち114検体が検出され，検出率は，55.1%となった．このうち，アデノウイルス，A群，C群ロタウイルスはそれぞれ11，101，1で，混合感染(アデノウイルスとA群ロタウイルス）が1検体であった．流行は主に10月と11月であった．

以上をまとめると，下痢症を引き起こすアデノウイルス，A群とC群ロタウイルスの流行調査にmultiplex PCRは迅速で，便利な実験室診断法であると考えられる．

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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

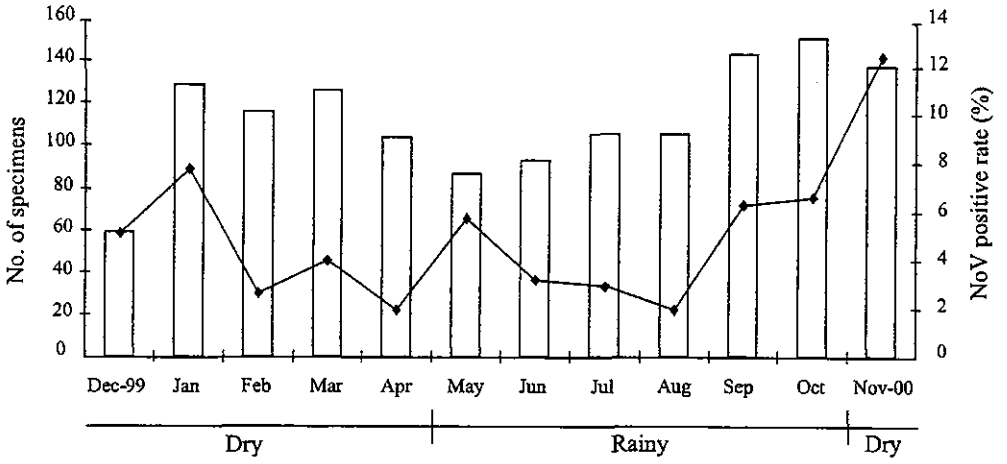


Fig. 1. Monthly distribution of NoV-positive specimens from children with acute sporadic gastroenteritis in Ho Chi Minh City, Vietnam. The two different climatic seasons (dry and rainy) in Ho Chi Minh City are shown. No. of specimens, □; positive rate (%), -◆-

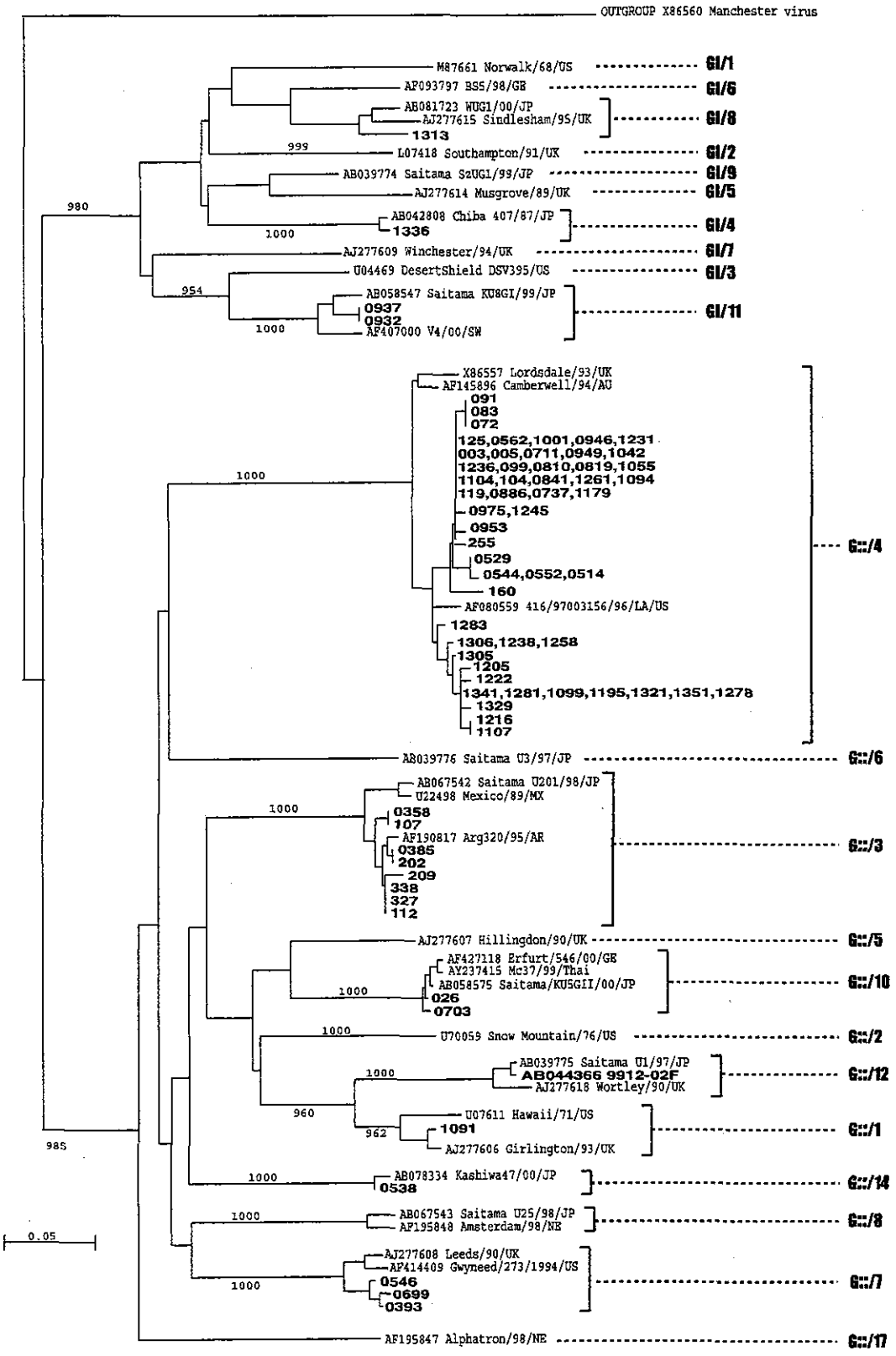
($\chi^2 = 15.5$, $P < 0.005$). This trend suggested that NoV prevailed at the end of the rainy season and the first half of the dry season.

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
1000
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
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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
AB044366 9912-02F
AJ277618 Wortley/90/UK
1000
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

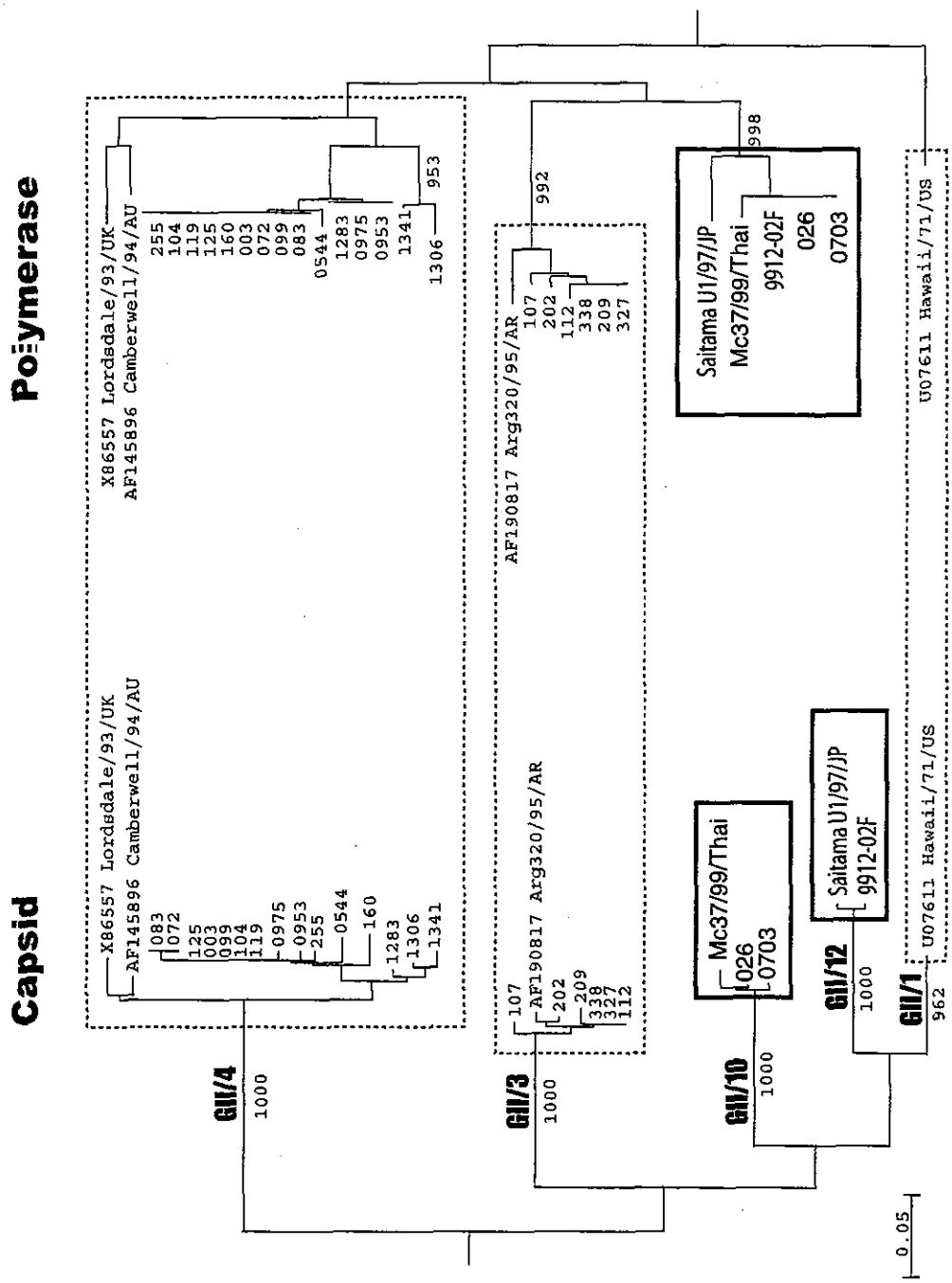


Fig. 3. Phylogenetic trees of 23 NoV GII strains. The left panel shows a tree based on the partially sequenced capsid (264 bp) regions, and the right panel shows that of RdRp (298 bp). The scale represents nucleotide substitutions per site. The strains boxed by broken lines are ones whose genotypes are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

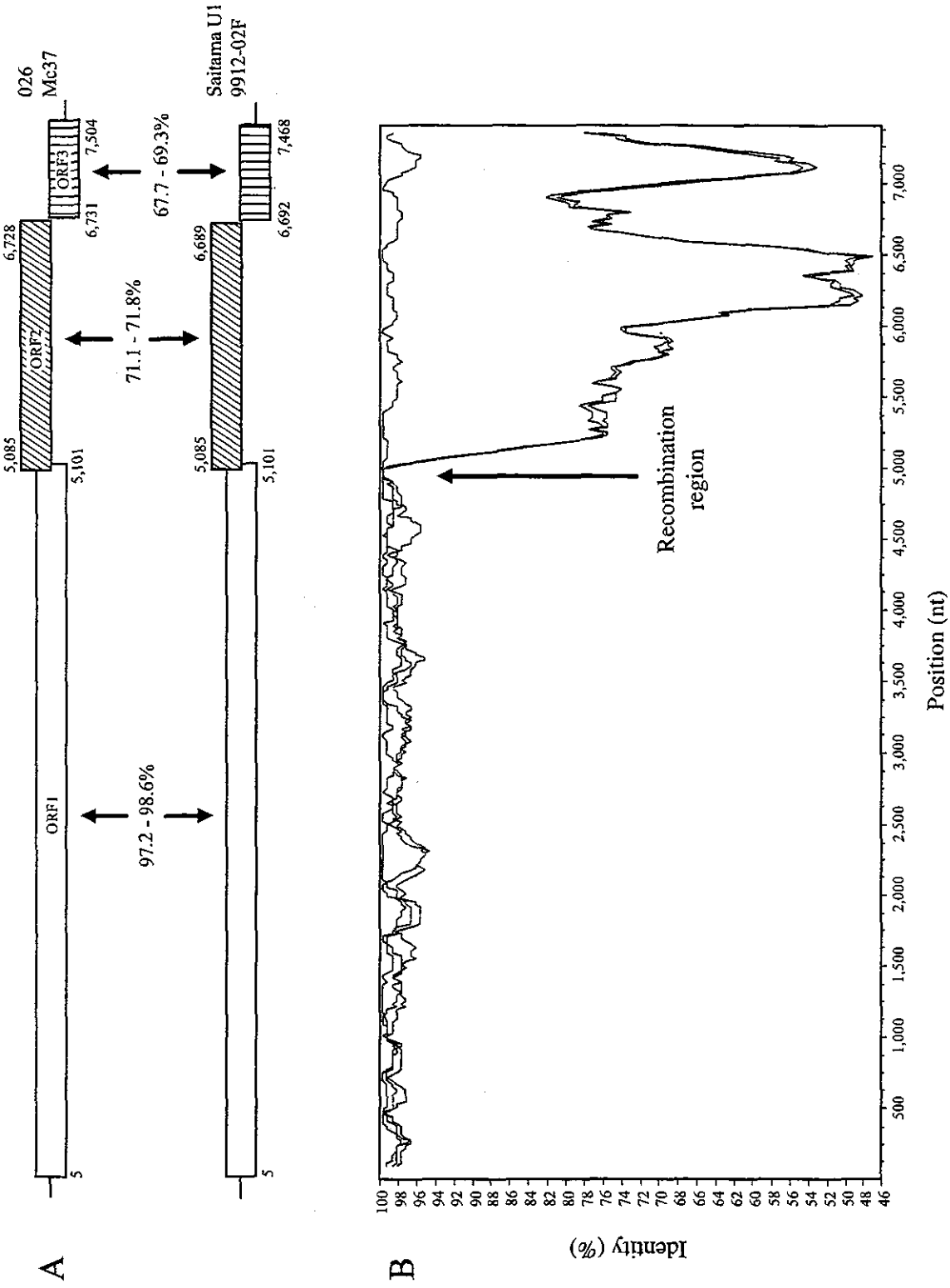
In 15 strains belonging to GII/4 genotype (Lordsdale cluster) and six strains belonging to the GII/3 genotype (the Saitama U201 cluster in which the Arg320 strain is included), the genotype was maintained in both RdRp and capsid regions (Fig. 3). Two other strains from GII/10, the 026 and 0703 strains,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