TABLE 1
Primer pairs used to amplify NoVs and SaVs in this study

Primer	Target virus	Polarity	Sequence position (5' to 3') reference strain	Amplicon size	Target region
G1SKF	NoV GI	+	5342-5361 (Norwalk 68)	330bp	Polymerase and capsid junction
G1SKR	NoV GI	−	5653-5671 (Norwalk 68)		
COG2F	NoV GII	+	5003-5028 (Lordsdale)	387bp	Polymerase and capsid junction
G2SKR	NoV GII	−	5367-5389 (Lordsdale)		
SLV5317	SaV	+	5083-5105 (Manchester)	434bp	Polymerase and capsid junction
SLV5719	SaV	−	5494-5516 (Manchester)		

NoV GI, norovirus GI; NoV GII, norovirus GII; SaV, sapovirus.

SaVs can be divided into five genogroups (GI to GV), among which, GI, GII, GIV and GV are identified within humans [19]. The SaV GI, GIV and GV genomes contain three ORFs, whereas the SaV GII genome contains two ORFs. ORF1 encodes all the non-structural proteins, including RdRp, and the major capsid protein (VP1). ORF2 encodes a small protein, and ORF3 encode a protein of unknown function [20].

Normally, in both NoV and SaV, the genogroup/genotypes are generally maintained across the three ORFs. A recombinant NoV or SaV can be defined as one that clusters with two distinct groups of strains when two different regions (normally the capsid and polymerase) of the genome are subjected to phylogenetic analysis. Since the first NoV recombinant, Snow Mountain strain [21], was reported, various naturally occurring recombinants in different types have been identified [22–25]. Likewise, the identification of SaV recombinants have been reported elsewhere [26–28].

In Vietnam, NoVs and SaVs were identified from several epidemiological surveillances, and are considered as the important agents of viral gastroenteritis in the country [29, 30]. The first Vietnamese NoV recombinants were reported from a surveillance during 1999–2000 [30], since then, neither data about calicivirus infections nor recombinant virus has been reported. A hospital-based surveillance was conducted in Ho Chi Minh City during 2005–2006 that investigated the presence of common viral agents causing diarrhea in children, has been described elsewhere [31]. In this study, we reported in details the detection of NoVs and SaVs in the surveillance mentioned above, and described the molecular characteristics of NoV and SaV strains detected. The clinical manifestations and the evaluation of disease severity in patients were also included.

Materials and Methods

Patients

Patients with acute gastroenteritis who either visited the out-patient ward or were admitted to the Department of Gastroenterology, Children's Hospital 1, Ho Chi Minh City from December 2005 to Novemer 2006, were recruited in the surveillance. Patients were examined by pediatricians, and the clinical symptoms of dehydration were assessed based on the WHO guideline [32]. A 20-point Vesikari's score was used to evaluate the disease severity in patients [33].

Fecal samples collection and virus detection

A total of 502 fecal samples were collected from studied patients (one specimen from each patient). The fecal specimens from the outpatients were collected at the out-patient ward or from the inpatients within 24 h after admission and stored at −20°C until use. They were prepared as a 10% suspension in distilled water and the viral RNA genomes were extracted from the fecal suspension with a QIAamp Viral RNA Mini kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instruction. The presence of NoVs and SaVs in fecal specimens was determined by RT-multiplex PCR [34]. Three primer pairs, G1SKR–G1SKF, COG2F–G2SKR and SLV5317–SLV5749 [34] were used to amplify NoVs GI, NoVs GII and SaVs, respectively (Table 1). PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min, and then visualized under ultraviolet light. The results were recorded by photography.

Nucleotide sequencing and phylogenetic analysis

All of NoVs and SaVs detected in this study were subjected to nucleotide sequencing by using the Big Dye Terminator Cycle Sequencing kit version 3.1 and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc.) according to the manufacturer's instruction. Primer pairs mentioned above were used as sequencing primers, generating a partial nucleotide sequence, including both polymerase region and the capsid region [34]. Similarities of the sequenced strains with other strains were assessed by BLAST search using the default options (DNA DataBank of Japan). Multiple sequence alignments were

TABLE 2
Monthly distribution of NoVs and SaVs detected from children with acute gastroenteritis in the Children's Hospital 1, Ho Chi Minh City, during 2005–2006

Seasonality	Dry season					Rainy season						Dry	Total (%)
	Dec 05	Jan 06	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	
No of specimens	20	30	43	57	30	53	32	17	30	32	60	98	502 (100)
No (%) of NoVs	2 (10.0)	1 (3.3)	2 (4.7)	4 (7.0)	0 (0)	3 (5.7)	2 (6.3)	0 (0)	6 (20.0)	4 (12.5)	5 (8.3)	3 (3.1)	32 (6.4)
No (%) of SaVs	0 (0)	1 (3.3)	2 (4.7)	0 (0)	1 (3.3)	1 (1.9)	0 (0)	0 (0)	1 (3.3)	0 (0)	0 (0)	0 (0)	6 (1.2)

NoV, norovirus; SaV, sapovirus. 62.5% of NoVs were identified in the rainy season.

calculated using the CLUSTALX program, and the phylogenetic trees were constructed by the neighbor-joining method with the MEGA 3.1 software package [35], and using different NoVs and SaVs sequences available in GenBank for comparison and as outgroups.

Accession numbers
The selected nucleotide sequences of Vietnamese NoVs and SaVs strains described in this study have been deposited in GenBank under accession numbers EU137732–EU137739.

Results

Detection of NoVs and SaVs
Among 502 fecal specimens collected during the 1-year surveillance, NoVs GII were determined in 32 (6.4%) specimens, and SaVs were detected in six (1.2%) specimens. Fifteen and four specimens showing positive with NoV and SaV, respectively, were found to be in mixed infection with other viral pathogens. There was no NoV GI found in this study. Regarding seasonal pattern, NoVs could be identified through the year, except in April and July. Twenty out of 32 (62.5%) of the NoVs were detected during the rainy season, which usually begins in May and ends in October in the southern part of Vietnam, including Ho Chi Minh City. Conversely, four out of six (66.7%) of SaV strains were identified during the dry season (Table 2).

Characteristics of the NoV and SaV-positive patients
The Table 3 showed the characteristics of positive cases with NoVs and SaVs. Twenty-eight and four patients showing positive with NoVs and SaVs, respectively, had adequate medical records for further analyses. To characterize the age distribution, all patients were classified into five different age groups (<6, 6–11, 12–23, 24–35 and >35 months old). NoV patients were neither found in <6 nor in >35 months of age, while 27 out of 28 NoV patients were between 6 and 23 months of age. Similarly, three out of four SaV cases were classified into either 6–11 or 12–23 months age group. Majority of NoV patients (22/28, 78.6%) were male, however, three out of four SaV patients were female. Although the surveillance was conducted in a children's hospital in Ho Chi Minh City, only 11/28 (39.3%) cases lived in the city, the remaining 17/28 (60.7%) of NoV patients came from various provinces in the southern part of Vietnam.

Clinical signs and symptoms of NoV infections
Seventeen patients showing monoinfection with NoV [31] were selected for analysis of the clinical manifestations, among them 15 medical records were enough data for further analyses. The main clinical signs and symptoms observed in children with NoV infection were diarrhea (100%), watery stool (93.3%), vomiting (66.7%), highest temperature $\geq$38.5°C (33.3%), coughing (26.7%) and coryza (6.7%). The mean duration of diarrhea and vomiting were 4.4 ± 3.9 days and 1.5 ± 1.7 days, respectively, and the maximum episodes of diarrhea and vomiting were 6.5 ± 2.5 times per day and 3.3 ± 2.8 times per day, respectively (Table 4).

Evaluation of severity in patients showing monoinfection with NoV by using a 20-point numerical score showed that the mean severity score of NoV positive patients was 9.8 ± 3.6. The severity scores were analyzed further by age groups, gender, place of living (Ho Chi Minh City and non-Ho Chi Minh City residents), time of collection (during rainy and dry season) and status of patients (hospitalized and non-hospitalized patients) (Table 3). Obviously, the mean severity scores of patients belonging to some groups were observationally lower than those of other groups (e.g., patients who were 12–23 months old, or patients who lived in Ho Chi Minh City); however, the difference was not statistically significant ($p > 0.05$). The only significant difference was observed between inpatients and outpatients, with the mean severity scores in each group being 10.82 ± 3.49 ($N = 11$) and 7.0 ± 2.45 ($N = 4$), respectively ($p < 0.05$). A comparison of the mean severity scores between monoinfection cases and

TABLE 3
Attributes of NoV positive cases[a] and mean severity score of patients in each group

	Distribution of patients by										
	Age (months)					Gender		Place of living		Patient status	
	<6	6–11	12–23	24–35	>35	Male	Female	HCMC[b]	Others	Inpatient	Outpatient
No. (%) of NoV cases	0 (0)	10 (35.7)	17 (60.7)	1 (3.6)	0 (0)	22 (78.6)	6 (21.4)	11 (39.3)	17 (60.7)	21 (75)	7 (25)
Vesikari's score[c]		11.4 ± 4.28 (*N* = 5)	8.67 ± 3.16 (*N* = 9)	12.0 (*N* = 1)		9.7 ± 3.97 (*N* = 10)	10.0 ± 3.16 (*N* = 5)	8.2 ± 1.92 (*N* = 5)	10.6 ± 4.06 (*N* = 10)	10.82 ± 3.49[d] (*N* = 11)	7.0 ± 2.45[d] (*N* = 4)

[a]Data based on 28 complete medical records.
[b]HCMC, Ho Chi Minh City.
[c]Data based on 15 medical records of patients who showed monoinfection with NoV.
[d]$p < 0.05$.

TABLE 4
Clinical signs and symptoms of patients who showed monoinfection with NoV during a one-year surveillance in Ho Chi Minh City, 2005–2006

Signs and symptoms	NoV infection cases
Diarrhea	100%
Watery stool	93.3%
Vomiting	66.7%
Temperature ≥ 38.5°C	33.3%
Coughing	26.7%
Coryza	6.7%
Mean duration of diarrhea	4.4 ± 3.9 days
Mean duration of vomiting	1.5 ± 1.7 days
Maximum episodes of diarrhea/day	6.5 ± 2.5 times per day
Maximum episodes of vomit/day	3.3 ± 2.8 times per day

mixed infection cases was also performed, however, the difference was not statistically significant (data not shown).

Only one medical record from two patients showing monoinfection with SaV was available, therefore, description of the clinical features of SaV infection in this study was not performed.

Phylogenetic analysis of NoV strains and identification of various recombinations

All of the 32 NoV strains detected in this study were successfully determined nucleotide sequence with the amplified fragments, which included both polymerase and capsid region. Phylogenetic analysis based on the capsid region revealed that 16/32 (50%) NoV strains clustered within the GII.4, and 13/32 (40.6%) strains belonged to the GII.3b cluster, according to the classification reported by Phan *et al.* [14]. One strain, HCMC91, clustered together with GII.12 NoV strains (96% nucleotide identity with the Chitta strain), and other two strains, HCMC204 and HCMC311, belonged to the GII.6 cluster (95% nucleotide identity with the SaitamaU17 strain). Interestingly, these two Vietnamese GII.6 strains did not group with any GII.6 NoV strains from sublineage a to d, therefore, clustered into a novel sublineage, tentatively called GII.6e (Fig. 1).

To verify the sequence identities of the GII strains, an additional phylogenetic analysis of Vietnamese NoV strains and other reference strains based on the polymerase region was performed (Fig. 2). All of the 16 capsid-based GII.4 NoV strains maintained their genotype in the polymerase region, however, other strains bored a different either genotype or subgenotype when polymerase-based grouping was carried out. Twelve out of the 13 capsid-based GII.3b NoV strains clustered into the GII.3a lineage,

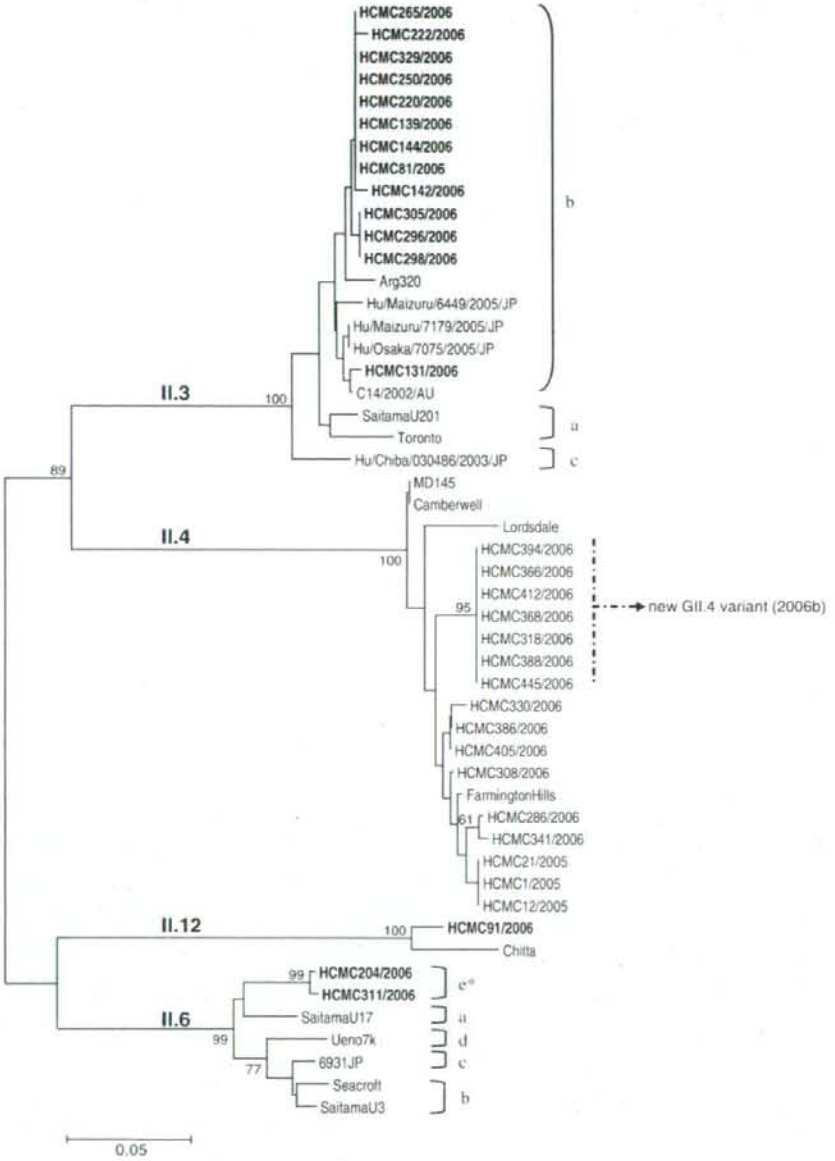

FIG. 1. Phylogenetic tree of the capsid region of 32 Vietnamese NoVs and other reference NoV strains available from GenBank. Vietnamese recombinant strains are in bold face. Genotypes and subgenotypes are indicated. Bootstrap values >75% are shown at the brach nodes.

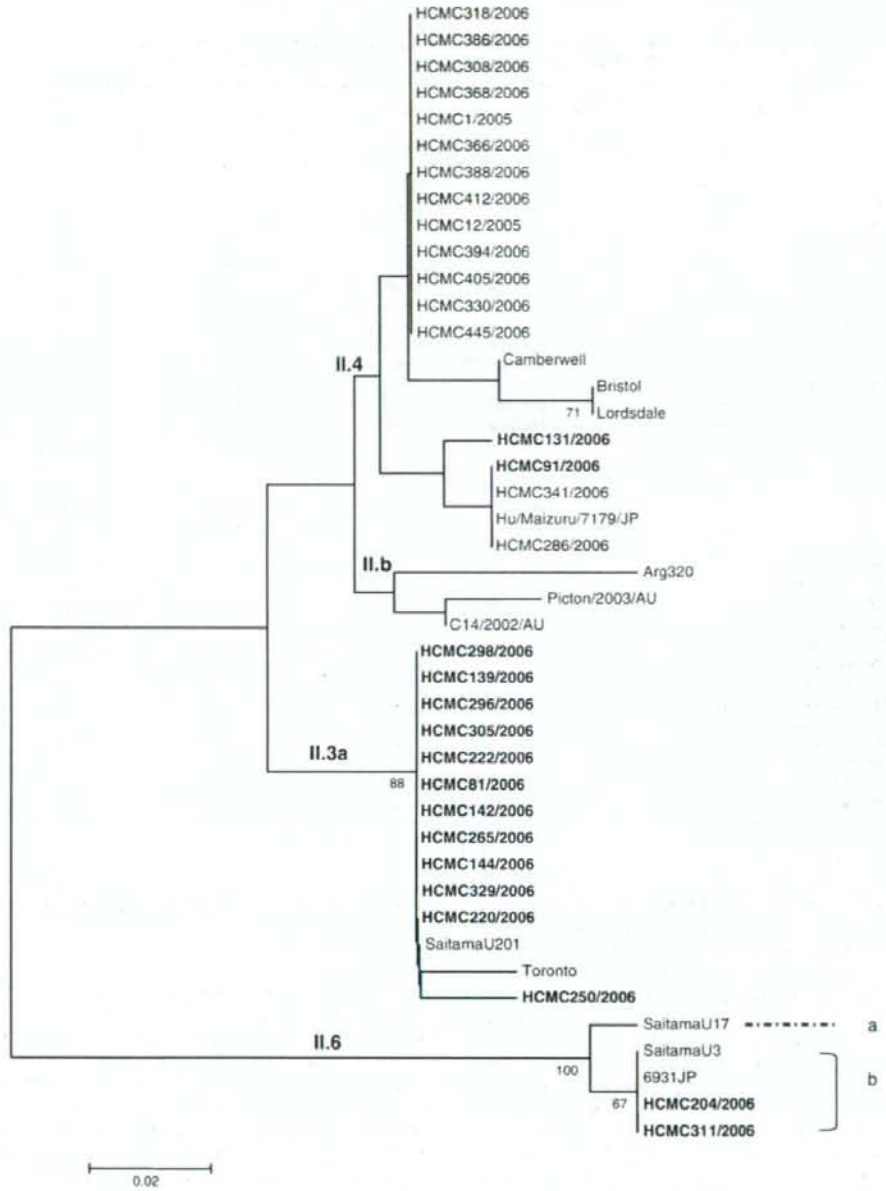

FIG. 2. Phylogenetic tree of the polymerase region of 32 Vietnamese NoVs and other reference NoV strains available from GenBank. Vietnamese recombinant strains are in bold face. Genotypes and subgenotypes are indicated.

TABLE 5
Molecular characteristics of NoVs strains detected among patients with acute gastroenteritis in the Children's Hospital 1, Ho Chi Minh City, during 2005–2006

Strain	Collection date[a]	Polymerase		Capsid		Type of recombination
		Genogroup genotype	Representative	Genogroup genotype	Representative	
HCMC1	Dec	II.4		II.4		
HCMC12	Dec	II.4		II.4		
HCMC21	Jan	II.4		II.4		
HCMC81	Feb	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC91	Feb	II.4	Lordsdale	II.12	Chitta	Intergenotype
HCMC131	Mar	II.4	Lordsdale	II.3b	Arg320	Intergenotype
HCMC139	Mar	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC142	Mar	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC144	Mar	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC204	May	II.6b	SaitamaU3	II.6e	N/A	Intersubgenotype
HCMC220	May	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC222	May	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC250	Jun	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC265	Jun	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC286	Aug	II.4		II.4		
HCMC296	Aug	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC298	Aug	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC305	Aug	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC308	Aug	II.4		II.4		
HCMC311	Aug	II.6b	SaitamaU3	II.6e	N/A	Intersubgenotype
HCMC318	Sep	II.4		II.4		
HCMC329	Sep	II.3a	Toronto	II.3b	Arg320	Intersubgenotype
HCMC330	Sep	II.4		II.4		
HCMC341	Sep	II.4		II.4		
HCMC366	Oct	II.4		II.4		
HCMC368	Oct	II.4		II.4		
HCMC386	Oct	II.4		II.4		
HCMC388	Oct	II.4		II.4		
HCMC394	Oct	II.4		II.4		
HCMC405	Nov	II.4		II.4		
HCMC412	Nov	II.4		II.4		
HCMC445	Nov	II.4		II.4		

N/A, not applicable.

whereas the other strain, HCMC131, grouped with other GII.4 strains when polymerase-based grouping was performed. This type of recombination, GII.3b/GII.4, was similar to that of the NoV recombinant strain 5017/04/JP, which was reported formerly [36]. Similarly, the capsid-based GII.12 strain, HCMC91, bored a different genotype, GII.4 when a BLAST search was performed in the polymerase region. This strain also shared best identity, 96%, with the well-known GII.4/GII.12 recombinant strain SaitamaU1 [22] in both the polymerase and capsid region, demonstrating that HCMC91 was also a recombinant virus. Regarding two capsid-based GII.6e strains, HCMC204 and HCMC311, the polymerase-based phylogenetic tree clearly showed that they clustered together with other NoV strains into the GII.6b sublineage, therefore, these two Vietnamese strains were GII.6b/GII.6e recombinant strains. Altogether, half of the NoV (16/32) strains identified in this study were determined as recombinant viruses (Table 5).

Phylogenetic analysis of SaV strains and the identification of a novel recombination

Results of nucleotide sequencing of the 434 bp PCR product allowed us to analyze the molecular characteristics of both polymerase and the capsid region of SaV strains detected. Among six Vietnamese SaV strains, genotype GI.1, GI.2 and GII.1 were identified in two, one and one strain, respectively, and all of these four SaV strains maintained the same genogroup/genotype across polymerase and the capsid region (Fig. 3). However, the remaining two strains, HCMC86 and HCMC180, showed different genotypes when the polymerase-based and capsid-based phylogenetic analyses were conducted. These two SaV strains shared 100% nucleotide identity,

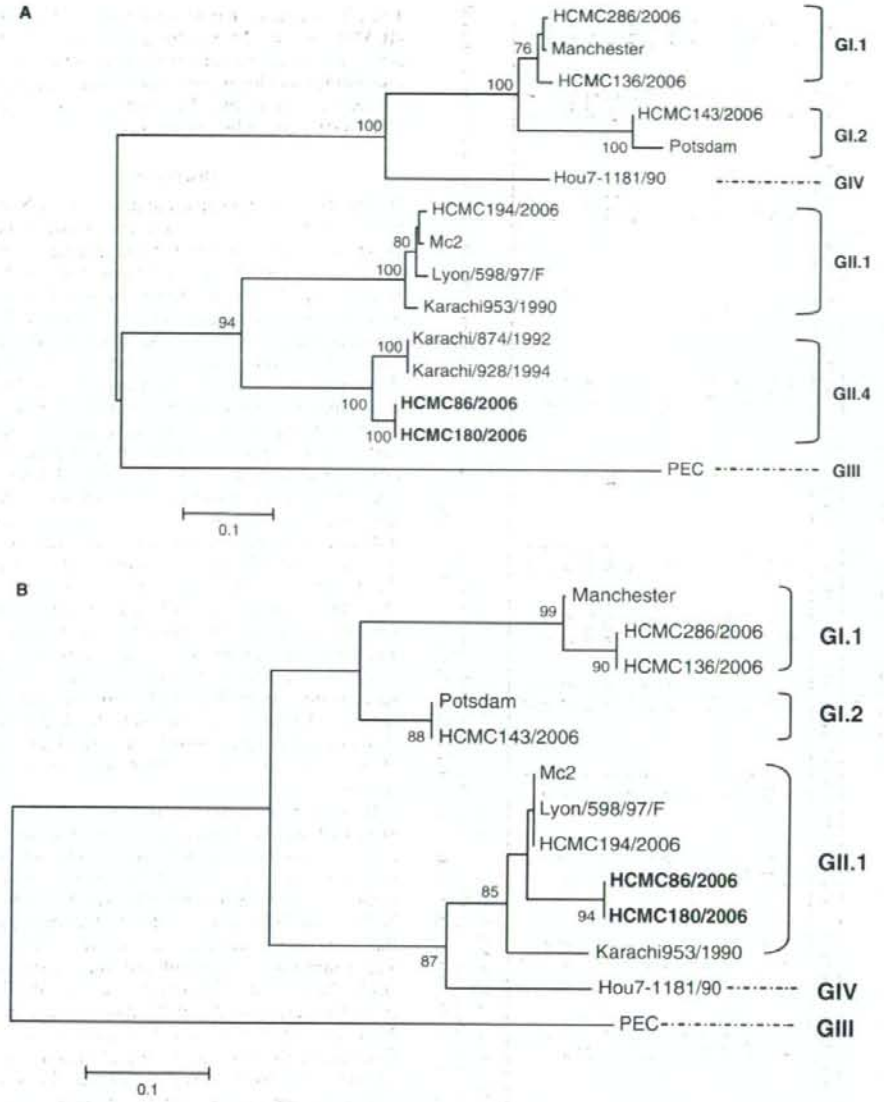

FIG. 3. Phylogenetic tree of the (**A**) capsid and (**B**) polymerase region of 32 Vietnamese SaVs and other reference SaVs. Two Vietnamese SaVs strain, HCMC86 and HCMC180, showed different genotypes when polymerase-based and capsid-based phylogenetic trees were constructed.

which indicates that they are the same strain. Nucleotide comparison showed that HCMC86 and HCMC180 had best identities (89.8–94.2%) with GII.1 strains in the polymerase region; however, they had higher homology with two Pakistani GII.4 SaV strains (Karachi/874 and Karachi/928) than GII.1 strains (94.2% vs. 67.7–68.1%) when a capsid-based comparative analysis was performed (Table 6).

TABLE 6
Percentage nucleotide homology of the polymerase and capsid region[a] of Vietnamese SaV strains and other reference strains

	I.1			I.2		IV	II.1			II.4				III
	Manchester	HCMC286	HCMC136	Potsdam	HCMC143	Hou7-1181	Mc2	HCMC194	Lyon 598	HCMC86	HCMC180	Karachi 874	Karachi 928	PEC
Manchester		99.2	97.4	80.8	81.5	65.7	48.9	49.6	49.6	49.6	49.6	48.5	48.5	45.4
HCMC286	97.1		97.4	81.2	81.9	66.0	48.9	49.6	49.6	49.2	49.2	48.2	48.2	45.4
HCMC136	97.1	100		80.4	80.4	65.3	48.9	49.6	49.6	48.5	48.5	48.2	48.2	45.4
Potsdam	84.0	81.1	81.1		97.1	63.1	46.0	45.3	46.0	44.3	44.3	42.9	42.9	41.4
HCMC143	84.0	81.1	81.1	100		63.8	47.1	46.4	47.1	46.0	46.0	44.6	44.6	40.7
Hou7-1181	73.9	72.4	72.4	73.9	73.9		46.7	47.1	47.5	49.2	49.2	47.1	47.1	40.8
Mc2	72.4	69.5	69.5	76.8	76.8	85.5		98.9	98.2	68.1	68.1	67.3	67.3	43.5
HCMC194	68.1	65.2	65.2	72.4	72.4	81.1	95.6		97.8	68.1	68.1	67.3	67.3	43.2
Lyon 598	72.4	69.5	69.5	76.8	76.8	85.5	100	95.6		67.7	67.7	67.0	67.0	44.6
HCMC86	71.0	71.0	71.0	75.3	75.3	84.0	94.2	89.8	94.2		100	94.2	94.2	45.7
HCMC180	71.0	71.0	71.0	75.3	75.3	84.0	94.2	89.8	94.2	100		94.2	94.2	45.7
Karachi 874[b]	N A	N A	N A	N A	N A	N A	N A	N A	N A	N A	N A		100	45.0
Karachi 928[b]	N A	N A	N A	N A	N A	N A	N A	N A	N A	N A	N A	N A		45.0
PEC	50.7	50.7	50.7	55.0	55.0	50.7	52.1	49.2	52.1	52.1	52.1	N A	N A	

Best identities results of HCMC86 and HCMC180 are in shaded.
[a]Nucleotide homology of polymerase region is shown in the lower left, and capsid region is in the upper right. Genogroups and genotypes are also indicated.
[b]The nucleotide sequences of the polymerase region of Karachi 874 and Karachi 928 are not available.

The phylogenetic analysis also indicated clearly that HCM86 and HCM180 clustered into two different genotypes when polymerase-based and capsid-based nucleotide phylogenetic trees were constructed. Altogether, these two Vietnamese SaV strains are GII.1/GII.4 recombinant strains.

Discussion

In this study, we reported the detection of NoVs and SaVs among diarrheic children in the Children's Hospital 1, Ho Chi Minh City, during 2005–2006. With the overall detection rate of 6.4% and 1.2%, respectively, NoVs and SaVs continued to be viral agents causing acute gastroenteritis in children in the southern part of Vietnam. Although these detection rates were slightly lower than those of the studies in developed countries [37, 38], the results in this study were similar to those of the epidemiological studies conducted previously at the same hospital [29, 30], and also comparative with other surveillances in other developing countries [39, 40]. Despite difference of time, the detection of NoVs and SaVs with similar proportions in the southern part of Vietnam indicated that these viruses have circulated stably in the area. NoV GI was not found in this study, and this result was in agreement with the previous study [29]. The absence of NoV GI in epidemiological surveillance was also reported elsewhere [18, 34]. The primer sets using in this study have been used to screen caliciviruses in other surveys, and they successfully identified NoV GI in the studied samples. Therefore, the inability to detect NoV GI strains in this study might have resulted from the absence of this virus within the collected fecal specimens.

In temperate climate countries, NoVs are usually identified in the winter time [36, 38], whereas in tropical countries, the seasonal pattern of NoVs is not clear. In this survey, NoVs was found all year round, except in April and July. Moreover, 62.5% of NoVs were detected from May to October, indicated that this virus prevailed during the rainy season. This result was concordant with that of the previous study during 2002–2003 [29], and slightly different from the result of the 1999–2000 survey, in which, NoVs prevailed at the end of the rainy season and the first half of the dry season [30]. However, the results of the 1999–2000 survey based on the specimens that were negative for other common viral agents, therefore, the absence of NoVs strains, if any, which were mixed infection with other viruses, might make the feature of monthly distribution of NoVs incomplete.

NoV GII.4 was the most common (50.0%) genotype among NoV strains detected in this study. Previous studies in Ho Chi Minh City also found NoV GII.4 in 78% and 82.1% of samples [29, 30], confirming the predominance of this genotype.

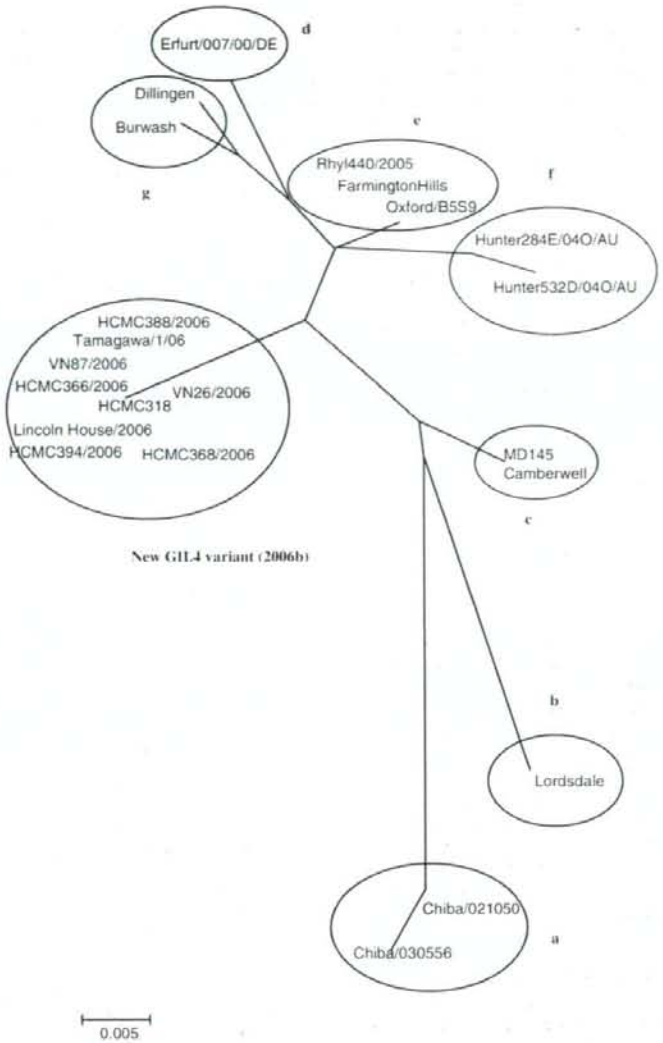

FIG. 4. Unrooted phylogenetic tree of new NoV GII.4 variant identified in this study and other GII.4 lineages. Vietnamese NoVs detected from another study [41], VN26 and VN87, as well as other reference strains, Tamagawa 1 06 and Lincoln House 2006, are included. The classification is based on Phan *et al.* [14].

However, 7 out of 16 Vietnamese GII.4 strains in this study belonged to a distinct cluster which has been determined as a novel GII.4 variant, 2006b [41] (Figs 1 and 4). These strains were firstly identified in September, and continued to be detected until the end of the surveillance, suggesting that these viruses have been continuing to prevail in this area in the coming year. Different genotypes of NoVs and SaVs were determined in this study, and among them, several genotypes have not been reported formerly in Vietnam (NoV GII.6, SaV GI.2 and GII.4). Of interest, SaV GII.4 was only

reported in two unique Pakistani strains collected in 1992 and 1994, respectively [42]. On the other hand, the 'new variant' designated GII.b NoVs, which has been detected in Europe in the beginning of 2000s and then identified in Asia [36, 38, 43, 44], could not be found in this study. A larger number of specimens, as well as an attempt to collect fecal samples from different places in Vietnam is needed for confirming the absence of this virus in the country.

Although detected in several epidemiological studies, and being considered as important viral agent causing acute gastroenteritis in young infants and children, this was the first time, to our knowledge that the clinical manifestations of NoV infections were described in Vietnamese pediatric patients. The clinical features of NoV-associated acute gastroenteritis observed among patients in this study were similar to those of other reports, including diarrhea with watery stool, vomiting and fever [18, 20]. Although the results of this study were comparable to another study conducted in Japan [18], the mean duration of diarrhea and maximum episodes of diarrhea per day in Vietnamese children were observationally higher than those of Finnish children (4.4 days vs. 2 days, 6.5 times/day vs. 4 times/day) [45]. The difference might be explained by the population studied. In this study, we collected samples from patients who sought to the hospital, whereas the survey carried out in Finland was a community-based study. Therefore, although both were classified as moderately severe diseases (8–10 points) [45], the mean severity score in Vietnamese patients was obviously higher than that of Finish children (9.8 vs. 8).

A comparative analysis was performed in order to see the difference in severity among several groups of patients, however, only the mean scores were statistically different between inpatients and outpatients. This situation was also observed among astrovirus positive patients described previously [31].

The clinical manifestation of SaV infection in this study could not be demonstrated because only one medical record among two SaV monoinfection cases was available. This patient suffered from an 8-day diarrhea, with maximum episode of diarrhea was 20 times per day and high fever up to 39°C. This feature was much different from other reports, which described SaV-associated diarrhea to be a mild disease. More clinical data on larger number of patients are needed in order to identify properly the clinical features of SaV infection in Vietnamese children.

RNA recombination plays a key role in virus evolution and it shapes a good deal of the virus diversity [46]. Recombinant NoV strains were increasingly found in epidemiological surveillances throughout the world [22, 23, 38, 43], including Vietnam [30]. In this survey, various types of recombination in NoVs were identified. Of interest, the GII.6b (polymerase)/GII.6e (capsid) recombination was first reported in this study. Similarly, the recombinant GII.1/GII.4 SaV strain detected in this survey has not been described elsewhere. Half of NoV strains, and one out of six SaV strains were identified as recombinant viruses, thus indicates that recombination is not a rare event, and the caliciviruses circulating in Vietnam have a trend to be more diverse.

The results of this study highlight the impact of caliciviruses in diarrheal diseases among children in Ho Chi Minh City, and are the first to describe the clinical manifestations of NoV infections in Vietnamese children. The data of nucleotide analysis from this study could provide useful information for knowledge on caliciviruses characteristics.

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Genetic Diversity of Norovirus, Sapovirus, and Astrovirus Isolated From Children Hospitalized With Acute Gastroenteritis in Chiang Mai, Thailand

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Norovirus (NV), sapovirus (SV), and human astrovirus (HAstV) are important causes of acute gastroenteritis in infants and young children. This study investigated the prevalence of NV, SV, and HAstV infections in children hospitalized with acute gastroenteritis in Chiang Mai, Thailand from May 2000 to March 2002. Fecal specimens were tested for NV, SV, and HAstV by reverse transcription polymerase chain reaction (RT-PCR) using degenerate specific primers. These viruses were characterized further by sequence and phylogenetic analyses of the partial capsid gene. From 296 fecal specimens tested, 13.5% (40 of 296) were positive for NV, SV, and HAstV. Of these, NV most predominant, with a prevalence of 60% (24 of 40), of which 17.5% were NVGI and 42.5% were NVGII. Of note, one specimen was positive for both NVGI and SV. SV was detected in 25%, while HAstV was detected in 17.5%. Analysis of nucleotide and amino acid sequences revealed that NVGI strains comprised GI/3, GI/4, GI/6, GI/7, and GI/13 genotypes. Among NVGII strains, approximately half of them belonged to genotype GII/4 (Lordsdale virus cluster), followed by GII/3, GII/10, GII/1, GII/6, GII/8, and GII/15. Analysis of SV sequences revealed that SVGI (Manchester virus) was more common than SVGII (London virus). The SV genotypes detected in this study belonged to SVGI/1, SVGI/4, SVGI/5, SVGII/1, and SVGII/2, whereas the HAstV belonged to genotypes HAstV-1, HAstV-2, HAstV-3, and HAstV-5. The findings suggest that NV, SV, and HAstV are important enteric viruses cocirculating among hospitalized children in Chiang Mai, Thailand. ***J. Med. Virol. 80:1749–1755, 2008.*** © 2008 Wiley-Liss, Inc.

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

INTRODUCTION

Acute gastroenteritis is one of the most common diseases in infants, children, and adults worldwide. During the first 5 years of life, every child will contract diarrheal disease, which enhances the risk of dehydration and nutritional deficiency [Jiraphongsa et al., 2005]. In addition to rotavirus (RV), human caliciviruses and astrovirus have emerged as etiologic causes of acute gastroenteritis in this age group. Norovirus (NV) and sapovirus (SV) are classified into the family *Caliciviridae* and are members of nonenveloped, positive-sense, single-stranded RNA viruses. Their genome contain approximately 7,300–8,300 nucleotides long, and a genome-linked protein (VPg) at the 5′ terminus and a poly A tail at the 3′ terminus [Bertolotti-Ciarlet et al., 2003]. Human astrovirus (HAstV) is a member of the family *Astroviridae*, and has a small (28–30 nm in diameter), round, nonenveloped characteristic. The genome is a positive-sense, single-stranded RNA of approximately 6,800 nucleotides in length [Schnagl et al., 2002].

NVs can be divided into five distinct genogroups based on the variation in the capsid gene sequences, in which strains belonging to GI, GII, and GIV are found in humans, whereas GIII and GV are found in cows and mice, respectively [Zheng et al., 2006]. Recently, NVs were classified into 8, 17, 1, 1, and 1 genotypes in GI, GII, GIII, GIV, and GV, respectively [Zheng et al., 2006]. SVs are divided into five genogroups (GI to GV) based on the

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difference of capsid gene sequences, in which GI, GII, GIV, and GV are found in humans, while GIII is found in porcine species [Farkas et al., 2004]. Recently, Akihara et al. [2005] reported that SV strains are classified further into 16 genetic clusters/genotypes (8 in GI, 5 in GII, 1 each in GIII, GIV, and GV) based on the differences of partial capsid amino acid sequences. HAstVs can be divided into eight distinct serotypes or genotypes based on the antigenic difference or variation in the capsid gene sequences: serotype/genotype 1 to 8, in which a high concordance between results of serotyping and genotyping was observed [Sakamoto et al., 2000].

In Thailand, epidemiological studies of NV, SV, and HAstV are less frequent than those of RV. The study conducted in Chiang Mai in 2000 and 2001 [Hansman et al., 2004] reported that NV infection in children hospitalized with diarrhea was 7.6%, while SV infection was 3.8%. One specimen (0.95%) was an NV/SV mixed infection. Later, during 2002 and 2003, Guntapong et al. [2004] reported 11 NV and 9 SV single infections, and 3 were NV/SV mixed infections from a total of 80 stool specimens collected from children hospitalized with acute gastroenteritis in 5 different geographical areas of Thailand (Sa Kaeo, Chanthaburi, Songkhla, Nong Khai, and Tak). For HAstV, the frequency of detection rate ranged from 8.6% to 14% in hospitalized children [Herrmann et al., 1991; Echeverria et al., 1994]. In 2004, a report from Bangkok demonstrated that HAstV was the cause of a neonatal gastroenteritis outbreak, which occurred in the nursery of a maternity ward at Ramathibodi Hospital. HAstV was detected in 4 of 13 (30.7%) diarrheic neonates and 1 member of the nursery staff who had diarrhea [Sirinavin et al., 2006].

This study reports the prevalence and molecular epidemiology of norovirus, sapovirus, and astrovirus infections in sporadic gastroenteritis among hospitalized children in Chiang Mai, Thailand from May 2000 to March 2002.

MATERIALS AND METHODS

Specimen Collection

Two hundred ninety-six fecal specimens were collected from children hospitalized with diarrhea in four different hospitals and one private clinic in Chiang Mai province between May 2000 and March 2002. The ages of the subjects ranged from neonate up to 5 years old.

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

Viral RNA was extracted from 10% fecal supernatant using the QIAamp viral RNA Mini Kit (Qiagen, Hilden, Germany). For RT, the viral RNA was reverse transcribed according to the manufacturer's instruction (Fermentas, Lithuania Glen Burnie, MD). The presence of NV (GI and GII), SV, and HAstV was detected by RT-PCR using the protocol described previously [Yan et al., 2003]. A forward primer, G1-SKF (nt 5,342–5,361) 5′-CTGCC-CGAATTYGTAAATGA-3′, was used in combination with the reverse primer, G1-SKR (nt 5,653–5,671) 5′-CCAACCCARCCATTRTACA-3′, for the amplification of NVGI, which specifically generated a PCR amplicon of 330 bp. For NVGII identification, a forward primer, COG2F (nt 5,003–5,028) 5′-CARGARBC-NATGTT-YAGRTGGATGAG-3′, was used in combination with the reverse primer, G2-SKR (nt 5,367–5,389) 5′-CCR-CCNGCATRHCCRTTRTACAT-3′, which generated a PCR product size of 387 bp. For SV detection, a 434 bp fragment was generated using the forward primer, SLV5317 (nt 5,083–5,105) 5′-CTCGCCACCTA-CRAW-GCTTGGTT-3′, and reverse primer, SLV5749 (nt 5,516–5,494) 5′-CGGRCYTCAAAVSTACCBCCC-CA-3′. For amplification of HAstV, a forward primer, PreCAP1 (nt 4,235–4,255) 5′-GGACTGCAAAGCAG-CTTCGTG-3′, was used in combination with the reverse primer, 82b (nt 4,934–4,953) 5′-GTGAGCCACCAGC-CATCCCT-3′, which generated a PCR product size of 719 bp. All of the positive samples were analyzed further for their genotypes by nucleotide sequence and phylogenetic analyses.

Sequence and Phylogenetic Analyses

The PCR products were purified by the QIAquick Gel Extraction Kit (Qiagen) and sequenced by using the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer-Applied Biosystems, Inc., Foster City, CA) on an automated DNA sequencer (ABI 3100; Perkin Elmer-Applied Biosystems, Inc.). The primers employed for amplification of the partial capsid genes were also used as sequencing primers. The nucleotide sequences obtained were translated into amino acid sequences using the GeneDoc program, and compared to those of NV (GI and GII), SV, and HAstV reference strains available in the GenBank using the BLAST program. The genotypes of NV (GI and GII) and SV were classified based on the recent capsid region classification scheme of Zheng et al. [2006] and Akihara et al. [2005], respectively. The serotypes/genotypes of HAstV were assigned in comparison with those of the reference strains by analysis of their amino acid sequences.

Nucleotide Sequence Accession Numbers

The partial nucleotide sequences of the capsid gene were deposited in GenBank under the accession number EU363852–EU363875 for NV strains, EU363876–EU363885 for SV strains, and EU363886–EU363892 for HAstV strains. The following capsid gene sequences of reference strains published in the GenBank were used in the phylogenetic analysis: NVGI: Boxer/01/US (AF538679), Chiba/00/JP (AB042808), DSV395 (U04469), Hesse (AF093797), Musgrove/89/UK (AJ277614), Norwalk/68 (M87661), Saitama T36GI/01/JP (AB112133), Southampton (L07418),

Winchester/94/UK (AJ277609); NVGII: Amsterdam/98-18/98/NET (AF195848), Bristol (X76716), CS-E1/02/US (AY502009), Erfurt/546/00/DE (AF427118), Fayetteville/98/US (AY113106), Hawaii (U07611), Hillingdon/90/UK (AJ277607), J23/99/US (AY130762), Leeds/90/UK (AJ277608), M7/99/US (AY130761), NongKhai-22/Thai (AY646866), NongKhai-51/Thai (AY646867), SaKaeo-14/Thai (AY646868), Seacroft/90/UK (AJ 277620), Snow Mountain virus (AY134748), Tak-62/Thai (AY646877), Tiffin/99/USA (AY502010), Toronto (U02030), VA97207/97 (AY038599), Wortley/90/UK (AJ277618); SV: 4408/Maizuru/JP (AB180209), 8/DCC/Tokyo/JP/44 (AB236377), Arg39 (AY289803), Chanthaburi-74/Thai (AY646854), Chiba/000671T/99 (AJ412805), Cruise/US (AY289804), Hou7-1181/90 (AF435814), Houston/90 (U95644), Karachi/874/92 (AB181129), Karachi/876/93 (AB181132), Karachi/877/90 (AB181133), Karachi/878/90 (AB181228), Karachi/938/94 (AB181248), Karachi/1021/92 (AB181230), Karachi/1026/92 (AB181134), London/92 (U95645), Lyon/598/97/F (AJ271056), Manchester/93 (X86560), Mex340/90 (AF435812), Moscow/2196/02/RF (AY 538722), Moscow/4536/02/RF (AY538716), NongKhai-24/Thai (AY646856), NongKhai-50/Thai (AY646853), PEC (Porcine Enteric Calicivirus) (AF182760), Sapporo/82/JP (U65427), Songkhla-6/Thai (AY646857), Stockholm/318/97/SE (AF194182), Tak-69/Thai (AY 646864).

RESULTS

Prevalence and Distribution of Norovirus, Sapovirus, and Human Astrovirus Infections

A total of 296 fecal specimens were collected from children hospitalized with diarrhea, 40 (13.5%) of which were positive for NV, SV, and HAstV. Of these, NV was detected in 24 (8.1%) of the fecal specimens tested. Seven of these (2.4%) were identified as GI genogroup and 17 (5.7%) as GII genogroup. Among the NVs detected, NVGII was more predominant (70.8%) than NVGI (29.2%). SV was detected in 10 (3.4%) specimens and one of these was positive for both NVGI and SV. In addition, HAstV was found in 7 (2.4%) of the specimens tested (Table I). NVGI, NVGII, SV, and HAstV were detected in 27 of 187 (14.4%) specimens in the first period (May 2000–April 2001) and 14 of 109 (12.8%) in the following period (May 2001–March 2002) (Table I). The monthly distribution of NVGI, NVGII, SV, and HAstV infections in children hospitalized with diarrhea is shown in Figure 1. In the year 2000, NVGII infection was detected at a high peak (~25%) in November. In 2001, infections of almost all NVGI, NVGII, SV, and HAstV tended to occur in the first 7 months of the year, except NVGII, which remained detectable in October. In January 2002, NVGI and GII were detected, while only NVGII was detected in February and HAstV in March. However, SV infection was not detected during this period (January–March 2002). The age of children at infection ranged from 4 months to 5 years old. Among those children, who were infected with NV, SV, or HAstV, 85% were 2 years of age and younger. When the children were grouped into 0–5, 6–11, 12–24, and >24 months of age, no significant difference in the rate of infection with NV, SV, or HAstV was observed among each age group.

Sequence and Phylogenetic Analyses of Noroviruses

All of the NV strains were characterized further for their genotypes by sequencing of the partial capsid genes. The genotypes were classified according to the classification scheme of [Zheng et al., 2006]. Six NVGI strains detected in this study were classified into four distinct GI genotypes as follows: 2 strains of GI/4, 2 strains of GI/6, and 1 strain each of GI/3, and GI/7. In addition, 1 NVGI strain, CMH308/01, could not be classified into any genotype based on the scheme described by Zheng, however, it belonged to GI/13, with a percentage deduced amino acid sequence identity of 100%, according to the classification scheme of [Okada et al., 2005] (Fig. 2).

All 17 NVGII strains were classified further into seven distinct GII genotypes, comprising GII/1, GII/3, GII/4, GII/6, GII/8, GII/10, and GII/15 (Fig. 2). NVGII/4 was found to be the most predominant genotype (9 of 17; 52.9%) of the NVGII strains detected in this study. The GII/3 and GII/10 genotypes were detected at an equally prevalent rate of 11.7% (2 of 17 strains). In addition, a relatively low detection frequency of GII/1, GII/6, GII/8, and GII/15 at 5.8% (1 of 17) was observed.

Sequence and Phylogenetic Analyses of Sapoviruses

A total of 10 SV strains were analyzed and classified further into two distinct genogroups, GI and GII,

TABLE I. The Prevalence of NVGI, NVGII, SV, and HAstV Detected in Children Hospitalized With Acute Gastroenteritis in Chiang Mai, Thailand, From May 2000 to March 2002

Date of specimen collection	No. of specimens tested	No. of specimens positive for virus (%)				
		NVGI	NVGII	SV	HAstV	Total (%)
May 2000–Apr 2001	187	5 (2.7)	11 (5.9)	8 (4.3)	3 (1.6)	27 (14.4)
May 2001–Mar 2002	109	2 (1.8)	6 (5.5)	2 (1.8)	4 (3.7)	14 (12.8)
Total (%)	296	7 (2.4)[a]	17 (5.7)	10 (3.4)[a]	7 (2.4)	40 (13.5)[a]

[a]One specimen was positive for both NVGI and SV.

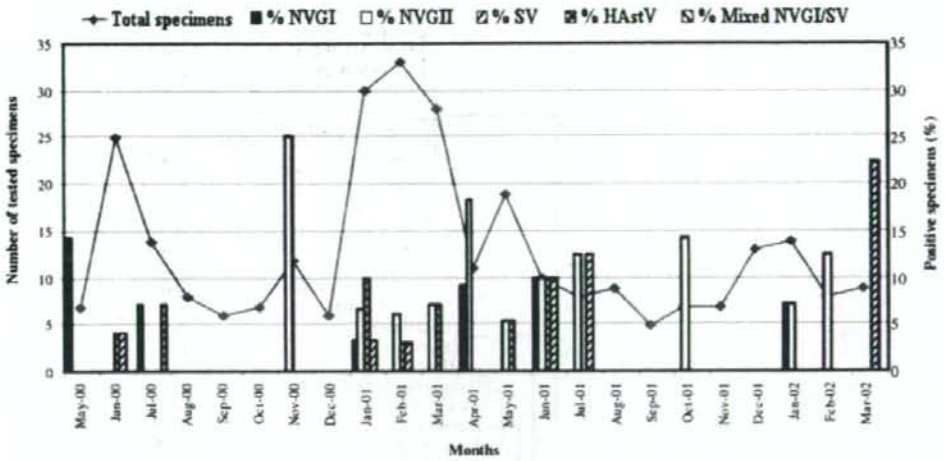

Fig. 1. Monthly distribution of NVGI, NVGII, SV, and HAstV infections among children hospitalized with diarrhea in Chiang Mai, Thailand, from May 2000 to March 2002 [number of tested specimens, —◆—; positive specimens (%), □].

according to the recent SV capsid region classification scheme described by Akihara et al. [2005]. It was observed that SVGI was a more common genogroup (80%) than the SVGII (20%) detected in this study. Eight strains of SVGI were classified further into three genotypes, that is, four strains were GI/1, three were GI/4, and one was GI/5. Moreover, two strains of SVGII were also classified into GII/1 and GII/2 genotypes (Fig. 3).

Sequence Analysis of Human Astroviruses

Seven HAstV strains detected in the present study, two of each isolate were HAstV-1, HAstV-2, HAstV-5, and one isolate was HAstV-3. The detection rate of the HAstV in children with acute gastroenteritis was rather low.

DISCUSSION

The present study describes the prevalence of NV and SV infection in children hospitalized with acute gastroenteritis in Chiang Mai city during May 2000–March 2002. The prevalent rates are in good agreement with those reported by Hansman et al. [2004] which also conducted the study in Chiang Mai during July 2000–July 2001. However, the prevalence of NV and SV are somewhat lower when compare to the follow-up study conducted recently in Chiang Mai from March 2002 to December 2004 [Khamrin et al., 2007] as well as to the study conducted in five other regions of Thailand by Guntapong et al. [2004]. The discrepancy of the prevalent rates between our study and others might be due to the difference in the duration and/or geographical area where those studies have been conducted.

Like other studies [Schnagl et al., 2000; Buesa et al., 2002; Oh et al., 2003; Boga et al., 2004; Hansman et al., 2004], the findings showed that NVGI strains are less common (29.2%) than NVGII (70.8%). It should be noted that in 2002 and 2003, NVGI disappeared completely from five other regions of Thailand [Guntapong et al., 2004]. In addition, NVGI was also undetectable in Chiang Mai area during 2002 and 2004 [Khamrin et al., 2007]. For NVGII, GII/4 has been reported as a major cause of global outbreaks and sporadic cases of gastroenteritis [Foley et al., 2001; White et al., 2002; Lau et al., 2004]. This study in Chiang Mai area found that GII/4 circulated as the most predominant genotype (37.5%), which is similar to those reported by Hansman et al. [2004]. However, a study conducted by Guntapong et al. [2004] in five other regions of Thailand during 2002 and 2003 reported a relatively high GII/4 at the incidence of 64.3%. Interestingly, in the following three consecutive years from 2002 to 2004 [Khamrin et al., 2007], GII/4 increased to 62.8% in Chiang Mai region, which similar to the finding of Guntapong et al. [2004]. For SV infection, SV genogroup I (GI) has been reported worldwide as the most predominant strain [Okada et al., 2002; Phan et al., 2004; Akihara et al., 2005; Phan et al., 2005, 2006]. SVGI/1 strains were previously reported as the most predominant genotype, followed by GII/1 strains and one isolate belonging to an intragenogroup recombinant strain in Chiang Mai during 2000 and 2001 [Hansman et al., 2004; Katayama et al., 2004]. In our study, SVGI was also detected at a very high incidence (80%), with GI/1 as the most predominant strain and followed by GI/4, GI/5, GII/1, and GII/2 strains. The study conducted in the other regions of Thailand by Guntapong et al. [2004] during 2002 and 2003 reported a higher detection rate of SV (15.0%). SVGI/1 was the most prevalent genotype, while the other two strains belonged to SVGV and a novel genotype in the SVGII

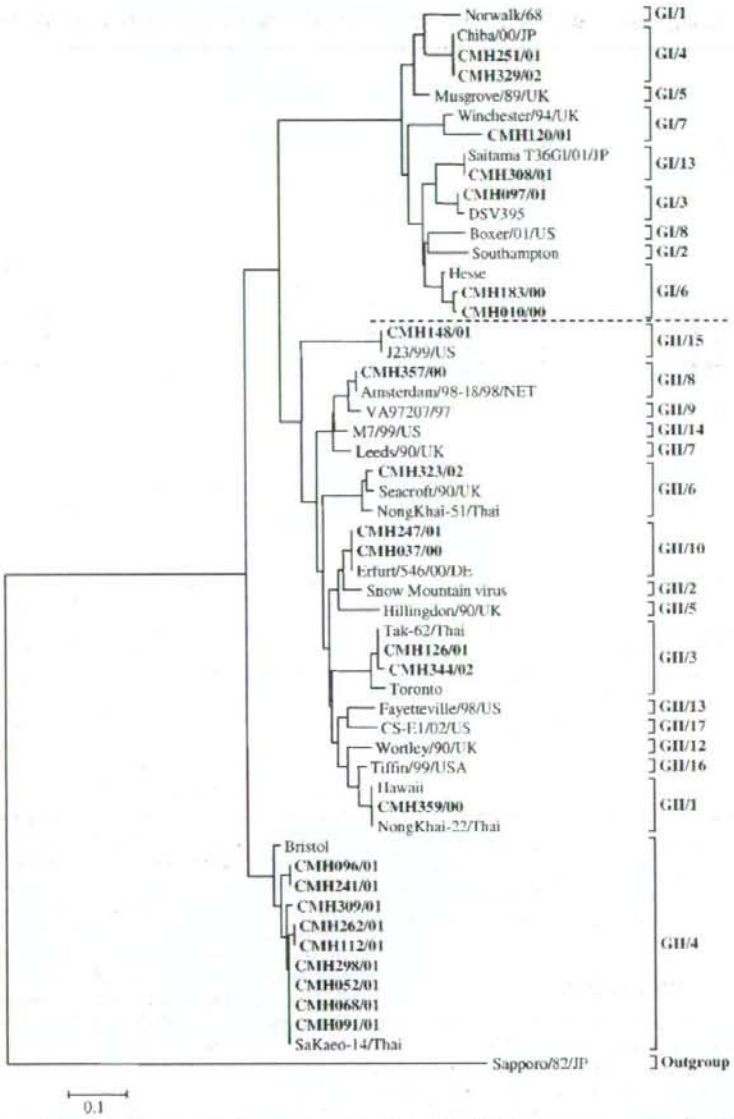

Fig. 2. Phylogenetic analysis of partial capsid deduced amino acid sequences of NVGI and GII strains detected between 2000 and 2002 in Chiang Mai, Thailand. The tree was constructed by multiple alignment of 7 NVGI and 17 NVGII positive sequences (indicated in boldface), 29 reference sequences, and 1 outgroup sequence. In the phylogenetic tree, NVGI strains were classified into eight distinct genotypes from 1 to 8 and NVGII strains were classified into sixteen distinct genotypes from 1 to 10 and 12 to 17 (excepting genotype 11 of porcine NVGII). Sapporo/82/JP was used as an outgroup strain for phylogenetic analysis. Bootstrap values are 1,000 replicates based on neighbor-joining and distance methods. Genotypes or genetic clusters are divided by brackets.

cluster, respectively. Another study in Chiang Mai region from 2002 to 2004 by Khamrin et al. [2007] reported a rather low detection rate of SV (1.2%) in which two strains belonged to SVGI genogroup (SVGI/1, SVGI/2) and other strain belonged to SVGIV genogroup. The data from our study and others [Guntapong et al., 2004; Khamrin et al., 2007] reveals that the detection rate, genogroup and genotype of NV and SV strains circulating in several regions of Thailand vary from time to time. However, the predominant NVGII/4 and SVGI persist in these regions over a number of years.

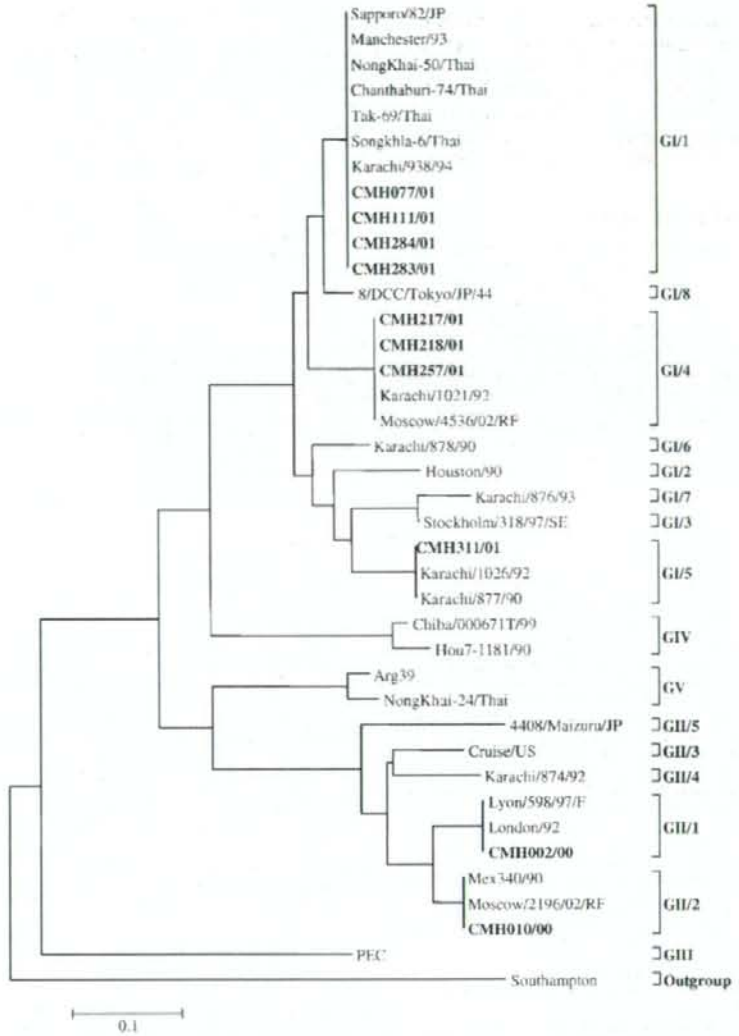

Fig. 3. Phylogenetic analysis of partial capsid deduced amino acid sequences of SV strains detected between 2000 and 2002 in Chiang Mai, Thailand. The tree was constructed by multiple alignment of 10 SV positive sequences (indicated in boldface), 27 reference sequences, and 1 outgroup sequence. In the phylogenetic tree, SV strains were classified into genogroup I, II, IV, and V. SVGI was classified further into eight genotypes and SVGII into five. SVGIII (Porcine enteric calicivirus, PEC) was also analyzed. Southampton was used as an outgroup strain for phylogenetic analysis. Bootstrap values are 1,000 replicates based on neighbor-joining and distance methods. Tree is unrooted. Genotypes or genetic clusters are divided by brackets.

Epidemiological data of HAstV as a causative agent of gastroenteritis in Thailand is rather limited. In 1991, Herrmann et al. first detected HAstV serotype 2 at 8.6% in children hospitalized with diarrhea in Bangkok. Later, Echeverria et al. [1994] reported the detection of HAstV at 14% in children hospitalized with diarrhea in Ratchburi province in the central part of Thailand. In 2004, Sirinavin et al. [2006] reported an outbreak of HAstV with a detection rate of 30.7% in neonates at a nursery in the maternity ward of Ramathibodi Hospital, Bangkok. However, the detection of HAstV infection in the previous studies based on serological assays [Herrmann et al., 1991; Echeverria et al., 1994; Sirinavin et al., 2006]. Our study is the first report that describes the distribution of HAstV genotypes circulating in Chiang Mai city. The data suggest that HAstV infection is less common in children with acute gastroenteritis compared to rotavirus and norovirus infection

in this area. These percentages are similar to those reported from other regions of the world such as Australia [Mustafa et al., 2000], Germany [Oh and Schreier, 2001], and Spain [Guix et al., 2002].

In conclusion, this study describes the genetic diversity of NV, SV, and HAstV genotypes cocirculating in children hospitalized with diarrhea in Chiang Mai, Thailand.

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Short communication

Statistical analysis of attack rate in norovirus foodborne outbreaks

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Abstract

Norovirus (NoV), which causes foodborne gastroenteritis outbreaks, is one of the important viruses in public health. We statistically analyzed the attack rate in foodborne outbreaks caused by NoV. The attack rate in 95 oyster-associated outbreaks was significantly higher than that in 195 food handler-associated outbreaks ($P=0.007$). The difference in the number of NoV genotypes implicated is considered to be an important factor for this difference. The attack rate in 20 outbreaks associated only with GII/3 was higher than that in 143 other outbreaks ($P=0.247$), while the attack rate in 27 outbreaks associated only with GII/4 was lower than that in 136 other outbreaks ($P=0.004$), suggesting that GII/4 NoVs cause asymptomatic infection more frequently than do other NoV genotypes. Our results suggest that differences in implicated foods, susceptibility of the host to NoV infection, and pathogenicity of NoVs may influence the attack rate in NoV foodborne outbreaks.

Keywords: Attack rate; Foodborne outbreaks; Food handler; GII/4; Norovirus; Oyster; Statistical analysis

1. Introduction

In recent years, viruses have been increasingly recognized as important causes of outbreaks of foodborne disease (Fleet et al., 2000; Graczyk and Schwab, 2000; Koopmans and Duizer, 2004; Parashar and Monroe, 2001). Norovirus (NoV) is currently recognized as the most important foodborne virus, which causes gastroenteritis outbreaks. The foods affected can be classified into two distinct groups based on the route of contamination: one group includes bivalve shellfishes such as oysters, which are contaminated with NoV in their sea life (Boxman et al., 2006; Cheng et al., 2005; Nishida et al., 2003; Nishida et al., 2007; Saito et al., 2006; Ueki et al., 2004, 2005), and the other group includes various kinds of foods other than bivalve shellfishes, which are secondarily contaminated with NoV from infected food handlers during food processing and/or food serving. Despite the fact that oysters are the most important issue for the prevention and control of NoV in foods, there is no virological standard for oysters intended for raw consumption in Japan and other countries (European Commission Health & Consumer Protection Directorate-General, 2002; Nishida et al., 2003; Nishida et al., 2007). Although risk analysis based on scientific data must be performed before setting the virological standard, there is a lack of scientific data on attack rate, which are required to calculate the minimum virus amount needed for infection when oysters are eaten, as well as a lack of data on the numbers of infectious NoV particles in oysters involved in foodborne outbreaks. Although the infectious dose of NoV is estimated to be about 10 particles at least (URL: http://www.cdc.gov/ncidod/dvrd/revb/gastro/norovirus-factsheet.htm), the number is not necessarily equal to that in outbreaks associated with oyster consumption and might depend on each individual or each virus strain. The attack rate may be influenced by factors other than amount of infectious virus particles ingested, such as host susceptibility and virus pathogenicity. In an initial investigation to obtain data that can be used for risk analysis for the prevention and control of NoV in food, we statistically compared the attack rates in oyster-associated outbreaks and food handler-associated outbreaks and the attack rates in outbreaks caused by different NoV genotypes.

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2. Materials and methods

2.1. Subjects

A total of 290 foodborne outbreaks that occurred between April 2001 and January 2005 in various areas of Japan were subject to statistical analysis. In all outbreaks, NoVs were detected by reverse-transcription, (nested) PCR and/or real-time PCR commonly performed in Japan (Kageyama et al., 2003; Kojima et al., 2002; Nishida et al., 2003; Nishida et al., 2007; Ueki et al., 2004; Ueki et al., 2005) with or without some modifications depending on laboratories, and NoVs were concluded to be the causal agent responsible for each outbreak. Among the 290 outbreaks, NoVs were genotyped in 163 outbreaks after partial sequencing of the capsid region (Nishida et al., 2003; Nishida et al., 2007). In most cases, sequencing was performed directly without cloning of the PCR products. Some PCR products, most of which were amplified from oyster samples, were cloned before sequencing because oysters may include various NoV strains. Phylogenetic analysis was performed for genotyping using the reference strains reported by Katayama et al. (Katayama, 2004).

2.2. Statistical analysis

We statistically compared the attack rate (ratio of the number of patients with symptoms such as nausea, vomiting, diarrhea, abdominal pain, and/or fever to the number of the individuals that have eaten foods suspected as being responsible for each outbreak) and the number of the involved patients in 95 oyster-associated outbreaks and 195 food handler-associated outbreaks by using the Mann–Whitney U test. The oyster-associated outbreaks included not only outbreaks in which NoV was detected from implicated oysters but also outbreaks in which oysters were included in the menu suspected as the food vehicle without the detection of NoV from the oysters. Food handler-associated outbreaks included outbreaks in which NoV was detected from food handlers in the facilities that were suspected as being responsible for the foodborne outbreaks. In some food handler-associated outbreaks, the food vehicle was not determined.

In 163 outbreaks in which NoVs were detected and genotyped, GII/4 and GII/3 were the genotypes most frequently associated with foodborne outbreaks (Table 1). We therefore compared the attack rates in outbreaks in which only GII/4 was detected (27 cases) and other outbreaks (136 cases) and the attack rates in outbreaks in which only GII/3 was detected (20 cases) and other outbreaks (143 cases) by using the Mann–Whitney U test.

3. Results

3.1. Comparison of oyster-associated outbreaks and food handler-associated outbreaks

The median attack rates were 58.3% in the 95 oyster-associated outbreaks and 47.2% in the 195 food handler-associated outbreaks (Table 2), the difference being statistically significant ($P=0.007$). This result indicates that the attack rate in oyster-associated outbreaks is higher than that in food handler-associated outbreaks. The median numbers of patients were 17 in the oyster-associated outbreaks and 40 in the food handler-associated outbreaks, indicating that the scale of food handler-associated outbreaks tends to be larger than that of oyster-associated outbreaks, though there was no statistical difference between them (Table 3).

Table 1
Number of foodborne outbreaks by NoV genotypes

Genotype	Cases detected single genotype		Cases detected two or more genotypes		Total
	Oyster-associated outbreaks	Food handler-associated outbreaks	Oyster-associated outbreaks	Food handler-associated outbreaks	
GII 3	4	16	20	6	46
GII 4	4	23	12	5	44
GII 5	2	3	17	5	27
GI 4	3	4	12	3	22
GII 12	1	2	9	3	15
GII 6	1	6	5	2	14
GI 8	0	1	7	3	11
GI 7	1	1	8	0	10
GII 15	0	2	8	0	10
GI 1	0	1	5	3	9
GII 14	0	0	8	1	9
GI 2	1	2	4	1	8
GII 2	1	6	0	1	8
GII 1	0	2	2	3	7
GII 8	0	2	3	1	6
GI 13	0	0	5	0	5
GI 11	0	0	2	2	4
GI 12	0	0	3	1	4
GI 14	0	0	4	0	4
GI 5	1	0	3	0	4
GI 9	0	0	3	0	3
GII 10	0	1	0	1	2
GII 11	0	0	2	0	2
Others	1	0	4	0	5

3.2. Comparison between different genome types of NoVs

We compared the attack rates in outbreaks caused by different NoV genotypes. The median attack rates were 41% in the 27 outbreaks in which only GII/4 was detected and 56.9% in the other 136 outbreaks (Table 4), the difference being statistically significant ($P=0.004$). This result indicates that the attack rate in GII/4 cases is lower than that in other NoV genotype cases. On the other hand, the median attack rates were 64.8% in the 20 outbreaks in which only GII/3 was detected and 53.2% in the other outbreaks ($P=0.247$), indicating that the attack rate in GII/3 cases is higher than that in other NoV genotype cases (Table 5).

4. Discussion

In this study, we showed that the attack rate in oyster-associated outbreaks was significantly higher than that in food