Norovirus and Sapovirus Infections among Children with Acute Gastroenteritis in Ho Chi Minh City during 2005–2006

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Summary

A molecular epidemiological study on common diarrheal viruses was conducted in a children’s hospital in Ho Chi Minh City between December 2005 and November 2006. Fecal samples were collected from 502 pediatric patients with acute gastroenteritis, and were screened for the presence of norovirus (NoV) and sapovirus (SaV). NoVs GII and SaVs were detected in 6.4% and 1.2% specimens, respectively, while there was no NoV GI found among studied samples. NoVs could be identified through the year, except in April and July, with the peak of detection rate (62.5%) during the rainy season. Conversely, four out of six (66.7%) of the SaV strains were identified during the dry season. Patients aged between 6 and 23 months were found to be more infected by NoVs. The overall mean severity score of norovirus-positive patients was 9.8, and no significant difference of severity scores among patients belonged to different age groups, gender and place of living. The results of phylogenetic analysis showed the diversity of caliciviruses circulating in the area, and various types of recombination were identified among NoVs and SaVs detected. These results provide important information on calicivirus infections among Vietnamese children.

Key words: norovirus, sapovirus, clinical manifestations, recombinant, Vietnam.

Introduction

Norovirus (NoV) and sapovirus (SaV) are members of the family Caliciviridae (other two genera are Lagovirus and Vesivirus). The NoV and SaV strains are determined as the major causes of non-bacterial acute gastroenteritis in infants and young children [1, 2].

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After being discovered through electron microscope in 1972 [3], NoVs were identified widely in epidemiological studies, and were the cause of outbreaks of gastroenteritis in various settings including hospitals [4, 5], schools [6, 7], cruise ships [8, 9], restaurants [10, 11] and day care centers [12, 13]. Sequence analyses of worldwide NoVs revealed that they are classified into seven distinct genogroups (GI to GVII), of these, GI, GII, GIV, GVI and GVII are known to infect humans [14]. NoV contains a positive-sense single-stranded RNA genome surrounded by an icosahedral capsid. The NoV genome contains three open reading frames (ORFs). The ORF1 encodes non-structural proteins, including NTPase, protease and RNA-dependent RNA polymerase (RdRp), OR2 encodes the capsid protein (VP1) and ORF3 encodes a minor structural protein (VP2).

SaV infects both children and adults, and have been found to cause outbreaks of gastroenteritis in kindergarten [15], hospital [16] and mental health care facility [17]. SaV- associated diarrhea is usually mild, compared to that caused by NoVs [18].
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

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 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target virus</th>
<th>Polarity</th>
<th>Sequence position (5’ to 3’): reference strain</th>
<th>Amplicon size</th>
<th>Target region</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1SKF</td>
<td>NoV GI</td>
<td>+</td>
<td>5342-5361 (Norwalk/68)</td>
<td>330bp</td>
<td>Polymerase and capsid junction</td>
</tr>
<tr>
<td>G1SKR</td>
<td>NoV GI</td>
<td>–</td>
<td>5653-5671 (Norwalk/68)</td>
<td>387bp</td>
<td>Polymerase and capsid junction</td>
</tr>
<tr>
<td>COG2F</td>
<td>NoV GII</td>
<td>+</td>
<td>5003-5028 (Lordsdale)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2SKR</td>
<td>NoV GII</td>
<td>–</td>
<td>5367-5389 (Lordsdale)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLV5317</td>
<td>SaV</td>
<td>+</td>
<td>5083-5105 (Manchester)</td>
<td>434bp</td>
<td>Polymerase and capsid junction</td>
</tr>
<tr>
<td>SLV5719</td>
<td>SaV</td>
<td>–</td>
<td>5494-5516 (Manchester)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NoV GI, norovirus GI; NoV GII, norovirus GII; SaV, sapovirus.
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 ≥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
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 bore 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 branch 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.
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, bore 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,
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).
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.
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 Finnish 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 is 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.

References

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