Molecular Epidemiology of Genogroup II Norovirus Infection Among Hospitalized Children With Acute Gastroenteritis in Suzhou (Jiangsu, China) From 2010 to 2013

Jian-Guang Fu, Jing Ai, Jun Zhang, Jing-Bin Wu, Xian Qi, Hong Ji, Miao Jin, Cheng Liu, Shen-Jiao Wang, Jun Shan, Chang-Jun Bao, Fen-Yang Tang, and Ye-Fei Zhu

1Key Lab of Enteric Pathogenic Microbiology, Ministry of Health, Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China
2Suzhou Center for Disease Control and Prevention, Suzhou, China
3Soochow University Affiliated Children’s Hospital, Suzhou, China
4Institute for Viral Diseases Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China
5The Second Affiliated Hospital of Nanjing Medical University, Nanjing, China

Noroviruses (NoVs) are the most common cause of acute gastroenteritis in both sporadic and outbreak cases. Genotyping and recombination analyses were performed in order to help getting more knowledge of the distribution and genetic diversity of NoVs in Suzhou, located in Jiangsu province of China. All stool samples were collected from hospitalized children younger than 5 years old with acute gastroenteritis. For genotyping, the open reading frame (ORF) 1 and ORF2 were partially amplified and sequenced. 26.9% of stool samples were positive for genogroup II NoVs. The most common genotype was GII.4 and its variants included Den Haag-2006b, New Orleans-2009, and Sydney-2012. The Den Haag-2006b variants predominated during 2010–2012. In 2013, it was replaced by the Sydney-2012 variant. The second most common genotype was GII.12/GII.3. NoVs could be detected throughout the year, with GII.4 and GII.12/GII.3 coexisting during the cold months, and GII.4 was the main genotype during the warm months. The highest prevalence of NoV was detected in young children aged <24 months. Patients infected with GII.4 had a higher chance of getting moderate fever than other NoV-positive patients, while those infected with GII.12/GII.3 tended to have a mild degree of fever. NoV is an important pathogen responsible for viral gastroenteritis among children in Suzhou. Analyses of NoV circulating between 2010 and 2013 revealed a change of predominant variant of NoV GII.4 in each epidemic season and intergenotype recombinant strains represented an important part.

KEY WORDS: norovirus; genogroup II; molecular epidemiology

INTRODUCTION

Norovirus (NoV) is a major etiologic agent responsible for non-bacterial sporadic and epidemic gastroenteritis worldwide in humans [Patel et al., 2008; Glass et al., 2009; Ahmed et al., 2014]. It has been estimated that every year NoV causes 200,000 deaths in children <5 years of age in developing countries [Patel et al., 2008]. NoVs are of concern given the significant burden it places on public health, particularly in hospitals, schools and aged-care facilities.

NoVs are members of the Caliciviridae family and contain an ~7.5-kb single-stranded, positive-polarity RNA genome. NoV can be divided into six genogroups (GI–GVI) and only genogroup I, II, and IV have been...
found to infect humans [Caddy et al., 2014]. The human NoV genome contains three open reading frames (ORFs): ORF1 codes for six non-structural proteins including the viral RNA-dependent RNA polymerase (RdRp), while ORF2 and ORF3 encode the major capsid proteins VP1 and VP2, respectively [Zheng et al., 2006]. At least 39 ORF2-based genotypes have been described so far, however, genotype GII.4 has emerged as the dominant strain worldwide, responsible for 70–80% of sporadic NoV infections during the last 20 years [Siebenga et al., 2009; Hoa Tran et al., 2013]. Epidemic patterns of NoVs in human populations include epidemic outbreaks of disease every 2–3 years, punctuated by the emergence of an antigenically distinct GII.4 strain that appears to escape human herd immunity to the previous circulating strains [Siebenga et al., 2010; Vega et al., 2011; Lindesmith et al., 2012].

Genotyping is generally carried out by partial sequencing of the RdRP and the major structural capsid protein VP1 [Bull et al., 2006]. However, the majority of intergenotype and intragenotype recombination in NoV occur at a single location, the ORF1-ORF2 overlap, which is also the transcription start site for the viral subgenomic RNA [Bull et al., 2007]. Recombination events within a genogroup are common. Thus, genotyping of NoV should ideally be based on sequencing of the capsid and polymerase regions of the viral genome.

Our epidemiological surveillance was initiated in January 2010 to monitor potential variations of strains over time, in terms of frequency and diversity of NoV genotypes, particularly the potential emergence of new GII.4 variants. This study described and analyzed data of the epidemiological surveillance of NoV infection of children in Suzhou (Jiangsu, China) between 2010 to 2013.

**MATERIALS AND METHODS**

**Stool Specimens**

Fecal specimens were collected from hospitalized children with acute gastroenteritis in Suzhou Children’s Hospital during the period from January 2010 to December 2013 in the Suzhou district, Jiangsu province, China. Acute gastroenteritis was defined as follow: at least three diarrheic stools and/or vomiting per day, which is caused by bacteria, or viruses, or fungi and parasites, but cholera, dysentery, typhoid, and paratyphoid are not included. The age of the participants ranged from 0 month to 59 months. The number of monthly samples was not less than 25. All stool specimens were stored at −80°C.

**Sample Preparation, RNA Extraction, and Detection of NoV GII RNA**

A 10% (wt/vol) stool suspension with a total volume of 1 ml was prepared in RNase-free water and centrifuged for 5 min at 2,370g. Viral RNA was extracted from the supernatant by using a MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Viral RNA was tested for GII NoVs by using the QIAGEN Probe RT-PCR Kit (Qiagen, Hilden, Germany) on a 7,500 Real Time PCR platform (Applied Biosystems, Singapore) as described previously [Trujillo et al., 2006].

**Genotyping**

In an effort to identify NoV genotypes, samples were analyzed using RT-PCR directed at region A of the polymerase gene (ORF1, 319 bp) and using the primers p289IUB/p290IUB and G2SKF/G2SKR at region C of the capsid gene (ORF2, 344 bp), respectively [Kojima et al., 2002; Puustinen et al., 2012]. RT-PCR was performed using the QIAGEN One step RT-PCR Kit (Qiagen) according to manufacturer’s instructions and were sent to the Sangon Biotech (Shanghai, China) Company for sequencing.

**Recombinant Norovirus Detection**

Potential recombinants were investigated by amplifying a region of 1,095 bp in the ORF1/ORF2 junction of the viral genome using the primers p290IUB [Puustinen et al., 2012] and G2SKR [Kojima et al., 2002]. RT-PCR was performed as described above. The PCR products were purified and were sent to the Sangon Biotech Company for sequencing. Sequences representative of the main variants of the recombinant strains detected in this study were deposited in GenBank (accession numbers KR093991-KR094008).

**Phylogenetic Analysis**

Genotyping of NoV GII strains was conducted by phylogenetic analysis of the polymerase gene and capsid gene, using the neighbor joining algorithm with 1,000 bootstrap replicates and a Kimura2-parameter model in MEGA 5.1 [Tamura et al., 2011]. Recombinant sequence data was analyzed for signs of recombination using Simplot analysis [Lole et al., 1999].

**Statistical Analyses**

Data were typed into a database and analyzed using SPSS software version 17.0 (SPSS, Chicago, IL). Categorical variables were compared by Pearson χ² test, Fisher’s Exact Test or Linear-by-Linear. Comparison between the two groups pairwisely was calculated by Bonferroni correction method. P values <0.05 were considered statistically significant.

**RESULTS**

From January 2010 to December 2013, a total of 1,682 specimens of children <5 years old with acute gastroenteritis were collected (413 in 2010, 419 in 2011, 431 in 2012, and 419 in 2013). Four hundred and
fifty-four (26.9%) were positive for the presence of NoV by real time RT-PCR. This study conducted further genotyping of NoV-positive samples, and the genotype of the capsid and/or polymerase was obtained in 375 samples.

Genetic Analysis of NoVs Genogroup II

**Genotypes.** The genotype was obtained from two genes (RdRp and capsid) in 332 specimens (83.0%), from the polymerase gene alone in 9 (2.0%) and from the capsid alone in 34 (7.5%). The strains were grouped into four different genotypes, with 241 strains in GII.4 genotype (53.3% of the strains genotyped in both genes), 81 strains in GII.12/GII.3 genotype (17.9%), 10 strains in GII.other genotype (2.2%). In 43 cases (9.5%), the genotype for region A or C could not be determined (Table I). Among GII.4, the Den Haag-2006b (GII.4-2006b) strain remained the most common genotype (185/452, 40.9%), followed by GII.4-Sydney-2012 (GII.4-2012) strain (35/452, 7.7%), and GII.4-New Orleans-2009 (GII.4-2009) strain (21/452, 4.4%).

**Recombinant strains.** The phylogenetic tree was constructed based on partial RdRp gene and capsid gene using neighbor joining method (Fig. 1a and b). Of the total number of strains genotyped with two genes, 19.5% (87/452) corresponded to suspected intergenotype recombinant strains (genotypes of a discordant capsid and polymerase). Most of the recombinant strains were GII.12/GII.3 (n = 81, 17.9%), followed by GII.4-2006b/GII.3 (n = 3, 0.7%), GII.b/GII.3 (n = 2, 0.4%), and GII.b/GII.13 (n = 1, 0.2%). Recombinant strains were separated into three groups in RdRp region: GII.12, GII.4-2006b, and GII.b, while in the capsid region were mainly concentrated in GII.3 group. The identities among the strains of GII.12/GII.3, GII.4-2006b, and GII.b were 96.2–100%, 96.2–99.4%, 93.5–100% in RdRp gene, respectively. The GII.12/GII.3 strains were segregated into two and three distinct genetic groups in RdRp and capsid gene in this study, respectively. In RdRp gene, the GII.12/GII.3 strains in 2012 and 2013 formed Cluster I and the strains formed Cluster II in 2010 and 2011. With respect to the capsid gene, the GII.3 strains from GII.12/GII.3 in 2012 and 2013 and GII.2006b/GII.3 in 2012 formed Cluster I, GII.b/GII.3 and GII.2006b/GII.3 in 2011 formed Cluster II, GII.12/GII.3 in 2010 and 2011 formed Cluster III. The identity of nucleotide sequences of GII.12/GII.3 isolates between cluster I and II in RdRp gene was 96.2–96.9%, while the identity of nucleotide sequence among cluster I, II, and III in capsid gene was 94.9–97.1% (cluster I and II), 94.9–95.8% (cluster II and III) and 91.7–94.9% (cluster I and III), respectively.

**Time trend.** The most common two genotypes, GII.4-2006b and GII.12/GII.3 were found in 2010–2012.

<p>| Table I. Total Number of Norovirus Genogroup II Detected in Suzhou (Jiangsu, China), 2010–2013 |</p>
<table>
<thead>
<tr>
<th>Genotype</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GII.4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2006b (GII.4)</td>
<td>85</td>
<td>45</td>
<td>54</td>
<td>1</td>
<td>185</td>
</tr>
<tr>
<td>2009 (GII.4)</td>
<td>2</td>
<td>14</td>
<td>5</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>2012 (GII.4)</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>GII.12/3</td>
<td>17</td>
<td>28</td>
<td>17</td>
<td>19</td>
<td>81</td>
</tr>
<tr>
<td>GII.other</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>GII.2 (GII.other)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>GII.6 (GII.other)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>GII.b/GII.13 (GII.other)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>GII.b/GII.3 (GII.other)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>GII.4-2006b/GII.3 (GII.other)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>GII.NT (A or C)</td>
<td>23</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>43</td>
</tr>
<tr>
<td>GII.b/N (GII.NT-C)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>GII.12/N (GII.NT-C)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>GII.4-2006b/N (GII.NT-C)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>GII.4-2006b/N (GII.NT-C)</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>GII.4-2012/N (GII.NT-C)</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>GII.NT/GII.12 (GII.NT-A)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>GII.NT/GII.2 (GII.NT-A)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>GII.NT/GII.4-2006b (GII.NT-A)</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>GII.NT/GII.4-2009 (GII.NT-A)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>GII.NT/GII.6 (GII.NT-A)</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>GII.NT/GII.6 (GII.NT-A)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GII.NT/GII.7 (GII.NT-A)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GII.NT/GII.12 (GII.NT-A)</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GII.NT (A and C)</td>
<td>32</td>
<td>28</td>
<td>10</td>
<td>7</td>
<td>77</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>164</td>
<td>122</td>
<td>106</td>
<td>60</td>
<td>452</td>
</tr>
</tbody>
</table>

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*a53.1% here means 87 samples were tested GII.4 positive out of 164 (last line) NoV positive samples in 2010.
*b53.3% here means 241 samples were tested GII.4 positive out of 452 (last line) NoV positive samples between 2010 and 2013.
*c97.7% here means 85 samples were tested GII.4-2006b positive out of 87 (first line) GII.4 NoV positive samples in 2010.
*d76.8% here means 185 samples were tested GII.4-2006b positive out of 241 (first line) GII.4 NoV positive samples between 2010 and 2013.
*eThe genotype for region C could not be determined.
*fThe genotype for region A could not be determined.

Fu et al.
After August of 2012, strains of the new Sydney-2012 variant were detected and were the main circulating strains in October. From October 2012 to December 2013, the GII.12/GII.3 and GII.4-2012 had replaced GII.4-2006b and became the predominant genotypes.

According to the characteristics of the climate in China, 1 year is divided into four seasons, including spring (March–May), summer (June–August), fall (September–November) and winter (December–February). Different constitute ratio was shown in the distribution of NoV genotypes by season (Table II, Fisher’s Exact \( P = 0.000 \)). GII.12/GII.3 was the main genotype in winter (16/32), while GII.4 was the main genotype in the remaining three seasons.

**Clinical aspects.** Of the 332 NoV-positive samples genotyped with both genes, 212 were isolated from males and 120 were isolated from females, resulting in a male-to-female ratio of approximately 1.77:1 and considerably higher (2.52:1) with respect to GII.12/GII.3 (Table II). Of the 332 children with a genotype confirmed, the highest proportion of detection was in young children aged <24 months (93.7%, 311/322). Comparison between the two groups pairwise was performed by Bonferroni correction method. Analysis result showed that there was a statistical difference between 0–11 months and 12–23 months group in genotype distribution (\( \chi^2 = 22.852, P = 0.000 \)). GII.4 (87.5%) was the most common genotype accounted for the highest constituent ratio among children aged 12–23 months. However, GII.12/GII.3 (33.2%) showed higher constituent ratio in age group 0–11 months than 12–23 months (8.9%). The clinical feature of NoV that were detected in acute gastroenteritis patients, were diarrhea, vomiting and fever. Among 277 patients infected with NoV, 38.6% (107/277) reported only diarrhea, 5.4% (15/277) only vomiting, 56.0% (155/277) diarrhea and vomiting, and 23.1% (72/277) fever. Table II showed that of 72 patients with fever, 30 experienced mild fever (37.5–38.2°C) while the remaining 42 patients experienced moderate fever (≥38.3°C). There was a difference in patients presenting with mild fever and moderate fever among the GII.4 and GII.12/GII.3 case group. GII.4 patients presented a higher ratio of moderate fever (66.04%, 35/53) than other NoV-positive patients, while the result of GII.12/GII.3 suggested that it tends to be mild (41.18%, 7/17).

The statistic values with * were calculated by using Bonferroni correction method in the comparison between the 0–11 months and 12–23 months group. The other statistic values were calculated by using Fisher’s Exact Test.

**DISCUSSION**

In this study, NoV was detected in 26.9% of children with diarrhea, indicating that NoV is an important cause of sporadic acute gastroenteritis in children. However, the number of the NoV positive samples in 2013 was obviously different than those in the previous 3 years. As for the reason, the total detection rate of Genogroup II NoV in 2013 in China was only 14.8% (data unknown) and Jiangsu had a similar rate, which was significantly lower than that in 2012. In addition, from January 2010 to December 2013, only 10 specimens were positive for the presence of Genogroup I NoV by real time RT PCR (data not shown). Most genogroup II NoV strains detected in this study belonged to the genotypes GII.4 and GII.3 (53.3% and 17.9%, respectively), which were the most prevalent strains globally in children with sporadic acute gastroenteritis [Siebenga et al., 2009; Boon et al., 2011; Cho et al., 2014; Yu et al., 2014]. GII.4-2006b was the most predominant NoV variant from 2010 to 2012, which was rapidly replaced by Sydney-2012 variant in 2013, consistent with the worldwide epidemic [Eden et al., 2013; van Beek et al., 2013; Allen et al., 2014]. GII.4-2009 was only detected to a low rate in 2011 and 2012 in Suzou.

Naturally occurring recombination events are common in NoVs and the most common recombination site is the ORF1–ORF2 junction localized upstream of the capsid gene [Bull et al., 2007; Eden et al., 2013; Mans et al., 2014]. In the present study, the proportion of recombinant strains was high (19.5%). Because of different sensitivity of PCRs to different regions, some samples were positive only in ORF1 or ORF2 gene by RT PCR, and it was difficult to determine whether they were recombinant strains or not. We found 92.0% of the recombinants in our study consisted of a combination of GII.12/GII.3. Interestingly, most GII.3 NoVs detected in the 2000s were recombinant strains, which possessed a non-GII.3 RdRp genotype [Bull et al., 2007; Phan et al., 2007; Chhabra et al., 2009]. According to the present study, the GII.3 capsid gene was associated with three different RdRp genotypes: GII.12, GII.b, and GII.4-2006b. Genotype GII.b/GII.3 recombinant strains were a major cause of pediatric disease worldwide [Bruggink and Marshall, 2009; Chhabra et al., 2009]. In contrast, the recombinant GII.12/GII.3 strain appeared to be restricted to a specific geographic region, including China, Japan and South Korea [Phan et al., 2006; Han et al., 2011; Zeng et al., 2011]. China CDC reported that the prevalence of GII.12/GII.3 was second to GII.4 2006b and the recombinant was detected in several provinces [Jin et al., 2008]. The results of the recombinant strain showed that recombinant strains with a different ORF1 were associated with different GII.3 capsid sequences (Fig. 1a and b). This is consistent with the previous study that the acquisition of new RdRps may lead to a faster mutation rate and increased genetic diversity [Mahar et al., 2013]. GII.12/GII.3 strains ranked only second to GII.4 2006b, and the recombinant GII.3 strains appeared to be restricted to a specific geographic region, including China, Japan and South Korea [Phan et al., 2006; Han et al., 2011; Zeng et al., 2011].
P <2 years [Nataraju et al., 2011; Zeng et al., 2011; Oldak et al., 2012]. Analysis result showed that GII.12/GII.3 in the age group of 0–11 months had a higher constituent ratio than 12–23 months, and GII.4 had absolute predominance in the age group of 12–23 months. Whether NoV genotype is linked to age group in children is unclear. The number of hospitalized males and NoV infected males was obviously more than that in females in this study, which might indicate that males are more susceptible to NoV infection. NoV was detected throughout the year, but GII.4 and GII.12/GII.3 dominated together during the cold months and GII.4 became the predominant genotypes during the warm months (Table II). Although the result of 72 patients presenting with fever showed that GII.4-2006b patients and GII.12/GII.3 patients were different, there were so few cases on the fever presentation of GII.4-2012 infections that it was difficult to tell their relationships. More clinical studies are necessary to conclusively demonstrate the different clinical characteristics associated with infection with different NoV genotypes.

In conclusion, the present study documents the genetic diversity of the genogroup II NoV circulating in the Suzhou. Similar to findings worldwide, GII.4-2006b was found to be the predominant NoV genotype from 2010 to 2012, and was replaced by GII.4 Sydney in 2013. However, recombinant GII.12/GII.3 strains were considered as one of significant pediatric pathogens, highlighting the role of recombination in the evolution of NoVs. The data presented here highlights the importance of combined polymerase- and capsid-based NoV genotyping, and more in-depth studies of NoV epidemiology extended to more regions of Jiangsu are warranted, to better assess the prevalence of NoV strains responsible for acute gastroenteritis.

ACKNOWLEDGMENT
The authors would like to thank Dr. Qing Shen from Karolinska Institutet, Stockholm, Sweden for modifying our manuscript.

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