Original Article

Prevalence and Genetic Diversity of Norovirus in Outpatient Children with Acute Diarrhea in Shanghai, China

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SUMMARY: This study aimed to investigate the epidemiology of norovirus (NoV) associated-diarrhea among pediatric outpatients in Shanghai, and characterize the genotypes of circulating NoV strain. Stool samples were collected from 910 children with non-dysenteric diarrhea between August 2008 and July 2009. One-step real-time RT-PCR was used to screen for NoV genogroup I (GI) and genogroup II (GII). Genotypes were classified by sequence analysis of partial capsid and RNA-dependent RNA polymerase (RdRp) fragments. NoV was detected year round with high activity in July, August, September, and October. Of 910 specimens, 165 (18.13%) were positive for NoV; 4 (2.42%) were GI, and 161 (97.58%) were GII. Based on capsid sequences, 8 different genotypes were identified for 114 NoV strains, including GII.4 2006b (57.89%), GII.3 (30.70%), GII.6 (4.39%), GII.12 (3.51%), GII.14, GI.2, GI.4, and GI.5 (0.88% each). Based on the RdRp sequences of 86 NoV strains, NoV was genotyped as GII.4 (62.79%), GII.12 (30.23%), GI.g (2.33%), GII.2 (1.16%), GII.6 (1.16%), GII.7 (1.16%), and GI.4 (1.16%). The RdRp genotypes of 30 strains were inconsistent with the capsid genotypes, indicating potential NoV recombinants.

INTRODUCTION

Due to the extensive application of nucleic acid amplification tests to investigate the etiologic role of norovirus (NoV) in gastroenteritis since the mid-1990s, NoV has been recognized as a leading cause of acute non-bacterial gastroenteritis outbreaks, and a common viral agent of sporadic diarrhea in all age groups worldwide (1,2). A growing body of literature over the past 10 years has documented NoV as an important enteropathogen and cause of childhood diarrhea (1–4). NoV, a member of the Caliciviridae family, is a single-stranded, positive-sense RNA virus. Based on the sequence of the major structural capsid protein (VP1), NoV is classified into five genotypes (genotype I [GI] to genotype V [GV]). In humans, three genogroups (GI, GII, and GIV) have been detected, and GI and GII are the most common viruses causing human infections (5). Using the typing-tools developed by Kroneman et al. (6), NoV has been subdivided into 8 GI genotypes and 21 GII genotypes based on the complete VP1 sequence, and 14 GI genotypes and 31 GII genotypes based on the partial sequence of RNA-dependent RNA polymerase (RdRp).

Despite the great improvement in water supplies, hygiene, and sanitation during the past decade in Shanghai, acute diarrhea remains a major cause of illness in children seeking medical care. Based on our clinical observations, non-dysenteric diarrhea is the most frequent type of diarrhea. In addition to rotavirus, NoV was reported as a common pathogen, responsible for 8.9% to 10.3% of diarrhea cases among hospitalized children in China over the past few years (7–10). Over the past 5 years, pediatric hospitalizations in Shanghai, primarily for acute diarrhea, decreased significantly due to the rational utilization of medical resources and wide implementation of oral rehydration solution therapy. However, the epidemiological profile of NoV infection in children visiting outpatient clinics in Shanghai for diarrhea is not yet well understood.

We carried out a prospective study among pediatric outpatients with acute non-dysenteric diarrhea. Our aim is to determine the incidence of NoV-associated diarrhea in the outpatient setting, and the circulating NoV genotypes in Shanghai.

MATERIALS AND METHODS

Case definition and sample collection: This study was conducted between August 2008 and July 2009 in a tertiary teaching children’s hospital with 800 ward beds that delivers health care to approximately one-fourth of the local pediatric population. NoV infections were investigated among outpatient children with acute diarrhea.

Diarrhea was defined as the presence of three or more episodes of unusually loose or watery stools in a 24-h period (11). Enrollment criteria included outpatients (i) who visited sentinel hospital for acute diarrhea and...
resided in Shanghai 1 month prior to onset of diarrhea; (ii) who had no blood or pus in their stool; (iii) whose fecal specimens showed no leukocytes under microscopic testing; (iv) whose samples were sufficient for nucleic acid extraction; and (v) whose parents or guardians agreed to participate in the study. This study was approved by the Ethics Committee of the Children’s Hospital of Fudan University.

A total of 910 children aged between 1 month and 12.2 years were enrolled in this study, and 910 fecal specimens were obtained. The stool samples were kept in a sterile container and frozen at −20°C until RNA extraction. All samples were also tested for rotavirus with a Rotavirus Group A Diagnostic Kit (Beijing Wantaikai, Beijing, China).

Nucleic acid extraction: A 20% stool suspension was prepared in sterile normal saline. After centrifugation at 8,000 rpm for 5 min, 150 µL of supernatant was removed for RNA extraction using Trizol (Invitrogen, Carlsbad, Calif., USA). RNA was eluted in 25 µL of sterile diethyl pyrocarbonate (DEPC) water prior to reverse transcription-polymerase chain reaction (RT-PCR). In each RNA extraction, sterile water was used as a negative control.

Detection of NoV: A one-step real-time GI-GII multiplex TaqMan assay was used for screening NoV. The primer and probe sets were previously described by tiplex TaqMan assay was used for screening NoV. The primer and probe sets were previously described by Tiplex et al. (8). The reverse transcription-polymerase chain reaction (RT-PCR) was performed on the Applied Biosystems 7500 real-time PCR system. Amplification was performed in 96-well reaction plates with 5 µL of RT-PCR master mix, which was dispensed into a 96-well reaction plate. The quantitative RT-PCR was performed on the Applied Biosystems 7500 real-time PCR system. Amplification was performed according to the manufacturer’s instructions: (i) RT for 15 min at 50°C, (ii) denaturation for 2 min at 95°C, (iii) 40 cycles of 15 s at 95°C and 60 s at 60°C.

A negative control containing DEPC water and two positive controls containing RNA from NoV GI and GII were included in each PCR run. The result was considered positive if the sample produced cycle threshold (Ct) values of 40 or less when the positive and negative control reactions yielded the expected values.

RT-PCR amplification and sequencing of NoV: First-strand cDNA synthesis of NoV was performed using random primers with SuperScript™ III RT (Invitrogen) according to the manufacturer’s protocol.

PCR was carried out using KOD FX (Toyobo, Osaka, Japan) on a Bio-Rad PCR system. The previously described modified primer sets G1FF/G1SKR and G2FB/G2SKR were used to amplify 597 bp of GI and 468 bp of GII, respectively (12). The forward super primer (FSP: CAGGCCACGTGTGTTGACGCG) and reverse super primer (RSP: TTTTTTGGCTATGGTC TCTG) bound to the 5′ extremities of the degenerate primers G1FF/G1SKR and G2FB/G2SKR in order to sequence in both directions (14). The PCR cycling parameters were 95°C for 2 min, then 40 cycles of 98°C for 10 s, 48°C for 30 s, and 68°C for 30 s, and then a 7-min elongation at 68°C. Primer sets JV12/JV13 were used to amplify an RdRp fragment for phylogenetic analysis (15). PCR conditions consisted of preamplification for 2 min at 95°C, followed by 40 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 50°C and elongation for 30 s at 68°C, and a final extension for 7 min at 68°C.

PCR products were separated by electrophoresis on a 1.5% agarose gel. PCR products were purified from the gel using a DNA extraction kit (Sunnybio, Shanghai, China) and were sequenced in both directions using primers FSP/RSP and JV12/JV13 on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, Calif., USA) using dye-terminator chemistry.

Genotyping and phylogenetic analysis of NoV: The capsid genotypes of NoV were classified based on the nucleotide sequence of the capsid N/S, which included 295 bp for GI and 282 bp for GII, consistent with the classification scheme of Kageyama et al. (16). The polymerase genotypes of NoV were classified based on 326 bp or 327 bp RdRp sequences. Sequences were aligned using ClustalX software (17). Two phylogenetic trees with 1,000 bootstrap replicates were generated using the neighbor-joining method with MEGA software (version 3.1) (18). All nucleotide sequences of capsid and RdRp were genotyped using the Norovirus Automated Genotyping Tool (www.rivm.nl/mpf/norovirus/typingtool). The standardized classification of capsid and RdRp genotypes for all reference NoV strains was in accordance with NoroNet (http://www.noronet.nl/noronet). Sequence data for the reference strains were downloaded from GenBank.

Statistical analysis: A chi-square test was used to analyze proportion data.

RESULTS

Of 910 fecal samples, 165 (18.13%) were positive for NoV. Of the 165 positive samples, 4 (2.42%) were GI and 161 (97.58%) were GII. Rotavirus was identified in 268 (29.45%) specimens, and 27 samples were co-infect- ed with NoV and rotavirus. The 165 NoV-infected children were aged between 3 to 68 months with a median age of 12 months. A large majority of the NoV-infected (93.94%), (155/165) and rotavirus-infected children (92.91%), 249/268 were 3 years old or younger. The detection rates of NoV by month ranged from 8.64% to 40.48%. In September and October 2008 and July 2009, the prevalence of NoV was above the average level and was significantly higher compared to the other months (P < 0.05). Compared to the epidemic trend of rotavirus, high NoV activity occurred prior to the seasonal peak of rotavirus diarrhea (Fig. 1).

Of the 165 NoV-positive strains confirmed by real-time PCR, capsid and RdRp fragments were successfully amplified and sequenced in 114 and 86 strains, respectively. Of the 30 samples with Ct values > 31 in real-time PCR, 25 could not be amplified by conventional PCR assay. On the basis of capsid sequences, the 114 NoV strains were clustered into 8 genotypes including 66 (57.89%) GII.4 2006b, 35 (30.70%) GII.3, 5 (4.39%) GII.6, 4 (3.51%) GII.12, 1 (0.88%) GII.14, 1 (0.88%) GII.2, 1 (0.88%) GII.4, and 1 (0.88%) GII.5 (Fig. 2A). Based on the RdRp sequences of 86 NoV strains, 7


genotypes were identified, GI.4 (54, 62.79\%)), GII.12 (26, 30.23\%), GI.I.g (2, 2.33\%), GI.I.2 (1, 1.16\%), GI.I.6 (1, 1.16\%), GI.I.7 (1, 1.16\%), and GI.I.4 (1, 1.16\%) (Fig. 2B).

A total of 76 NoV strains were assigned both capsid and RdRp genotypes. The capsid and polymerase regions of 30 strains segregated into different genotypic clusters in the two separate trees, which indicated potential NoV recombinants; 23 strains were GII.12 polymerase/GII.3 capsid, 2 were GII.12 polymerase/GII.4 capsid, 2 were GII.g polymerase/GII.12 capsid, 1 were GII.7 polymerase/GII.6 capsid.

DISCUSSION

Accumulating studies have documented the prevalence of NoV in children with acute gastroenteritis in the range of 6\% to 48\% with an overall median of 14\% (3). Our 1-year surveillance data showed that NoV was responsible for 18.1\% of non-dysenteric diarrhea among outpatient children in Shanghai. Xu et al. reported that NoV was detected in 9% of children hospitalized with diarrhea in our hospital from 2001 to 2005 (10). Data from the present study, as well as previous studies, suggest that NoV is a common viral agent causing childhood diarrhea in Shanghai. The variation in detection rates is most likely related to the detection methods as well as the study year and target subjects. NoV is remarkably genetically diverse, and none of the reported RT-PCR assays are able to detect all strains (19,20). The primer and probe sets for GI and GII NoVs designed by Kageyama et al. (12) contain the highest nucleotide homology in the open reading frame (ORF)1-ORF2 region. Based on our findings and published literature, this protocol has been confirmed to have greater sensitivity and simplicity than conventional RT-PCR assays (12,21). Here, we used modified multiplex real time RT-PCR in a single reaction to screen for NoV, which is more convenient and suitable for routine diagnosis of a large number of clinical samples.

Recent studies showed that NoV plays an important etiological role in sporadic diarrhea in infants and young children (2,22). We found that NoV-associated diarrhea occurred mainly in children aged 3 years or younger, which is similar to rotavirus. Our finding is in agreement with studies from Spain, Japan, and India (23–25).

NoV epidemics usually appear in wintertime (26,27), although a summer peak is seen during outbreaks and in some regions (25,28). According to the existing data from multiple geographical areas of China, NoV becomes highly active in mid-autumn and winter or early spring (7–10). However, we observed high NoV activity between late summer and mid-fall, just preceding the seasonality of rotavirus. NoV activity decreases significantly in November when rotavirus diarrhea peaks. Whether the seasonal pattern of NoV is the same every year merits continuous monitoring.

We found that NoV strains circulating in Shanghai almost exclusively belonged to GII. Moreover, the GI.4 2006b variant was the most commonly detected from 2008 to 2009. In the spring of 2006, GI.4 2006b NoV was found to be circulating widely, leading to a large-scale outbreak of gastroenteritis in Europe in the summer and fall of 2006 (29). Since then, GI.4 2006b has spread worldwide (28,30). GI.4 2006b was detected in China in July 2006 and has accounted for 64.7\% of sporadic NoV gastroenteritis in seven provinces of China (7). In the past 15 years, four GI.4 variants have emerged globally, and have been linked to the worldwide outbreak of NoV gastroenteritis (28,31–35). Recently, a new GI.4 2007 variant was detected in India, and a new GI.4 2008 variant was found in Canada and South Africa (36–38). It is crucial to monitor for new variants in the local region.

The polymerase and capsid genotypes of 30 NoV strains differed, suggesting that these strains were possible genetic recombinants. Naturally occurring NoV recombinants have been increasingly identified worldwide (39), and GI.4 polymerase/GI.3 capsid and GI.14 polymerase/GI.6 capsid recombinants have been found in China (7). In this study, we observed multiple potential intergenotypic recombinants such as
Fig. 2. (A) Phylogenetic analysis of 112 NoV GII strains based on the N/S capsid sequences. NoV strains detected in Shanghai were designated by month/identification number/year. Local NoV strains detected in Shanghai which bear the same capsid and polymerase genotypes were highlighted with black dots. NoV strains with distinct capsid and polymerase genotypes were highlighted with black triangle. (B) Phylogenetic analysis of 85 NoV GII strains based on partial polymerase sequences.
GII.3/GII.4 2006b, GII.12/GII.3, GII.g/GII.12, and GII.7/GII.6. We need to more carefully verify these recombinants. Virus recombinants can affect phylogenetic grouping, confound molecular epidemiological studies, and it has major implications in vaccine design. Therefore, it is necessary to characterize NoV capsid and polymerase genotypes in order to classify NoV genotypes.

Our prospective study outlines the epidemiological picture of NoV infection and the NoV genotype diversity in Shanghai. Like influenza, the evolution of GII.4 NoV is epochal, and has occurred through serial changes in the capsid sequence, which is called an antigen shift. A NoV outbreak occurs when virus variants emerge to which the human population has no immunity (40). Ongoing surveillance of NoV activity and the circulating GII.4 variants are needed for the prediction and control of NoV outbreaks and vaccine development.

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Conflict of interest None to declare.

REFERENCES


