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Identification of Norovirus as the Top Enteric Viruses Detected in Adult Cases with Acute Gastroenteritis

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Abstract. To elucidate the importance of the norovirus and other enteric viruses, and the difference of the genetic relatedness on norovirus between the outbreak and sporadic cases, a total of 557 stool samples, consisting of 503 sporadic cases and 54 samples of 4 outbreaks were collected and tested for norovirus and other enteric viruses in Beijing, China, July 2007–June 2008. The data showed norovirus, rotavirus, astrovirus, and sapovirus, were detected in 26.6%, 6.1%, 1.8%, and 0.5%, respectively. Norovirus was detected almost throughout the surveillance period, norovirus co-infecting with rotavirus, astrovirus, and sapovirus, respectively, were identified both in outbreak and the sporadic cases. GII.4/2006 was identified as the predominant strain circulating both in outbreak and sporadic cases. The results showed that norovirus was rather the important agent than other enteric viruses affected adults with acute gastroenteritis; no significant genetic relatedness of the dominant strains was found between the outbreak and sporadic cases.

INTRODUCTION

Norovirus is one of the most important pathogens of acute nonbacterial gastroenteritis in both developed and developing countries, resulting in acute diarrhea, nausea, and vomiting.[1] Norovirus, a member of the family *Caliciviridae,* is a non-enveloped, positive-sense, single-strand RNA virus. Its genome contains approximately 7.7 kb in length, which is organized into three open reading frames (ORFs): ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase (RdRp), ORF2 encodes the major capsid protein VP1, and ORF3 encodes the minor structural protein VP2. According to nucleotide sequence analysis of the polymerase or capsid regions, norovirus is classified at least into five genogroups, GI–GV, each genogroup is further divided into distinct genotypes. GI, GII, and GIV have been found in humans, and GII seems to account for a majority of human cases.[2] Among the GII genotypes, GII.4 variants are described as the predominant strains spreading globally, resulting in pandemic outbreaks since the mid-1990s.[3–6]

In recent years, global public health concern caused by norovirus was raised referring to significant morbidity and occasional mortality,[7] which frequently occurred in schools, hospitals, cruises, and other semi-closed institutions.[8–11] The pathogen is highly contagious with a low infectious dose and spreads by ingestion of contaminated food and/or water, or inhaling the agent particle by contact with patients.[12] It has been deduced that approximately 90% of the outbreaks of acute nonbacterial gastroenteritis in the United States were caused by norovirus and 23 million cases were affected annually.[13,14] In addition to human costs, norovirus infections cause economic losses that approach or exceed US$650,000 in supplies, staff time off, and closed beds.[15] Although previous studies showed the disease was mild and self-limiting, recent studies have showed its ability in causing more severe complications than previously expected.[16,17]

Accordingly, the role of norovirus as the etiological agents in outbreaks and sporadic cases with acute gastroenteritis needs to be further defined, especially in adults, primarily because of overrepresentation of the studies in children and the low sensitivity of the detection assays used in previous studies.[18–21] Although the traditional reverse transcriptase-polymerase chain reaction (RT-PCR) method is more sensitive than electron microscopy or enzyme immunoassays, its insensitivity is still dramatically lower than real-time RT-PCR assays in the detection of norovirus. Because of the low dose of the virus in the stool, a more sensitive assay is needed. Recently, a research group successfully developed real-time RT-PCR assays based on improved primers targeting the ORF1-ORF2 junction region that detect norovirus GI and GII in the stool samples from outbreaks and sporadic cases,[22] and it had been illustrated that the assays had good sensitivity, specificity, and can be used as a routine diagnostic test.

In China, acute nonbacterial gastroenteritis is considered as a severe public health problem, and norovirus is demonstrated as the second important viral agent of acute gastroenteritis in children, which is lower than that of rotavirus according to many epidemiologic studies performed,[23,24] however, there were seldom studies conducted in adult populations to elucidate the importance of the norovirus and other enteric viruses.

From July 2007 through June 2008, we conducted hospital-based sentinel surveillance for acute nonbacterial gastroenteritis in a general hospital in Beijing; sporadic and outbreak adult cases with acute gastroenteritis were sampled and detected for norovirus and other enteric viruses. By doing so, the role of norovirus and other enteric viruses in adult patients with the acute gastroenteritis might be clarified, and the difference in the genetic relatedness of isolated strains between the outbreaks and sporadic cases could be defined as well.

MATERIALS AND METHODS

Stool samples. The surveillance period ran from July 2007 to June 2008 in a tertiary hospital, the general hospital of People's

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Liberation Army in Beijing. Stool samples were collected from two settings: outbreak and sporadic cases. The definition of a sporadic case of acute nonbacterial gastroenteritis was more than three times per day a loose or watery stool, and stool samples were collected from patients visiting an outpatient clinic and/or emergency room; the definition of outbreaks was defined as more than two hospitalized cases in the same ward that were epidemiologically linked with each other, and the cases were associated with 1) more than two episodes of vomiting, 2) three episodes of diarrhea, or both (1) and (2) within a 24-hour period. Trained medical staff administered the case findings and sample collection. Approximately 5 g (mL) stool were collected in a sterile plastic container, after proper labeling, the samples were sent to the laboratory as early as possible in a cool box and stored at −80°C until further tests. All of the samples were screened for norovirus by real-time RT-PCR, astrovirus and sapovirus by multiplex RT-PCR, and rotavirus by enzyme immunoassay (IDEIA, Oxiod, UK). Medical records were reviewed to obtain information about the patients for further analysis.

Viral RNA extraction and detection. Viral RNA was extracted from 10% fecal supernatant using the QIAamp Mini Elute Kit (Qiagen, Hilden, Germany). For RT, the viral RNA was reverse transcribed by M-MLV (Promega, Madison, WI) and a random primer (hexadeoxyribonucleotide mixture) according to the manufacturer's instructions. The RT step was carried out at 42°C for 1 hour, followed by 99°C for 5 min, then held at 4°C and stored at −80°C.[25]

Norovirus detection was performed by using the real-time RT-PCR method based on SYBR green chemistry to enhance the sensitivity of the evaluation for its low infectious dose, the positive was defined when Ct ≤ 30. Briefly, the presence of norovirus (GI and GII) was detected according to the protocol described previously[22] and modified; astrovirus and sapovirus were performed by the multiplex RT-PCR method with independent specific primers according to the protocols described[25] using cDNA as templates (see Table 1). Rotavirus A was detected by using the IDEIA rotavirus direct antigen detection kit (IDEIA, Oxiod, UK). The amplicons were analyzed by electrophoresis on 1.5% agarose gel, stained by ethidium bromide, and visualized with UV light.

Sequencing and phylogenetic analysis. The norovirus amplicons were purified using the Omega Gel extraction kit (BioTek Instruments, Inc., Winooski, VT). The purified products were sequenced with automated DNA sequence (ABI PRISM 373; Perkin-Elmer, Foster City, CA). The resulting sequences were analyzed using ClustalX (version 1.83) followed by phylogenetic analysis using MEGA (version 3.1). The statistical significance of the inferred phylogenies was estimated using bootstrap analysis with 1,000 pseudo-replicate data sets. The nucleotide sequences generated in the study were deposited in GenBank under accession nos. EU482094, EU482095, and GQ380642–GQ380655.

Statistical analysis. Differences in continuous variable levels or proportions were used by one-way analysis of variance (ANOVA) or χ^2 test, where appropriate. Two-tailed $P < 0.05$ was considered statistically significant. The SPSS version 13.0 software package was used for all analysis (SPSS, Inc., Chicago, IL).

RESULTS

General characterization of the study subjects. A total of 557 adult acute nonbacterial gastroenteritis cases were studied, including 503 sporadic cases and 54 cases from four outbreaks. The mean (±SD) age of the study subjects was 52 (±20) years and 307 (55.1%) patients were male. After excluding duplicated samples, a total of 557 samples were detected, from which 147 samples (26.4%) were positive for norovirus detection, consisting of GI (25) and GII (112). Other enteric viruses, including rotavirus 34 (6.1%,), astrovirus 10 (1.8%), and sapovirus 3 (0.5%,), were also detected. Co-infection was confirmed in both sporadic cases and outbreak cases. In the sporadic gastroenteritis cases, all 13 samples with co-infection were found for norovirus infection, eight were co-infected with rotavirus, three with astrovirus, and two with sapovirus. Two of the four outbreaks were co-infected with rotavirus. We then divided the subjects with norovirus infection into a sole-infection group and co-infection group, the comparison between two groups disclosed the mean age ± SD in the sole-infection group (55 ± 21 years of age) was lower than that in the co-infection group (59 ± 19 years of age), although the difference did not attain a significant level ($P > 0.05$). In addition, the proportion of males 67.2% in the sole norovirus infection group was not significantly higher than that of 53.8% in the co-infection group ($P > 0.05$). Furthermore, we compared the mean age (±SD) of co-infection between sporadic cases and outbreak cases, the mean age (±SD) in sporadic cases was 51 (±19) years, significantly younger than that of 72 (±21) years of age in outbreak cases ($P < 0.05$), and the proportion of male gender was significantly different distributed between sporadic and outbreak cases ($P < 0.05$).

TABLE 1
List of the specific primers used in this study*

Method	Virus	Target	Primers	Sequence (5′–3′)	Amplicon size
Real-time RT-PCR	Norovirus GI	ORF1-ORF2 conjunction	GI-F	CGYTGGATGCGNTTYCATGA	
			GI-R	CTTAGACGCCATCATCATTYAC	85
	Norovirus GII	ORF1-ORF2 conjunction	GII-F	CARGARBCNATGTTYAGRTGGATGAG	
			GII-R	TCGACGCCATCTTCATTCACA	98
Multiple RT-PCR	Norovirus GI	Capsid	G1-SKF	CTGCCCGAATTYGTAAATGA	
			GI-SKR	CCAACCCARCCATTRTACA	330
	Norovirus GII	Capsid	COG2F	CARGARBCNATGTTYAGRTGGATGAG	
			G2-SKR	CCRCCNGCATRHCCRTTRTACAT	387
	Astrovirus	Capsid	Mon269	CAACTCAGGAAACAGGGTGT	
			Mon270	TCAGATGCATTGTCATTGGT	449
	Sapovirus	Capsid	SLV-5317	CTCGCCACCTACRAWGCBTGGTT	
			SLV-5749	CGGRCYTCAAAVSTACCBCCCCA	434

* RT-PCR = reverse transcriptase-polymerase chain reaction; ORF = open reading frames.

Sporadic cases with acute gastroenteritis. The seasonal distribution analysis showed the detection of norovirus throughout the observation period except May 2008, and the two highest detection rates were observed in January and April, which was significantly higher than in other months. Rotavirus was found in only 5.9% of the samples, mostly in March and April 2008, whereas astrovirus and sapovirus were detected with a low level, and no obvious seasonal distribution trend could be observed, as shown in Figure 1.

Outbreaks gastroenteritis. Altogether 54 samples were collected from four outbreaks occurring from December 2007 to March 2008, norovirus was confirmed as the major agent responsible for the four outbreaks. Compared with the sporadic cases, the patients from the outbreak displayed an older age (71 versus 50 years of age) and were more afflicted with chronic diseases, including tumor, diabetes mellitus, and cardiac disease.

Genetic relatedness of norovirus between sporadic cases and outbreak cases. To further characterize the genetic relatedness of the norovirus strains from outbreaks and sporadic cases, a total of 16 representative strains selected randomly, including four strains of outbreaks and 12 strains of sporadic samples, were sequenced based on the partial capsid protein.

The sporadic strains of norovirus reported in the study were grouped into two different genogroups, GI and GII, and GII dominated. Within the GI genogroup, two strains (GQ380643 and GQ380644) were classified into GI.4 genotypes, 1 strain (GQ380642) were classified into GI.1 genotype. Among the GII genogroup, 7 of 9 strains were found to belong to GII.4 genotypes, clustering in the same linage within the reference strain GII.4/2006b, N.Hu/NSW696T/2006 (accession no. EF648519), which prevailed throughout the world since late 2006; furthermore, 1 strain (GQ380652) showed a high similarity in 97% with the reference strain GII.3/EU249134), 1 strain (GQ380651) could not be classified into any genotypes according to the present classification, and further study needs to be performed on the characterization of the ungenotyped strain. A comparison of the isolates from outbreaks indicated that they belonged to GII.4 by sequencing and phylogenetic analysis, and clustered in a lineage of GII.4 within the representative strain GII.4/2006b, as shown in Figure 2.

DISCUSSION

Acute nonbacterial gastroenteritis is one of the most important infectious diseases in China and severely affects infants and young children.[18–21,26] However, studies are seldom performed in adult populations for elucidating the importance of the norovirus and other enteric viruses. In this study, norovirus was identified in 26.4% of the studied adult subjects with acute nonbacterial gastroenteritis in Beijing between July 2007 and June 2008, though the rate was higher than that of the previous study in children in China;[23] this was in agreement with the published studies reporting that the prevalence rate varied between 3.5% and 47.3%.[27,28] The difference in the prevalence of norovirus infection may be due partly to the different sensitive methods used at distinct locations of the genome in different studies. In this study, the norovirus infection rate (26.4%) was higher than that of rotavirus A (6.1%), astrovirus (1.8%), and sapovirus (0.5%) in the adult populations; the data differed from the results of the previous studies performed with children in China and other countries,[3–6,23] in which the highest rate was detected for rotavirus A infection. The discrepancy of the highest prevalent rates between adults and children implied that norovirus affected adults more frequently than children, but not rotavirus A. Alternatively, although the sensitive method was only used on the detection of norovirus, the Ct value was defined as less than 30 cycles; this may counteract the query of the higher prevalence of norovirus than other enteric viruses. Moreover, the prevalence of other enteric viruses only showed an individual percent far lower than the prevalence of norovirus, and above all, we could deduce that norovirus was the top enteric virus than any other virus in adults.

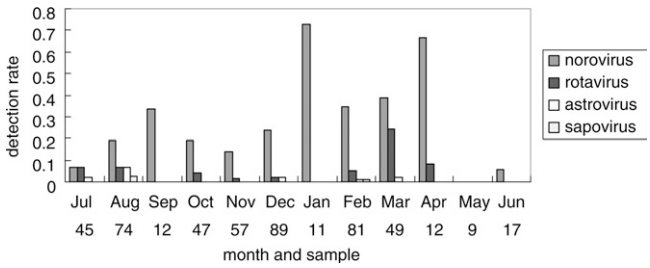

FIGURE 1. Seasonal distribution of norovirus, rotavirus, astrovirus, and sapovirus infection detected in adult sporadic cases with acute gastroenteritis in Beijing during July 2007 to June 2008.

Norovirus was detected almost continuously throughout the study period, and centralized in the winter and spring for outbreaks. In this study, norovirus mixed with other enteric viruses, comprised of rotavirus A, astrovirus, and sapovirus, respectively, were acquired both in outbreaks and sporadic cases, indicating that co-infection occurred in adults regularly, but no more than three virus infections were detected simultaneously. Significant associations of co-infection between sporadic and outbreak cases were found, and no significant distribution of age and male gender was reported between solo norovirus infection and co-infection patients. To date, the real importance of the mixed infections was not well elucidated, but it had been demonstrated that no significant differences were found in the clinical symptoms of the patients with multiple viral infections.[28,29]

The results of molecular epidemiology disclosed that GII.4/2006b accounted for the majority of sporadic cases and outbreaks during the surveillance period, no significant difference in the proportion and genotypes was discovered between outbreaks and sporadic cases, the data of this study was inconsistent with the results derived from which GII.4 predominated in outbreaks and GII.3 in sporadic cases,[30] the main reason for the disagreement results may be explained partly by the geographic and the temporal and seasonal variations, and probably by the emergence of the new circulating GII.4 variants. Previous studies showed that although multiple genotypes of norovirus were reported, a trail of distinct GII.4 variants were identified as the main agents involved in the global epidemics. After almost a 2 to 4 years interval, a new variant GII.4 emerged and displaced the previous one, denoting GII.4 norovirus evolution is epochal, with periods of stasis followed by the emergence of novel epidemic strains that involve in a linear manner over time.[15] Variant GII.4/2006b was first obtained in Spain in December 2005 and became the overwhelming predominant one since then, it was first isolated in China in July 2006 and then prevailed circulating in China.[23,31] To date, the

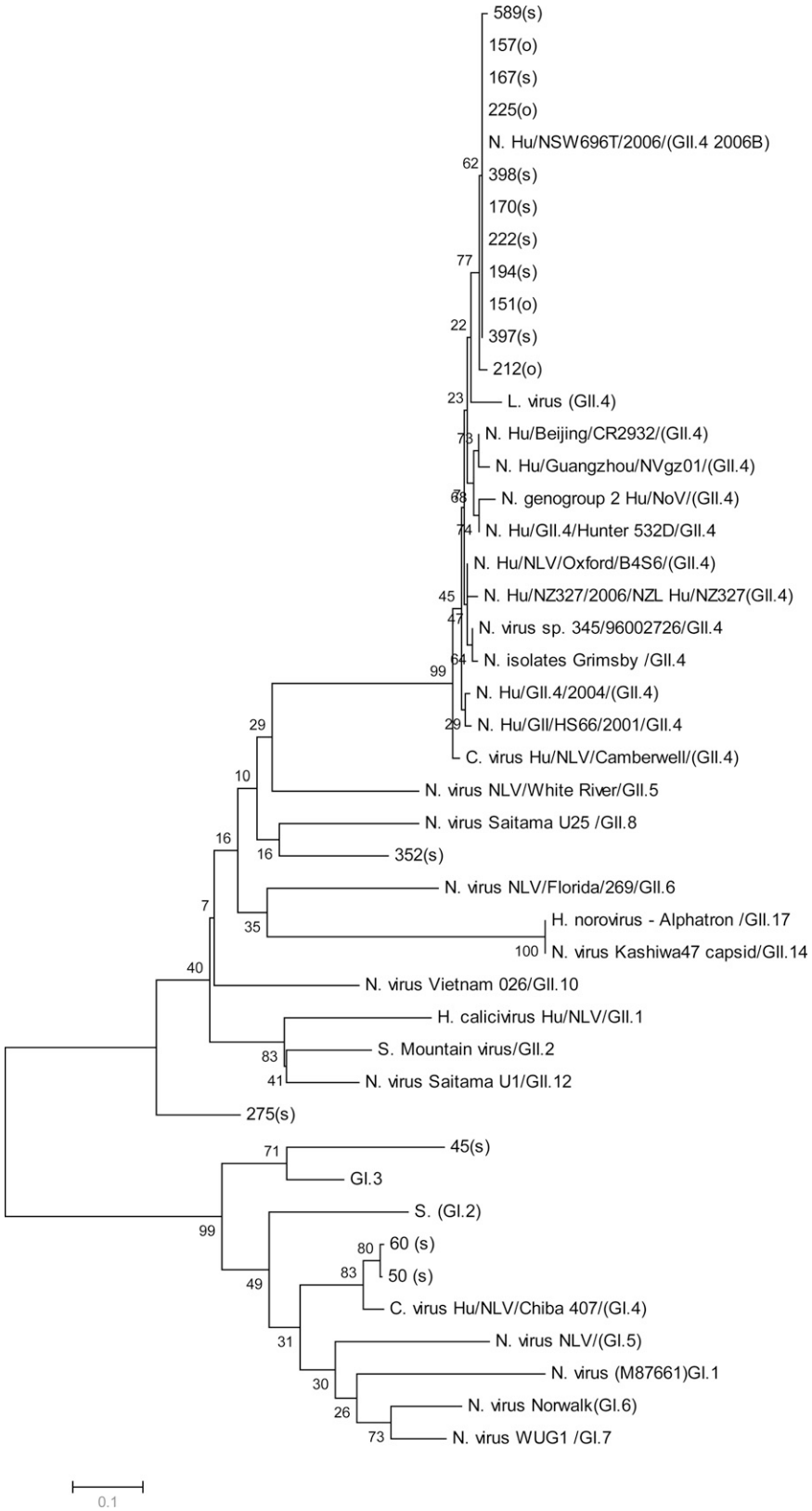

FIGURE 2. Phylogenetic analysis of the partial capsid protein of norovirus GII genogroup detected in the adult outbreaks and sporadic cases in Beijing from July 2007 to June 2008. S denotes sporadic case and O denotes outbreak. The accession nos. included in the tree were 589 (GQ380655), 398 (GQ380654), 397 (GQ380653), 352 (GQ380652), 275 (GQ380651), 225 (GQ380650), 222 (GQ380649), 212 (GQ380648), 194 (GQ380647), 170 (GQ38046), 167 (GQ380645), 60 (GQ380644), 50 (GQ380643), 45 (GQ380642), 151 (EU482094), 157 (EU482095), respectively.

reasons for the variants GII.4/2006 circulating globally are still not fully understood, but may involve herd immunity and the ability of the virus to evade immune surveillance, and other factors such as difference in stability, infectious dose, and/or host-related factors that may also play an important role.[32,33]

Limitations in the study should be considered. First, long-time surveillance on norovirus and other enteric viruses in adults need to continue for further elucidating the dynamic changes of norovirus and other enteric viruses, and the proportion and genotypes in the outbreaks and sporadic cases; second, recombination of norovirus between genogroups and genotypes occurred frequently as another important feature of the norovirus evolution,[31,33] therefore, recombination of the pathogen of norovirus should be considered in the study; third, detection on bacterial gastroenteritis should be considered, because bacterial infection might be a possible cause of acute gastro enteritis in our subjects.

In conclusion, the results of the study show that, in comparison with other enteric viruses, norovirus played a major role in acute nonbacterial gastroenteritis; no significant genetic relatedness of the dominant strains was found between sporadic cases and outbreaks. GII.4/2006b was identified as the predominant strain spreading throughout the year and predominated in the winter for outbreaks. Significant associations of co-infection between sporadic and outbreak cases were found, however, no significant associations of age and male gender were found with co-infection.

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