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RESEARCH ARTICLE

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# Molecular epidemiology and changes in genotype diversity of norovirus infections in acute gastroenteritis patients in Huzhou, China, 2018

Lei Ji[1] | Gang Hu[2] | Deshun Xu[1] | Xiaofang Wu[1] | Yun Fu[1] | Liping Chen[1]

[1]Microbiological Laboratory, Huzhou Center for Disease Control and Prevention, Huzhou, China

[2]Obstetrics and Gynecology, Huzhou Maternity and Child Health Care Hospital, Huzhou, China

**Correspondence**
Liping Chen, Microbiological Laboratory, Huzhou Center for Disease Control and Prevention, 313000 Huzhou, China.
Email: lipingchen1106@hotmail.com

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## Abstract

Norovirus is an important causative agent of acute gastroenteritis worldwide, affecting people of all ages. Stool samples collected from patients with clinical symptoms of acute gastroenteritis in all age groups at the diarrhea outpatient department of the First People's Hospital in Huzhou were analyzed to gain insight into the prevalence and genetic characteristics of norovirus. From January to December 2018, a total of 551 specimens were screened for norovirus by real-time reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was used for genomic amplification and sequencing of the RNA-dependent RNA polymerase and capsid gene of the positive samples. Genotypes of norovirus were assigned using the norovirus Noronet typing tool and phylogenetic analysis. About 100 (18.1%) specimens were identified as norovirus positive. GII genogroup was the main genogroup identified (83.0%; 83/100). About 42 (42.0%) samples were successfully sequenced and genotyped by RT-PCR. Since one of the samples was dual infection, so we got 43 virus finally. Nine norovirus GII genotypes and four norovirus GI genotypes were detected in Huzhou during our research period. The main two norovirus GII genotypes were GII.2[P16] (54.8%; 23/43) and GII.17[P17] (11.9%; 5/43). We characterized the molecular epidemiology of norovirus infection in acute gastroenteritis patients during 2018. GII genogroup was the main genogroup identified. The dominance norovirus genotype circulating in the population of Huzhou was GII.2[P16] in 2018.

**KEYWORDS**
acute gastroenteritis, epidemiology, GII.2[P16], norovirus

## 1 | INTRODUCTION

Acute gastroenteritis (AGE) is a leading cause of morbidity and mortality affecting both developing and developed countries.[1,2] Norovirus is an important causative agent of AGE worldwide, affecting people of all ages.[3] Norovirus AGE generally has an incubation period of 24 to 48 hours and is characterized by acute onset of nausea, vomiting, abdominal cramps, and diarrhea. This disease is usually self-limiting in healthy individuals, and symptoms typically resolve within 2 to 3 days.[4,5] The majority of norovirus transmissions are via the fecal-oral cycle

Lei Ji and Gang Hu contributed equally to this study.

through the ingestion of contaminated food or water or through direct contact with contaminated environmental surfaces or infected persons.[6] A low infectious dose and environmental stability are some of the attributes that facilitate effective norovirus transmission.[7]

Noroviruses are nonenveloped viruses with a single-stranded positive sense RNA genome ~7.5 kb in length.[8] The genome is organized into three open reading frames (ORFs): ORF1 encodes for a polyprotein required for replication such as NTPase, protease, and RNA-dependent RNA polymerase (RdRp); ORF2 encodes the viral protein 1 (VP1); and ORF3 encodes the viral protein 2 (VP2).[9] With a great genetic diversity, noroviruses has been classified into 10 genogroups (GI-GX) that are further divided into 49 confirmed capsid genotypes and 60 confirmed P-types.[8] Genotype GII.4 is further divided into variants.[10] Subtyping of GII.4 strains into variants will be based on phylogenetic clustering and that new GII.4 variants will only be recognized after they become epidemic in at least two geographically diverse locations.[11] New GII.4 variants were given the name of the city of the first full-length capsid sequence available in the public domain, for example, GII.4 Sydney.[11]

Over the past 10 years, the GII.4 variants has been predominant in Huzhou.[12-15] However, In the winter of 2014 to 2015, a novel GII.17 Kawasaki[P17] emerged and became predominant in Huzhou, and steadily replaced the previously circulating GII.4 Sydney[P31] strain.[14] The same situation also found in other cities in China.[16,17] For the first time that non-GII.4 norovirus become the predominant genotype in this area. Moreover, in late 2016, another uncommon norovirus recombinant genotype GII.2[P16] caused a sharp increase of norovirus activity in China[18] and also in Huzhou.[15] Then GII.17[P17], GII.4 Sydney[P31], and GII.2[P16] had cocirculated in Huzhou as the main three genotypes in the next year (2017).[15] Our study is part of continuous monitoring of epidemiological patterns and genetic characteristics of norovirus in Huzhou. After years of continuous monitoring, we find that the outbreak cases of norovirus is usually concentrated in a few epidemic types.[12] The study of sporadic cases can reflect the diversity and epidemic trend of norovirus in a certain area. Here, we describe the prevalence and genetic characteristics of norovirus in the sporadic AGE cases from January to December 2018 in Huzhou City here, Zhejiang, China.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethics statement

This study was approved by the ethics committee of Huzhou Center for Disease Control and Prevention (HJK20180326). Informed consent for the stool samples was obtained from the patients or their guardians. This study was part of a routine laboratory-based investigation. No human experimentation was conducted. All experiments were carried out using norovirus strains. The only human materials used were stool samples that had been sent to our laboratory for routine virological diagnosis of gastroenteritis. Informed consent for the stool samples was obtained from the patients or their guardians.

### 2.2 | Specimen collection

This study was part of a hospital-based local regional norovirus gastroenteritis surveillance program conducted at the First People's Hospital in Huzhou. This surveillance system is part of the initiative monitoring of foodborne diseases of China, with samples screened for *Salmonella*, *Vibrio parahaemolyticus*, *Shigella*, diarrheogenic *Escherichia coli*, and norovirus. Between January 2018 and December 2018, a total of 551 fecal samples were collected from patients (including children and adults, outpatients, and inpatients) with clinical symptoms of AGE. Results statistical analysis was carried out according to five age groups (≤5, 6-15, 16-40, 41-60, and ≥60 years). Stool samples were freshly collected and transported to Huzhou Center for Disease Control and Prevention on ice for immediate storage at −80°C before norovirus detection.

Surveillance subjects were patients who sought medical care at the diarrhea outpatient department of the First People's Hospital in Huzhou with clinical symptoms of AGE. AGE was defined as diarrhea (ie, three or more loose or liquid stools within a 24 hours period) and/or vomiting, abdominal pain, fever, and nausea.

### 2.3 | Viral RNA extraction and norovirus detection

Viral RNA was extracted from a 200 μL sample of the 10% stool-phosphate-buffered solution suspension with a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA extracts were subjected to real-time reverse transcription-polymerase chain reaction (RT-qPCR) or stored at −80°C until further use.

RT-qPCR was used for initial sample testing for norovirus GI and GII detection with genogroup-specific primers and probes as previously described.[12,19] Real-time RT-PCR was carried out using a One Step PrimeScript RT-PCR Kit (DRR064; TaKaRa Biotechnology) with the ABI 7500 (Applied Biosystems). The reaction was conducted with an initial RT step at 42°C for 30 minutes, followed by 95°C for 5 minutes and 40 cycles of qPCR at 95°C for 5 seconds and 55°C for 35 seconds.

### 2.4 | Genomic amplification for genotyping

Samples that tested positive for norovirus by RT-qPCR were analyzed using two PCRs directed at region A of ORF1 (the 3′ end of the polymerase gene) using the primers JV12Y/JV13I,[20] and at region C in ORF2 (the 5′ end of the capsid gene) using the primers G1SKF/G1SKR for GI and G2SKF/G2SKR for GII.[21] RT-PCRs were carried out using a One Step RNA PCR Kit (TaKaRa Biotechnology) with the amplification conditions of previous literature.[12] The expected amplicon size was 327 bp for region A, 330 bp for GI, and 344 bp for GII in region C. Fragment spanning the ORF1/ORF2 overlap region was amplified using the primers JV12Y and G1SKR/G2SKR for potential recombinant norovirus strains. RT-PCR was carried out using a One

Step RNA PCR Kit (TaKaRa Biotechnology). RT-PCR conditions were as follows: RT at 50°C for 30 minutes and denaturation at 95°C for 2 minutes, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 48°C, and 1 minute at 72°C. A final elongation step was performed for 10 minutes at 72°C. The expected amplicon size was 1120 bp for GI and 838 bp for GII. PCR products were visualized by 1.5% agarose gel electrophoresis. The sequencing and the edition of the sequences were conducted by TaKaRa Biotechnology (Dalian, China), and that the same primers used in RT-PCRs were also used for sequencing.

### 2.5 | Phylogenetic analysis

Genogroup and genotypes analyses were conducted with the aid of the on line norovirus Genotyping Tool (https://www.rivm.nl/mpf/norovirus/typingtool)[22] and the strains were named according to the time of detection. Phylogenetic trees were drawn for both the partial polymerase and capsid gene sequences focused on the main three GII genotypes that dominate in Huzhou: GII.2[P16], GII.17[P17], and GII.4 Sydney [P31].[12,14,18] In total, 29 strains were subjected to a phylogenetic analysis using the MEGA software with the ORF1 (RdRp) and ORF2 (VP1). The phylogenetic analysis was constructed using the neighbor-joining algorithm with the Kimura two-parameter model and supported statistically by bootstrapping with 1000 replicates with MEGA software (version 7.0) with 1000 bootstrap replicates.[23] Recombination analysis was conducted using the SimPlot program (version 3.5).[24] Reference strains were downloaded from GenBank.

### 2.6 | Statistics analysis

Statistic analysis was performed by SPSS version 19.0 software with a significance level of .05 (*P* value).

### 2.7 | Nucleotide sequence accession numbers

The sequences of the norovirus strains obtained in this study were deposited in the GenBank under the accession numbers MN186385-MN186391 and MN210047-MN210082.

## 3 | RESULTS

### 3.1 | Norovirus infections and clinical features

In total, 100 (18.1%) specimens were identified as norovirus positive. GII genogroup was the main genogroup identified (83.0%; 83/100), followed by dual infection with GI and GII genogroup (10.0%; 10/100) and GI genogroup (7.0%; 7/100). The detection rate remains low between January and May, and from July to September. In June and from October to December, norovirus detection rate is high and reached a peak in December. The highest detection rate was 39% in December. In contrast, the lowest norovirus infection rate was in August (4.0%), when only two samples were positive (N = 50).

Infection with norovirus was found in all age groups tested (≤5, 6-15, 16-40, 41-60, and ≥60 years) (Table 1). The highest detection rate was in the 16 to 40 year age group (65/272; 23.9%). The female-to-male ratio was 1.04 (51:49) in norovirus-positive patients. The clinical features of the norovirus-associated AGE patients were watery stool, abdominal pain, vomiting, and fever. There was no statistically significant difference between norovirus-positive and -negative AGE patients in terms of sex ($\chi^2 = 0.026$; $P = .872$). Among the five age groups, the detection rate in age group 16 to 40 was highest and in age group less than or equal to 5 was lowest ($\chi^2 = 48.62$; $P = .001$). We studied the symptoms of GII.2[P16] positive AGE patients (N = 23). The most common symptom is watery stool (23/23; 100%), followed by abdominal pain (9/23; 39.1%) and vomiting (8/23; 34.8%). Only one conducted fever (>38°C).

### 3.2 | Genotyping and distribution of norovirus

All samples that tested norovirus positive by real-time PCR were classified according to genotype. In total, 42 samples (42.0%) were successfully sequenced and genotyped by RT-PCR (Table 2). Since one of the samples was dual infection, so we got 43 virus finally. Nine norovirus GII genotypes and four norovirus GI genotypes were detected in Huzhou during our research period. The main two norovirus GII genotypes circulating in the population were GII.2[P16] (23/43) and GII.17[P17] (5/43). There were 10 recombinant strains detected in Huzhou in 2018 (GI.5[P4], GI.6[P11], GII.4 Sydney[P31], GII.17[P17], GII.7[P6], GII.3[P12], GII.13[P16], GII.2[P16], GII.1[P16], and GII.4 Sydney[P16]). Recombinant strains GI.5[P4] and GII.1[P16] were detected in Huzhou for the first time. Sequences covering the ORF1-ORF2 junction were obtained to study recombination breakpoints using SimPlot software. Bootscan plot analyses confirmed that all recombination points were located close to the ORF1-ORF2 junction, which corresponds to the previously proposed hotspot for NoV recombination.[25,26] At the breakpoint position, NS18524 and NS18512 were divided into two segments, respectively.

**TABLE 1** Epidemiological features of patients with acute gastroenteritis (AGE)

| | Tested cases | Positive cases | Positive rate | $\chi^2$ | *P* |
|---|---|---|---|---|---|
| Sex | | | | | |
| Male | 274 | 49 | 17.9 | 0.026 | .872 |
| Female | 277 | 51 | 18.4 | | |
| Age group | | | | | |
| ≤5 | 36 | 2 | 5.6 | 48.62 | .001 |
| 6-15 | 21 | 3 | 14.3 | | |
| 16-40 | 272 | 65 | 23.9 | | |
| 41-60 | 118 | 19 | 16.1 | | |
| >60 | 104 | 11 | 10.6 | | |
| Total | 551 | 100 | 18.1 | | |

**TABLE 2** Genotype distribution of identified norovirus strains in Huzhou from January to December 2018

| Genogroup | Genotype | Number |
|---|---|---|
| GI | GI.2[P2] | 2 |
| | GI.4[P4] | 1 |
| | GI.5[P4] | 3 |
| | GI.6[P11] | 1 |
| GII | GII.4 Sydney[P31] | 1 |
| | GII.7[P6] | 2 |
| | GII.3[P12] | 1 |
| | GII.15[P15] | 1 |
| | GII.1[P16] | 1 |
| | GII.13[P16] | 1 |
| | GII.2[P16] | 23 |
| | GII.4 Sydney[P16] | 1 |
| | GII.17[P17] | 5 |
| Total | | 43 |

In 2018, the dominance of GII.2[P16] was evident, and detected almost every month except August and September. GII.4 Sydney [P31] only detected in one case in January 2018. GII.17[P17] can be detected at low activity throughout the year.

## 4 | DISCUSSION

In this study, we investigated the epidemiology of norovirus infections and the genetic diversity of norovirus strains circulating in the sporadic AGE cases in Huzhou, Zhejiang in 2018. The main findings were as follows: (a) a variety of GI and GII genotypes were identified during our research time; (b) norovirus GII.2[P16] strain outnumbered GII.4 Sydney[P31] as the the predominant genotype[15]; (c) norovirus-positive rate was relatively low in 2018 compared with previous years.[12-14,18]

Noroviruses demonstrate a high genetic diversity, which mainly contributed to antigenic drift, point mutations, and RNA recombination.[27] Recombination is one of the major driving forces of virus evolution and recombination points are usually located near or within the ORF1/ORF2 overlap.[25,26] Thus, it is necessary to genotyping both sequences to find new recombinant variants.[28] Norovirus strains circulate in Huzhou exhibits great genetic diversity and at least 13 genotypes were identified based on both RdRp and capsid genes, with most of them were recombinant strains.[12,15] We have been reported many recombinant strains in Huzhou before.[12,15] New recombinant strains have been detected almost every year in the past few years. The recombinant strains GI.5[P4] and GII.1[P16] were the newly detected in 2018. Predominant genotypes appear alternately every year.

GII.4 norovirus has been circulating in the Huzhou area since 2008.[12] From 2008 to the 2014, GII.4 accounted for the majority of both sporadic cases and outbreaks of norovirus-associated AGE.[12,13] In winter 2014 to 2015, a new GII.17 variant (GII.17 Kawasaki[P17]) was first detected in Huzhou, and unexpectedly replaced the previous circulating GII.4 strains.[14] GII.17 became the most commonly detected norovirus strain in the Huzhou area in early 2015. However, GII.4 Sydney[P31] re-emerged in October 2015[29] and presented an alternate prevalence pattern with GII.17 until the appearance of GII.2[P16] at the end of 2016. The resurgence of the GII.4 variant in the post-GII.17 period caused a sharply increase in the number of sporadic norovirus infection cases in Huzhou in 2016 to 2017.[15] In 2017, GII.2[P16], GII.4 Sydney[P31], and GII.17[P17] were the main three genotypes cocirculating at the same time. As for 2018, the dominance of GII.2[P16] was more evident, and detected almost every mouth except August and September. GII.4 Sydney[P31] only accounted for one case in January. GII.17[P17] detected at low activity throughout the year. And the norovirus-positive rate was greatly reduced in 2018 compared with the same period of 2017.[15] These results were not consistent with the finding in Shanghai[30] which showed that by the end of 2018 the prevalence of GII.4 still remained higher than any other types. But our results was consistent with those obtained in the outbreak cases,[31] in which the dominant genotypes were GII.2[P16], fallowed by GII.17[P17] and GII.4 Sydney [P31]. This discrepancy might be due to the difference in sampling techniques, area representativeness, and not all norovirus cases are genotyped.[30] The prevalence pattern of GII.2[P16] in Huzhou was not consistent with the findings in Alberta, Canada, where GII.2[P16] predominant in the first 6 month of 2017, but was replaced, thereafter, by GII.4 Sydney[P16].[32] We have detected GII.4 Sydney[P16] for one case in 2018 and two in 2017.[15] There was no sign shows that GII.4 Sydney[P16] will become the dominant genotype in Huzhou yet. There are significant regional differences in the prevalence of norovirus infection.[33,34] The reason for the difference may be the distinct geography, target population, and study design.[35,36]

As a non-GII.4 norovirus, the predominance of GII.17 strains did not last long. The "static genotypes" theory of non-GII.4 norovirus explains the short appearance of their dominance.[37] GII.4 strains as an "evolving genotype" which can continuous evolve through accumulation of mutations to have the ability to re-emerge. Suppose GII.2[P16] be the second one which can dominant only for a short time. Then, which one will be chosen to replace the GII.2[P16] and be the next winner in Huzhou. Maybe the next one is GII.4 Sydney[P16] just like Canada or maybe the next one is just the recurrent GII.4 Sydney[P31]. Further investigations and continuous monitoring are necessary.

Our previous research found that children (≤10 years) and elderly individuals (>60 years) were more likely to be infected with GII.4.[14] In contrast, GII.17 seemed more likely to infect people aged 16 to 60 years, previously considered as noncompromised.[29] The result of GII.2[P16] was the same with GII.17. It is interesting that the young adults seemed more susceptible to the non-GII.4 norovirus like GII.17 and GII.2[P16]. Why these non-GII.4 norovirus abandon the delicate children and elderly and attack the young adults? This is a question we cannot answer yet.

Our result is limited by a small number of samples, single-site setting and short time of sample collection. Genotyping was only

successful for 42 (42%) samples detected. More than half of the specimens are not genotyped beyond the GI and GII classification. So the genotype diversity and dominant genotypes may not be the real situation. The majority of the samples that could not be typed had *Ct* values more than 30. In future studies, epidemiologic and virological surveillance should be broadened to better clarify the epidemiological patterns and genetic characteristics of norovirus strains in Huzhou, China.

In this study, we analyzed norovirus-associated sporadic AGE in Huzhou from January to December 2018 in Huzhou, China. A decrease in detection rate was found in 2018 and norovirus GII genogroup was the predominant genogroup identified in norovirus-positive patients with AGE. The predominant genotype of norovirus during our study period was GII.2[P16] and the circulation pattern of norovirus was distinct in 2018 without the coprevalence of multiple genotypes as in 2017.[15] Norovirus strains circulate in Huzhou exhibits considerable genetic diversity during the past few years.[12-15] The predominant norovirus genotypes have changed from GII.4 Sydney [P31] and GII.2[P16] in 2017 to GII.2[P16] in 2018. Monitoring of the norovirus genotypic shift and variation is important to elucidate the molecular epidemiology of norovirus and prevention of AGE.

## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interest.

## AUTHOR CONTRIBUTIONS

LJ and LPC participated in the design of the study and performed the statistical analysis. GH and DSX participated in the Norovirus detection. XFW and DSX participated in the genomic amplification for genotyping. YF and LJ participated in the sequence analysis and phylogenetic analysis. LPC drafted the manuscript. All authors read and approved the final manuscript.

## ORCID

*Liping Chen* https://orcid.org/0000-0002-5801-7791

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