

Original Article

Association of recombinant GII.P16-GII.2 norovirus strain with increased norovirus outbreaks during February-March 2017 in Changzhou, China

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Received December 26, 2017; Accepted September 5, 2018; Epub November 15, 2018; Published November 30, 2018

Abstract: Norovirus is the leading global cause of epidemic gastroenteritis and responsible for more than 90% of all viral gastroenteritis as well as 50% of gastroenteritis outbreaks worldwide. During February-March 2017, a sharp increase in the number of norovirus outbreaks was reported by the Changzhou Center for Disease Control and Prevention in China. A total of 250 anal swabs were collected from 24 outbreaks, and the genotypes were determined by sequencing analysis. The genetic diversity, epidemiological status, and characteristics of the norovirus strains were analyzed. A novel recombinant GII.P16-GII.2 norovirus strain was identified as the primary cause of these outbreaks. Phylogenetic trees showed that the novel GII.P16-GII.2 strains were similar to the GII.P16-GII.4 strains (GenBank accession nos. KX907727.1, USA, 2015; LC175468.1, Japan, 2016) for *RdRp* gene. It also showed similarity to the GII.P16-GII.2 recombinants (GenBank accession no. LC145787.1, Japan, 2012; KJ407074.2, USA, 2011) for *VP1* gene. Thus, the novel GII.P16-GII.2 recombinants might have evolved from GII.P16-GII.4 strains in 2015-2016 and GII.P16-GII.2 in 2011-2012.

Keywords: Norovirus, epidemic gastroenteritis, genetic diversity

Introduction

Noroviruses, the leading cause of nonbacterial acute gastroenteritis outbreaks [1], are highly contagious and capable of rapid spread. Noroviruses are positive-sense, single-stranded RNA viruses within the Caliciviridae family [2]. The genome contains three open reading frames (ORFs). ORF1 encodes the nonstructural proteins, including a RNA-dependent RNA polymerase (*RdRp*), ORF2 encodes a major capsid protein (viral protein 1 [*VP1*]), and ORF3 encodes a minor capsid protein (*VP2*) [3]. According to the *RdRp* and *VP1* gene sequences, noroviruses are classified into seven genogroups (GI-GVII) [4]. GI, GII, and GIV noroviruses can infect humans; GI and GII viruses are the most common and include a minimum of thirty-one distinct genotypes [4].

Reportedly, the GII.4 has been the most common norovirus genotype circulating worldwide

since 2002 [4, 5]. Similarly, in Guangdong, as well as the other regions of China, GII.4 noroviruses have caused the majority of outbreaks. Several other genotypes, such as GI.3, GI.6, GII.6, and GII.21, were occasionally detected in sporadic cases; however, they rarely caused large outbreaks [6].

Furthermore, during the winter of 2014-2015, in some parts of Asia, a new GII.17 strain emerged as the major cause of acute gastroenteritis outbreaks [7-9], suggesting that a non-GII.4 norovirus might become the predominant genotype. In winter 2014-2015, a new GII.17 variant (GII.P17-GII.17 Kawasaki) was first identified in Guangdong; it caused a substantial increase in the number of acute gastroenteritis outbreaks [8]. Epidemics caused by this lineage were detected almost simultaneously in several other provinces of China, and sporadic cases were reported worldwide [10]. After June 2016,

the number of GII.17 outbreaks decreased and were replaced by GII.4 outbreaks.

Surprisingly, in late 2016 in China, the number of norovirus outbreaks increased significantly in the same period during the previous four years (56 in 2016 vs. 6 in 2013, 11 in 2013, 36 in 2014, and 14 in 2015). A recombinant GII.2 virus caused 79% (44/56) outbreaks in 2016 [3]. Since then, the sharp increase in the number of outbreaks and the unique GII.2 genotype has been under intensive focus.

During February-March 2017, the number of norovirus outbreaks increased sharply in the Changzhou city, China. Several hundred students from many schools developed symptoms of diarrhea and vomiting. An epidemiological investigation was conducted to determine the cause of the pathogen, infection sources, route of transmission, and risk factors. In the present study, the genetic diversity, epidemiological status, and characteristics of the norovirus strains were analyzed.

Materials and methods

Case definitions

According to the scheme for monitoring the aggregation and outbreak of viral gastroenteritis in Jiangsu Province (2017 edition), acute gastroenteritis outbreaks were defined as follows: more than 20 patients with vomiting and diarrhea were associated with a common source of infection within 1 week or ≥ 1 cases of deaths. In the absence of mortality, at least 2 cases were confirmed by laboratory diagnosis.

Specimen collection

According to the Noroviruses Monitoring Technology Plan in Jiangsu province, anal swabs from cases and healthy controls were collected during each outbreak. These samples were transported to our laboratory under cold conditions ($<0^{\circ}\text{C}$) within 24 h, and all the samples were tested within 24 h. In this study, a total of 250 anal swabs were collected from 24 outbreaks during February-March 2017. The present study was approved by the Ethics Review Committee of the Changzhou Center for Disease Control and Prevention.

Source of epidemiological data

In China, all acute gastroenteritis outbreaks must be reported to the Infectious Disease Report Information Management System and Emergency Public Health Event Management Information System. In the present study, information was collected using a standardized form that included basic demographic information (sex, date of birth, and address), case classification (probable or confirmed), severity (mild or severe), death status, date of onset of symptoms, date of diagnosis, date of death (if applicable), and virus type for confirmed cases.

Detection of viral RNA from specimens

Viral RNA was extracted from 50 μL anal swabs using the MagMAXTM-96 Viral RNA Isolation Kit and MagMAXTM Express (Thermo Fisher, USA) according to the manufacturer's instructions. The RNA from each sample was quantified using a commercially available real-time PCR kit (Diagnostic Kit for Human Noroviruses, Jiangsu Shuoshi Biological Technology Co., Ltd, Taizhou, China) according to the manufacturer's protocols. The results were classified into two categories: GI-positive or GII-positive.

The norovirus-positive RNA was subjected to RT-PCR using the OneStep RT-PCR kit (Qiagen, Hilden, Germany); the *VP1* and *RdRp* genes of norovirus were amplified by RT-PCR assays. The primers used for *VP1* were as follows: forward, 5'-CTGCCCCGAATTGTAAATGA-3' and reverse, 5'-CCAACCCARCCATTRTACA-3'; whereas, those for the *RdRp* gene were as follows: forward, 5'-GTGCACACTGCAGCAGCC-3'; reverse, 5'-CGTCATTCGACGCCATCTTCAT-3', as described previously [3, 11]. The RT-PCR was performed under the following conditions: a reverse transcription step at 50°C for 30 min and PCR involving an initial activation step at 95°C for 15 min, followed by 40 cycles each at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. Subsequently, the PCR products were analyzed by electrophoresis on 1% agarose gels. The second PCR reaction was carried out using Q5 (Qiagen, Hildesheim, Germany) to elevate the DNA concentration. The resulting amplicons were subjected to direct sequencing. The nucleotide sequences of these samples were

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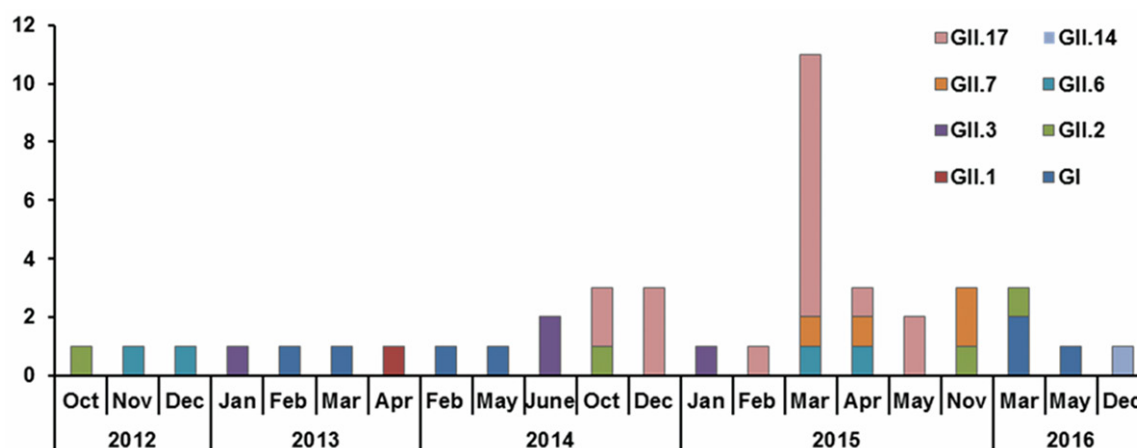


Figure 1. Reported monthly GI and GII norovirus outbreaks, Changzhou, China, 2012-2016.

Table 1. Norovirus detection in acute gastroenteritis outbreaks, Changzhou, China, 2012-2016

Detection	Outbreaks, no. (%)				
	2012	2013	2014	2015	2016
Total norovirus	38 (66.7)	28 (59.6)	68 (59.7)	111 (52.9)	50 (43.9)
GII.2/GII.2 mixed*	19 (50.0)	0 (0.0)	9 (13.2)	9 (8.1)	14 (28.0)
Non-GII.2	19 (50.0)	28 (100.0)	59 (86.8)	102 (91.9)	36 (72.0)
Total acute gastroenteritis	57	47	114	210	114

*Outbreaks involving either only GII.2 or GII.2 with other GI or GII genotypes.

submitted to the GenBank database with the accession numbers MF149067 to MF149079 and MF737444 to MF737456 for VP1 and RdRp genes, respectively.

Genotyping and phylogenetic analysis of noroviruses

To construct a phylogenetic tree, different genotype sequences of reference GII.P16 and GII.2 strains, first reported by various countries or regions, were obtained from the GenBank database at the National Center for Biotechnology Information (NCBI). In the present study, a total of 138 GII strains' sequences, were analyzed to determine the evolution of the viruses. The sequences of these strains were screened using the online Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to search for the maximally similar sequences. Multiple sequence alignments were performed using the ClustalW program. The robustness of the groupings was assessed using bootstrap resampling of 1,000 replicates, and phylogenetic trees were examined using the MEGA 7.0 program. All nucleotide

sequences of capsid were genotyped using the Norovirus Automated Genotyping Tool (www.rivm.nl/mpf/norovirus/typingtool). The sequence data for the reference strains were downloaded from GenBank.

Results

Epidemiological features

In Changzhou city, norovirus outbreaks are highly seasonal; a majority (>90%) are reported during October-March. During 2012-2016, a total of 43 outbreaks occurred in Changzhou. 7/43 (16.3%) outbreaks were caused by GI noroviruses, while 36/43 (83.7%) were GII, including GII.1, GII.2, GII.3, GII.6, GII.7, GII.14, and GII.17. Notably, a GII.17 variant [18/24 (75%)] was identified in Changzhou that was predominant during the October 2014-May 2015 norovirus seasons. After November 2015, the number of GII.17 outbreaks decreased (**Figure 1**). Overall, during 2012-2016, the GII.2 strains norovirus had been detected only sporadically (**Table 1**). A distinct difference was observed in the annual GII norovirus infections in different

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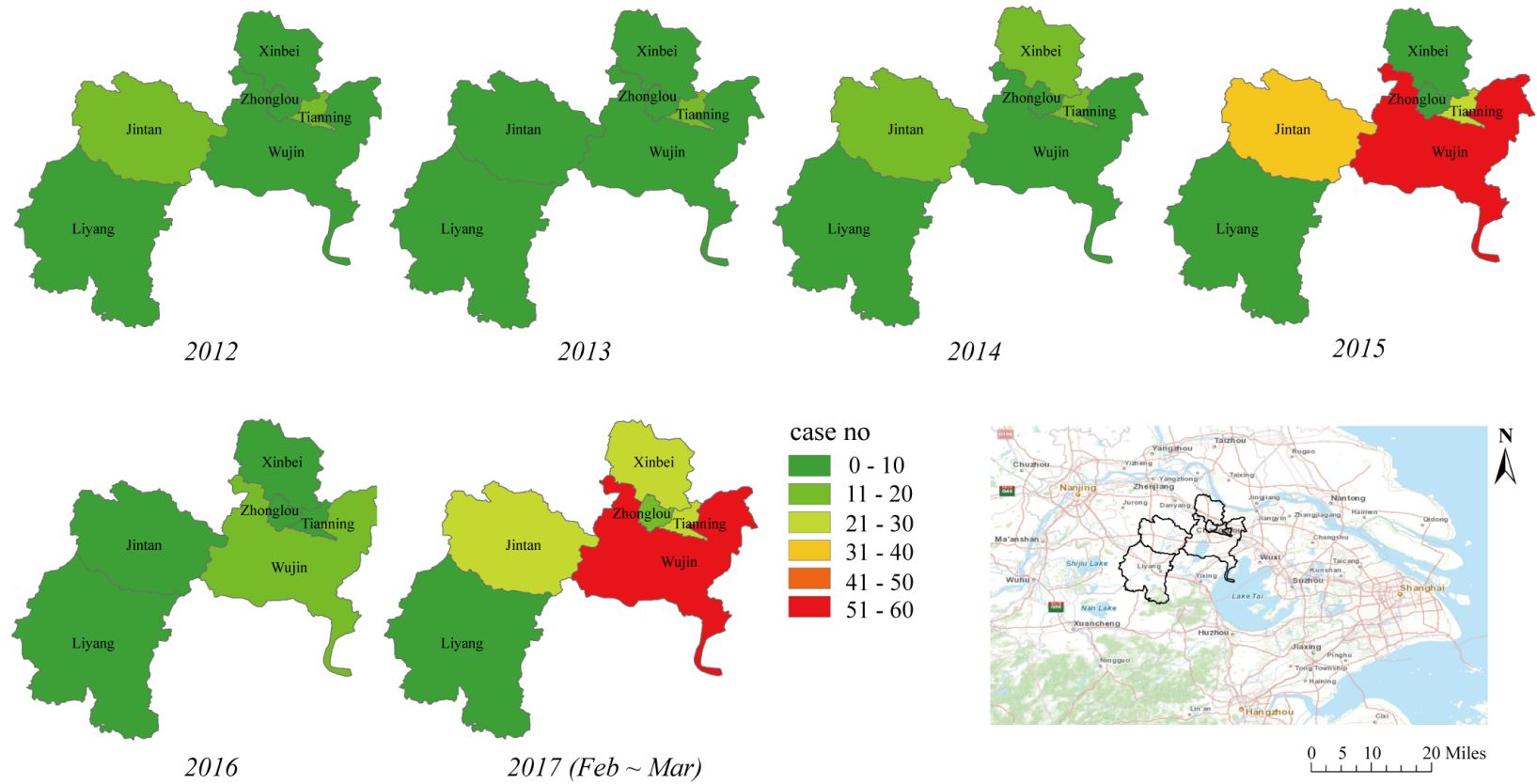


Figure 2. Annual GII norovirus infections in the districts of Changzhou city from 2012 to 2017.

Table 2. Settings of norovirus outbreaks, Changzhou, China, February-March, 2017

Setting	Outbreaks, no. (%)	Positive specimens, no. (%)
School		
Kindergarten	9 (42.9)	62 (70.5)
Primary school, grades 1-6	7 (33.3)	46 (59.0)
Junior high school, grades 7-9	2 (9.5)	11 (52.4)
Senior high, grades 10-12	2 (9.5)	12 (80.0)
Children welfare institution	1 (4.8)	10 (90.9)

districts of the Changzhou city. From 2012 to 2014, as well as 2016, the infections in each district were low with average severity. However, in 2015, the GII norovirus infections were increased as a whole; especially in the Wujing and Jintan districts (**Figure 2**).

However, a steep rise in the norovirus outbreaks, from February to March 2017, was reported in Changzhou, which was higher than the past five years. The majority of the infections also occurred in the Wujing district, and secondly in Jintan, Xinbei, and Tianning (**Figure 2**). A total of 250 anal swabs were collected from 24 outbreaks during these two months. The genotypes were determined by sequencing analysis. GII genotypes caused the majority of outbreaks (23/24) and one outbreak (1/24) was associated with a GI genotype. Among the 23 GII-positive samples, the GII.2 (n=21), GII.14 (n=1), and GII.17 genotypes (n=1) were identified. Furthermore, GII.2-associated outbreaks mainly occurred in the school (95.2%). Especially, 42.9% of the GII.2 outbreaks occurred in kindergartens, 33.3% in elementary schools, 9.5% in junior high schools, and 9.5% in senior schools (**Table 2**). Further genotyping results showed that the 21 GII-associated outbreaks were caused by GII.P16-GII.2 norovirus.

Phylogenetic analysis

In order to establish the genetic relationships among these GII.P16-GII.2 norovirus strains, the sequences of the *VP1* (GII.2) and *RdRp* (P16) genes of representative strains, detected in this study, were analyzed and compared to the reference strains that were first reported by various countries and regions. Of the 138 GII.P16-GII.2 strains, complete *VP1* gene sequences and *RdRp* region of thirteen different sequences were amplified and sequenced;

these served as representative results.

The phylogenetic trees were inferred from GII.P16 *RdRp* and GII.2 *VP1* gene sequences. In the *RdRp* gene tree, all strains formed three major clusters. The GII.P16 (GenBank accession no. AY772730.1, Germany, 2000) and GII.P16-GII.17 (GenBank accession no. KJ196286.1, Japan, 2002) strains formed one cluster. GII.

P16-GII.3 recombinants (GenBank accession no. KF944110, Russia, 2011; KT779557.1, Russia, 2012), GII.P16-GII.13 (GenBank accession no KJ145322.1, Taiwan, 2013), as well as, GII.P16-GII.2 recombinants (GenBank accession nos. LC145787.1, Japan, 2012; KJ407074.2, USA, 2011) formed another cluster. The last cluster was formed by thirteen GII.P16 strains, identified in this study, and eight reference strains reported by various countries and regions. Strikingly, in the case of the *RdRp* gene, our GII.P16-GII.2 strains were more similar to the GII.P16-GII.4 strains (GenBank accession nos. KX907727.1, USA, 2015; LC175468.1, Japan, 2016) than to the GII.P16-GII.2 recombinants (GenBank accession nos. LC145787.1, Japan, 2012; KJ407074.2, USA, 2011) (**Figure 3A**).

In the *VP1* trees, the GII.2 strains formed two major clusters. The strains of USA in the years 1975 and 1976 formed one cluster. The other strains collected from the year 2005 to present formed another cluster. Interestingly, except the reference strains, the *VP1* gene of the strains, in this study, were more similar to the GII.P16-GII.2 recombinants (GenBank accession nos. LC145787.1, Japan, 2012; KJ407074.2, USA, 2011) (**Figure 3B**). Thus, it was speculated that these virus strains were mutated in the *RdRp* gene region and formed new GII.P16-GII.2 variations worldwide during the winter of the year 2016.

Discussion

Norovirus is a leading cause of gastroenteritis epidemics. Previous reports showed that GII.4/Sydney/2012 had been the major circulating norovirus genotype worldwide. However, during the late winter and early spring of 2014/2015, GII.17 became the predominant cause of norovirus outbreaks in China, and GII.2 norovirus

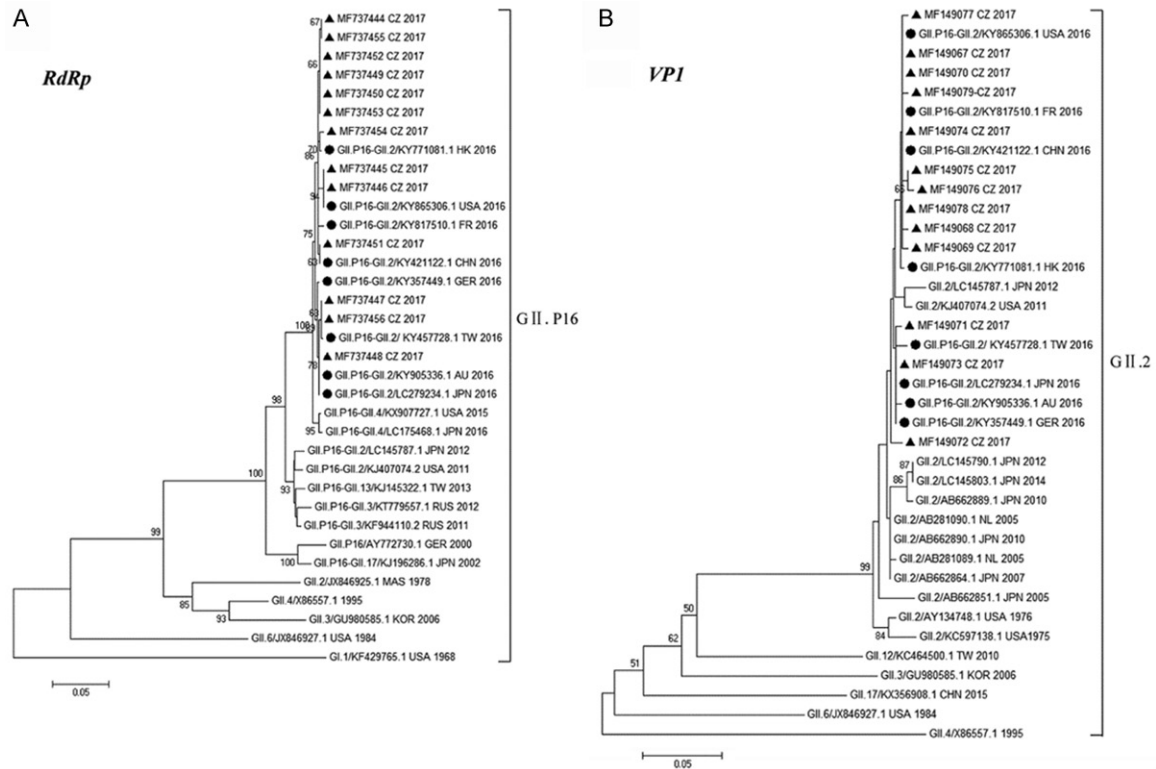


Figure 3. Phylogenetic trees for newly identified GII.2 noroviruses in Changzhou, China, Feb-Mar, 2017, reconstructed based on *RdRp* (A) and *VP1* (B) with a representative norovirus using the neighbor-joining method with datasets of 1,000 replicates in MEGA 7.0 software (<http://www.megasoftware.net>). For both trees, norovirus GII.2 reference sequences were downloaded from GenBank; accession number, country, and year are shown. Strains indicated by filled triangles (▲) and circles (●) are the current GII.P16-GII.2 strains and reference strains, respectively. PS: GER, Germany; HK, Hong Kong; JPN, Japan; TW, Taiwan; RUS, Russia; USA, United States of America; AU, Australia; CHN, China; CZ, Changzhou; KOR, Korea; MAS, Malaysia; RdRp, RNA-dependent RNA polymerase; VP, viral protein. Scale bars indicate the nucleotide substitutions per site.

was rarely found in every season [10, 12]. According to the statistics, a total of 43 outbreaks occurred in Changzhou during 2012-2016. GII, including GII.1, GII.2, GII.3, GII.6, GII.7, GII.14, and GII.17, was the most common (83.7%) pathogen in the outbreaks. Consistent with the other cities in mainland China, a GII.17 variant (75%) was identified as the predominant genotype during October 2014 to May 2015. Nevertheless, the timing and genetic subtype of viral diarrhea caused by norovirus in Changzhou were relatively consistent with that in the other cities in China. GII.2 strains norovirus had been detected only sporadically during 2012-2016. However, the recent outbreaks illustrated the potential of GII.2 to convert from a sporadically detected strain into an unprecedented dominating strain.

Abundant noroviruses caused gastroenteritis from February-March 2017 in Changzhou that was markedly higher than the previous years. Almost all these outbreaks occurred in kinder-

gartens and schools, and mostly in the Wujing district. The genotyping results showed that 21 outbreaks were caused by GII.P16-GII.2 norovirus. The emergence of novel GII.P16-GII.2 recombinant norovirus strains during the gastroenteritis outbreak was reported in Germany [13], Japan [14], and several other regions of China [15], several months ago. Similarly, during the 2016-2017 norovirus season in Germany, the GII.P16-GII.2 strains also were found in nearly half of the outbreaks (31/65), and the remaining were sporadic cases (29/65) [13]. During August to December 2016 in China, GII.P16-GII.2 recombinants were identified in 48 outbreaks [3].

Before GII.P16-GII.2 strains appeared, the GII.P16 polymerases were found in combination with other strains, such as GII.17, GII.4 Sydney (GenBank accession no. LC175468), GII.10 (GenBank accession no. KC110854), GII.3 (GenBank accession no. KF944110.2), GII.16 (GenBank accession no. AY682551), and GII.13

(GenBank accession no. KM036380) [16-19]. In 2014, GII.P16-GII.2 recombinant strains also were detected sporadically, accounting for only 2/170 (1.1%) GII strains in South Korea [19]. In Changzhou, the sharp increase in the number of outbreaks and the unique GII.2 genotype prompted us to further characterize these viruses.

We retrieved the sequences of *VP1* (GII.2) and *RdRp* (GII.P16) genes from GenBank. Eight GII.P16-GII.2 recombinants reported firstly in various countries and regions such as Germany, Japan, Australia, France, Taiwan, and China and so on were chosen as reference strains. The phylogenetic tree showed that for the *RdRp* gene, 13 GII.P16-GII.2 strains in our outbreaks were similar to the GII.P16-GII.4 strains (GenBank accession nos. KX907727.1, USA, 2015; LC175468.1, Japan, 2016). However, for the *VP1* gene, 13 GII.P16-GII.2 strains were similar to the GII.P16-GII.2 recombinants (GenBank accession nos. LC145787.1, Japan, 2012; KJ407074.2, USA, 2011). Thus, we speculated that the novel GII.P16-GII.2 recombinants might have evolved from GII.P16-GII.4 strains in 2015-2016 and GII.P16-GII.2 in 2011-2012.

In summary, GII.P16-GII.2 recombinant norovirus strain is primarily responsible for an epidemic in Changzhou China during spring 2017. Thus, a continuous surveillance is essential in order to assess whether the emerging new epidemic GII.P16-GII.2 norovirus strain can become the predominant strain. Furthermore, its effect on the size of the outbreak, duration of the disease, and herd immunity of the population are yet to be investigated.

Acknowledgements

This work was supported by the National Natural Science Foundation of Jiangsu province (No BK20151176), Jiangsu Provincial Medical Youth Talent (No QNRC2016309), Jiangsu Preventive Medicine Association Project (No Y2013016), Changzhou High-Level Medical Talents Training Project (No 2016CZBJ004), Changzhou Technology Science Project (No CJ20140042, No CJ20160037, No CE2016-5042) and Project of Changzhou Municipal Commission of Health and Family Planning (No QN201726, No WZ201610).

Disclosure of conflict of interest

None.

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