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Should we pay attention to recombinant norovirus strain GII.P7/GII.6?

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ABSTRACT

Background: Recombinant norovirus strain GII.P7/GII.6 has been circulating in Asia and around the world for at least 20 years, but has been responsible for relatively few outbreaks.
Methods: We used statistical analyses, real-time reverse transcription – PCR, and genome sequence analyses to investigate an outbreak of gastroenteritis, identifying the pathogen, the risk factors associated with the outbreak, and the molecular features of GII.P7/GII.6 strains.
Results: An outbreak of gastroenteritis was reported at a school involving 12 students and lasting 6 days, from September 13 to September 18, 2017. Epidemiological studies suggested that norovirus was transmitted from person to person and not via contaminated food or drinking water in this outbreak. Using a sequence analysis of the junction region between open reading frames 1 and 2, the pathogen was identified as a recombinant norovirus (strain GII.P7/GII.6).

The full-length genome of the outbreak strain shared 86%–97% identity with those of other GII.P7/GII.6 strains. Phylogenetic trees were constructed from partial open reading frame 1 (ORF1) and ORF2 sequences from the outbreak strain and GII.P7/GII.6 norovirus sequences available in GenBank. On the ORF1 tree, the partial sequences of ORF1 were grouped into cluster A (with GII.6), cluster B (with GII.7), and a separate cluster (C), based on the GII.6 and GII.7 reference strains. The ORF2 tree showed all GII.P7/GII.6 strains formed a cluster together with GII.6 strains. Amino-acid substitutions and insertions/deletions were common in the capsid protein, especially in it's P2 and P1 domains. The outbreak was controlled within several days using appropriate measures.
Conclusions: Because it may play a prominent role in future outbreaks, recombinant norovirus strain GII.P7/GII.6 should be monitored with routine surveillance.

Introduction

The genus *Norovirus* is highly diverse, and can be classified into seven genogroups (GI to GVII) and more than 40 genotypes [1,2] using a dual system based on the nucleotide sequences of the genes encoding the RNA-dependent RNA polymerase (RdRp) and VP1 [3]. Although norovirus infection is usually mild, it is the primary cause of nonbacterial acute gastroenteritis outbreaks and is responsible for nearly half of all cases of gastroenteritis. It is estimated that approximately 200,000 people die each year from norovirus infections, almost all of whom are children in the developing world [4]. Since 2002, GII.4 viruses have been the commonest genotype circulating globally [5,6]. *Norovirus* strain GII.P16-GII.2 has only been detected in the last decade [7], but has already caused epidemics in China and Japan [8,9] and spread to other countries [10,11].

Human norovirus has a ~7.5-kb single-stranded positive-sense RNA genome, which encodes three open reading frames (ORFs). ORF 1 encodes a large nonstructural polyprotein, which is cleaved by a viral protease (3C) into six nonstructural proteins p28, NTPase, p22, VPg, 3C-like protease (3CLpro), and RNA-dependent RNA polymerase (RdRp). ORF2 encodes the major capsid protein (VP1), and ORF3 encodes the minor capsid protein (VP2). VP1 can be structurally divided into a shell (S) domain and a protruding (P) domain. The P domain of VP1 bears the major antigenic sites and can be further subdivided into the P1 subdomain (residues 226–278 and 406–520) and the P2 subdomain (residues 279–405).

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The P2 subdomain is the target of neutralizing antibodies and interacts with histo-blood group antigen (HBGA) ligands to mediate viral entry into the epithelial cells of the gastrointestinal tract [12,13]. The P2 subdomain is the most exposed region on the viral particle and has been consistently identified as a site of polymorphic epitopes in epidemic norovirus GII.4 strains [14]. Additional solvent-exposed residues at VP1 positions 333, 340, 356, 368, 372, 407, and 412–413 are also predicted to be potential antibody epitopes [13,15]. Recombinant norovirus strain GII.P7/GII.6 has been detected in several countries. Amino-acid substitutions and deletions and insertions in the polyprotein, VP1, and VP2 are very common across both time and geographic regions [16–18]. A school in Fengtai district, Beijing, China, reported a gastroenteritis outbreak to the Fengtai Center for Disease Prevention and Control on September 17, 2017. Epidemiological studies, pathogen detection, and a bioinformatic analysis indicated that the incident was an acute outbreak of gastroenteritis caused by a recombinant norovirus strain, GII.P7/GII.6. Effective measures were taken and the outbreak was controlled within several days.

Methods

Case definition, epidemiological data, and sample collection

In this outbreak, clinical cases were defined as school students or staff members with at least one of the following symptoms over the period between September 13 and September 24: (i) diarrhea (watery or mushy stools) three or more times within 24 h, (ii) vomiting, (iii) nausea, (iv) fever, or (v) abdominal pain. A 'laboratory-confirmed case' was defined as a clinical case whose stool or rectal swab tested positive for norovirus with real-time reverse transcription (RT)–PCR.

A questionnaire was used to collect demographic information and information on clinical symptoms, the date of disease onset, and food and water consumption from September 10 to the end of the outbreak. Details of outpatient services, dates of recovery, and dates of specimen collection were also recorded. The questionnaire was administered to all clinical cases and to 20 intimate contacts of the cases. The intimate contacts were defined as individuals who had sat beside the cases in the classroom or had played with the cases within 5 days before the investigation or the teachers of the cases' class.

On September 18, medical workers collected two stool samples and 10 rectal swab samples from clinical cases, three rectal swabs each from intimate contacts of the cases, and six swabs from the desks and doorknobs of the classrooms. On September 19, stool samples were obtained from three clinical cases, one intimate contact of a case, and a pantry man employed by the food company that serviced the school.

RNA extraction

The surface swabs and rectal swabs were immersed in 2 mL of Dulbecco's modified Eagle's medium. A 1 mL suspension (10% w/v) of each stool sample was prepared in phosphate-buffered saline. RNA was extracted from a 140 μL sample of each stool suspension or swab solution with the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

Pathogen identification

The RNA samples were screened for the presence of *Rotavirus* and *Norovirus* genetic material using real-time RT–PCR detection kits produced by Jiangsu Shuoshi Bio-Tech Co., Ltd. (Jiangsu, China), according to the manufacturer's instructions. The ORF1/2 junction region of six norovirus-positive samples was PCR-amplified with the primers MON431 (5′-TGGACIAGRGGICCYAAYCA-3′) and G2SKR (5′-CCRCCNGCATRHCCRTTRTACAT-3′), yielding 544-bp amplicons. The complete genomic sequence of one virus was also determined (for primers and PCR conditions, see Supplementary Table S1). The amplicons were purified with the QIAquick PCR Purification Kit (Qiagen), following the manufacturer's instructions. The purified PCR products were sequenced with an ABI PRISM® 3730 DNA Analyzer by BeijingTianyi Bio-Tech Co., Ltd.

Sequence analyses

The sequences were genotyped with a web-based genotyping tool (http://www.rivm.nl/mpf/norovirus/typing tool) [16] and BLAST (http://blast.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed in MEGA 7.0 using the neighbor-joining method, with 1000 bootstrap replicates to determine branch support. A SimPlot analysis was performed, setting the window width and the step size to 200 bp and 20 bp, respectively [17]. Different methods implemented in the Recombination Detection Program v.4.16 (RDP4) were also used [21]. Amino-acid substitutions in the norovirus proteins were analyzed with a sequence alignment in ClustalW and BioEdit.

Statistical analyses

The spectrum of symptoms experienced by the cases over the course of the outbreak was summarized as frequencies and proportions. All statistical analyses were performed with SPSS 16.0 and MS Excel.

GenBank accession numbers

The nucleotide sequences determined in this study were uploaded to GenBank under accession numbers: MG674720–MG674725.

Ethics approval

No ethics approval was required because this study was undertaken as part of a public health response to an acute incident. However, each study participant (or their guardian) provided their verbal informed consent for their information and samples to be used in this study.

Results

General and demographic information

The school had six grades and nine classes, with 30–36 students in each class. The total enrolment was 280 children, aged 6–12 years, who were under the care of 22 staff members, including one school doctor. The lunch foods for both the students and staff were provided by a local food company. Lunches were distributed to students and staff room by room. All the students ate their lunches in their own classroom. Boiled water, cooled to a temperature of 35–40 °C, was provided for all students and staff for drinking. Both the classrooms and teachers' offices were located in the same two-story building. The school had two playgrounds: a small playground for grade 1 students and a larger playground for students in grades 2–6. The school's hygiene standards were overall very good, with sterilization and disinfection performed daily by a specialized company.

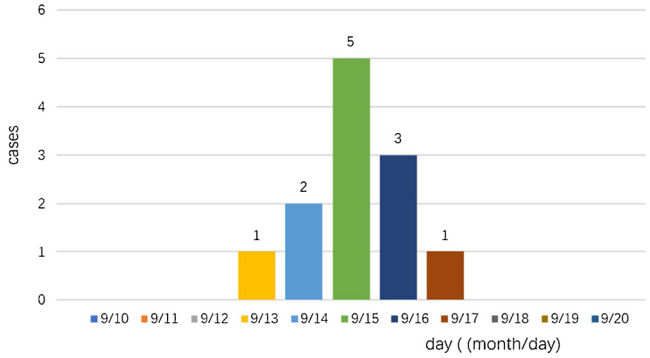

Fig. 1. Epidemiological data for the outbreak caused by norovirus GII.P7/GII.6. Graph showing the number of cases reported every day; the outbreak began on September 13, peaked on September 15, and ended on September 17.

Epidemiological analysis

From September 13–18, 12 students from three different classes reported symptoms of gastroenteritis. Ten cases occurred in one grade 1 class (class 1A), one case occurred in a second grade 1 class (class 1B), and one case occurred in a grade 4 class. The case with earliest symptom onset was a 6-year-old girl in class 1A. She reported abdominal pain around 09:00 on September 13, but insisted on attending her classes, and then had four bouts of diarrhea later that night. She felt well the morning of September 14 and went to school. Both the case and her parents (both of whom reported no symptoms) indicated that the student had not recently consumed suspected tainted water or foods, had not attended a social gathering within the last 5 days, and had not had close contact with anyone experiencing symptoms of acute gastroenteritis. Thereafter, other cases were reported to teachers, and the school doctor reported the outbreak to the Fengtai Center for Disease Control and Prevention on September 17. Details of the incidence of cases during the outbreak are shown in Fig. 1.

The mean age of the 12 cases was 6.5 years (6.5 ± 0.3). Six cases were boys and six were girls. Among the 12 cases, 11 (91.7%) experienced vomiting and other common symptoms of gastroenteritis, including nausea (75.0%), diarrhea (50.0%), abdominal pain (66.7%), and fever (33.3%). Most of the cases occurred in grade 1 class 1A (10/36 students), whereas one case was a girl in the nearby grade 1 class 1B and one case was a girl in grade 4 on the upper floor of the school. The girl in class 1B was familiar with the index case and with the girl in grade 4. There were 13 girls and 22 boys in class 1A. The first case sat near the door and most other cases mainly sat in the center of the classroom. Ten of the 12 cases (five girls and seven boys) studied in this one classroom. Seven cases received clinical treatment, but no students were hospitalized.

No risk factors associated with food or water were identified with epidemiological studies and statistical analyses (data not shown). All cases vomited in the school washroom or at their family home. The intimate contacts among the students in class 1A were not clearly identified, because they were very young and were unable to provide detailed recollections beyond a period of 2–4 days.

Identification of viral pathogens and their genome sequences

Two stool samples and eight rectal swab samples from cases and two rectal swab samples from close contacts tested positive for norovirus GII, whereas all desk and doorknob swabs were negative. *Rotavirus* was not detected in any sample. Six 544-bp amplicons spanning the ORF1/2 junction region were sequenced and one complete viral genome from the stool sample containing the highest amount of norovirus RNA was sequenced. The sequences of all six PCR amplicons were identical. The norovirus genotype was classified as GII.P7/GII.6 based on the sequence of the ORF1/2 junction region. The full norovirus genomic sequence (MG674720) determined in this study shared 99% nucleotide sequence identity with an isolate (KU935739) identified in 2015 in Zhengzhou, China; 97% identity with an isolate (KX268709) from the USA; 90% similarity with an isolate (MF140680) from the Netherlands; and 86% similarity with an isolate (KX752057) detected in 2009 in Beijing, China (data not shown). The sequence similarities of the 368-bp ORF1/2 junction region were 95% between the norovirus strain detected in this outbreak (MG674720) and the strain associated with an outbreak in Australia (KT970473), and 92% between the strain MG674720 and a strain detected in an outbreak in Brazil (KR074172) [18,19], as calculated with BLASTn.

Phylogenetic and recombination analyses

The nucleotide sequences of ORF1 and ORF2 of the outbreak strain were classified as two distinct genotypes, GII.P7 and GII.6, with an automated genotyping tool. The sequence of the ORF1/2 junction region of one isolate (Fengtai5 MG674721) was analyzed further with SimPlot to identify sites of potential genomic recombination. ORFs 1 and 2 of MG674721 shared a high degree of sequence homology with GII.P7 strain (AB258331) and the GII.6 strain (AB039778), respectively. The recombination breakpoint was located near the ORF1/2 overlap region at position 750 of MG674721, where the two strains had identical sequences (Fig. 2). A SimPlot analysis revealed the presence of a recombination breakpoint at positions 170–180 in the sequences (Fig. 2). This position corresponds to nucleotides (nt) 5018–5028 of reference strain JX989075. The same recombination breakpoint was also detected with the RDP4 program. The recombination event was confirmed as a significant ($p < 0.01$) with a Bootscan analysis in both the SimPlot and RDP4 programs (Fig. 2).

We constructed two phylogenetic trees from the partial ORF1 and ORF2 sequences of the GII.P7/GII.6 norovirus strains available in GenBank (information regarding these sequences is shown on the tree). The partial sequences of ORF1 were grouped into cluster A (with GII.6), cluster B (with GII.7), and a separate cluster C, based on the GII.6 and GII.7 reference strains. Cluster A included the outbreak strain, strains from the USA, China, and Thailand, and GII.6 strains (GenBank: AB682736), whereas cluster B included strains from Spain, the Netherlands, and China, as well as GII.7 strains (GenBank: AF414409),two isolates from Thailand formed another cluster C which distant from clusters A (GII.6 strains) and B (GII.7 strains) (Fig. 3a). On the ORF2 tree, all GII.P7/GII.6 strains formed a cluster together with GII.6 strains (Fig. 3b).

Amino-acid substitutions

Several conserved positions in four other sequences (B to E) differed from the corresponding sites in the Fengtai outbreak strain(A): position 70 (R to K), position 73 (I to T), position 490 (T to I), position 750 (T to A), and position 787 (I to T) (Table 1). The strains A, B, and D showed a high amino acid identity in VP1, whereas strains C and E differed from them and from each other. The sequence variation was high in VP1, especially in the P2 subdomain (residues 279–405), which is known to interact with neutralizing antibodies and HBGA ligands. There were only two substitutions between the sequence A and B, and four substitutions between the sequence A and D in VP1 and VP2. Three insertions and many substitutions were observed between A, B, and D with C strains, and two insertions and many substitutions were also observed with sequence E both in the P1 domains. Substitutions were also common in the P2 domain between the A, B, and D with

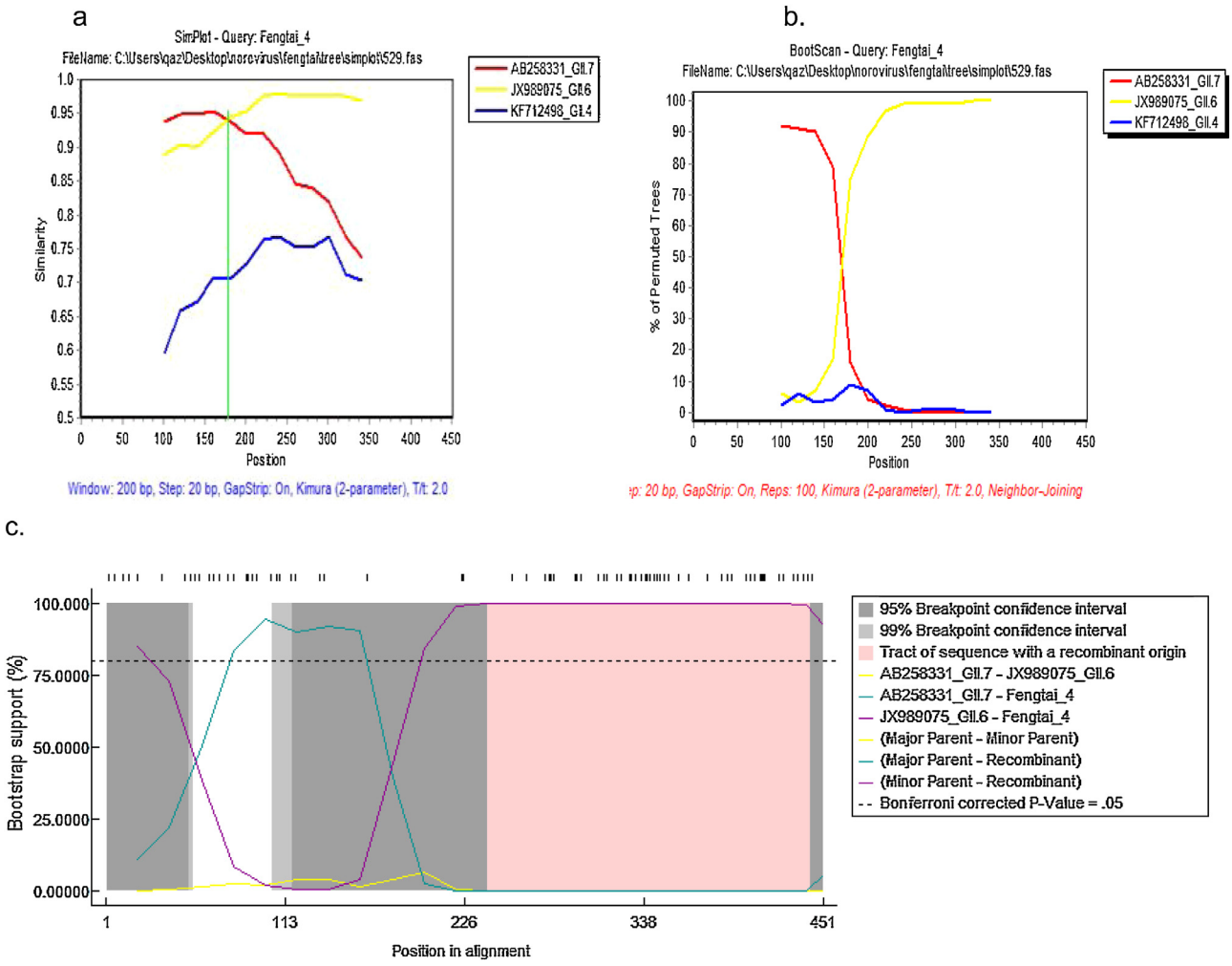

Fig. 2. SimPlot and Bootscan analyses of recombinant norovirus GII.P7/GII.6 detected in the outbreak. (a) SimPlot analysis of recombinant norovirus GII.P7/GII.6 detected in the outbreak. On the similarity plot, the y-axis gives the percentage identity; and the site at which the two parental norovirus strains, genotypes GII.6 (JX989075) and GII.7 (AB258331), share equal identity with the recombinant (crossed by the vertical blue lines) is the predicted site of recombination. (b) Bootscan analysis of recombinant norovirus GII.P7/GII.6 detected in the outbreak using the SimPlot software version 3.5.1. The y-axis gives the percentage bootstrap support values of the permuted trees. GII.17 strain (KF712498) was used as the outgroup sequence. (c) Bootscan analysis of recombinant norovirus GII.P7/GII.6 detected in the outbreak, using the RDP software version 4.0. Bootscan evidence for the recombination event based on pairwise distances. In all Simplot and Bootscan analyses, a sliding window of 200-bp and a step size between plots of 20 bp were used.

C or with E strains (Fig. 4). The sequences of other strains shared low similarities with the Beijing 2009 isolate, and the sequence identity between the Fengtai outbreak strain and the Beijing 2009 strain was only 90% in VP1 (Fig. 4 and Table 1).

Control and prevention

All appropriate measures were taken according to known prevention and control strategies for norovirus [20]. Standard outbreak control measures were undertaken, such as hand washing, health education, and disinfection of the classroom and washroom. The cases were quarantined at home and were only allowed to return to class after an asymptomatic period of 3 days, whereas their intimate contacts were monitored by the school doctor.

Discussion

Recombinant norovirus strain GII.P7/GII.6 has been circulating in Asia for at least 20 years [21]. The GII.P7/GII.6 genotype has been identified throughout the world in the past 5 years, including in the USA [22], China, Japan, and Vietnam [23], South Africa and Burkina Faso [24,25], Uruguay [26], Australia [18], and Finland [27]. However, all of these noroviruses were detected in stools from cases of sporadic infection. To date, there have been only two reports of a GII.P7/GII.6 strain associated with a disease outbreak, in Brazil and Australia [18,19]. Here, we have reported the first outbreak caused by this recombinant strain in China, and the third such outbreak reported worldwide. Over the past 5 years, the number of norovirus GII.P7/GII.6 sequences available in GenBank has increased greatly, and most of them are from China. Therefore, routine surveillance must be undertaken to monitor this recombinant virus in China.

Norovirus can cause outbreaks in enclosed or semi-enclosed environments because it can spread through a variety of routes, including the fecal–oral route (usually through the ingestion of contaminated food or water), aerosol formation, and other intimate contacts, and can be stable for weeks outside the host. Human infection can be caused by as few as 18 virus particles [28,29]. In the outbreak described here, epidemiological analyses indicated that the food and water that the children had consumed were uncontaminated. The environment may have been contaminated, although we detected no norovirus in the desk or doorknobs swab. The risk factors for norovirus infection and the origin of this outbreak are not clear, but it was not transmitted by contaminated food or water. Close contact and aerosol formation may

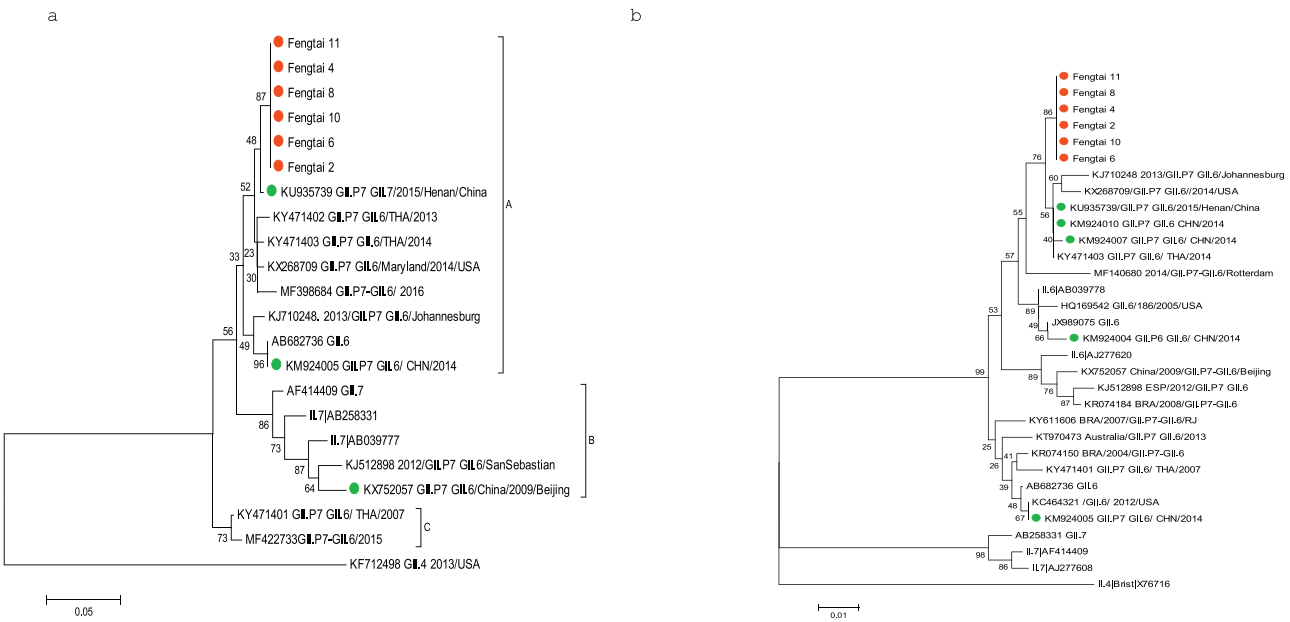

Fig. 3. Phylogenetic analyses of partial nucleic acid sequences of the norovirus GII.P7/GII.6 polymerase and VP2 genes. (a) Phylogenetic tree constructed based on partial ORF1 sequences (239 bp), nucleotides (nt) 4830–5068, of norovirus Fentai 4 (MG674720). (b) Phylogenetic tree constructed based on partial ORF2 sequences (219 bp), nt 5080–5298, of norovirus Fentai 4 (MG674720). The trees were constructed with the neighbor-joining method with a bootstrap analysis of 2000 replicates, using MEGA version 6.0. Bootstrap values >70% are shown. References strains of norovirus genotypes are named according to GenBank, with their respective accession numbers. A GII.4 strain was used as the outgroup sequence. The scale bar at the bottom of the tree indicates distance. The red dots show the sequences from the outbreak, and the blue dots show the other sequences from China.

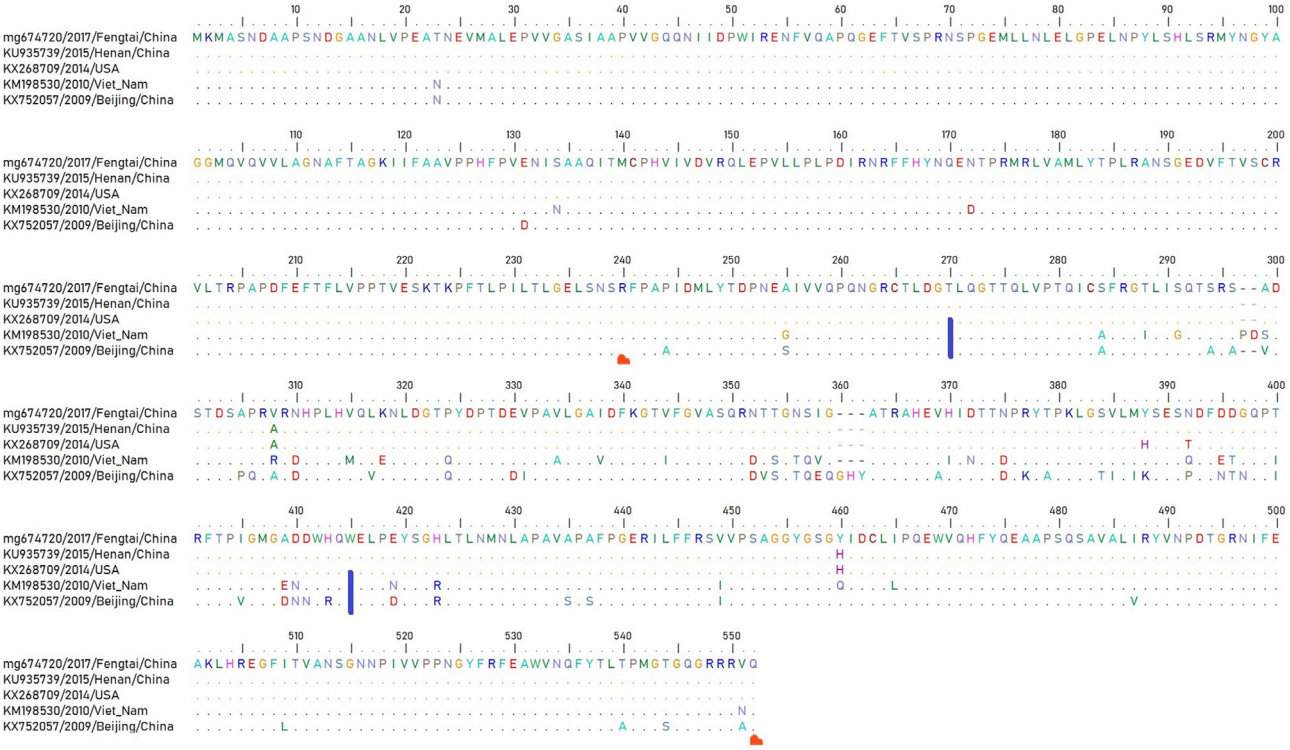

Fig. 4. Mutations in the VP1 proteins of the GII.P7/GII.6 norovirus strains. Amino-acid mutations and insertions/deletions among the isolates are very common in the P1 and P2 domains. indicates the P1 domain (not including the P2 domain); indicates the P2 domain.

have been the most plausible transmission routes. Timely intervention no doubt helped to reduce the extent and duration of the outbreak.

Over the 4 days from the onset of symptoms in the first case to the time that control measures were implemented, the outbreak involved only 12 cases. All of the cases occurred in just three classes, and 10 of 12 cases occurred in a single class. Therefore, recombinant norovirus strain GII.P7/GII.6 may not be easily transmitted or some of the students may have been protected by preexisting antibodies induced by previous infections with other norovirus genotypes within the preceding few years. This may have prevented a larger outbreak.

Table 1
The amino acids mutations in RDRP, VP1 and VP2 protein among several GII.P7/GII.6 norovirus strains.

Recombinant norovirus strains	Amino acids substitutions, deletions and insertions														
	RDRP				VP1				VP2						
	A (1697aa)	B (1697aa)	C (1697aa)	D (1697aa)	E (1697aa)	A (547aa)	B (547aa)	C (550aa)	D (547aa)	E (549aa)	A (257aa)	B (258aa)	C (−)	D (258aa)	E (−)
A	0[a]					0					0				
B	6	0				2	0				5(1 − :S)	0			
C	13	20	0			54(3 − :GHY)	54(3 − :GHY)	0			/	/	/		
D	13	7	42	0		4	2	54(3 − :GHY)	0		6(1 − :S)	3	/	0	
E	44	38	57	42	0	38(2 − :PD)	38(2 − :PD)	39(2 − :PD)	55(2 − :PD;3 + GHY)	0	/	/	/	/	0

A:MG674720/2017/fengtai/Beijing/China.
B:KU935739/2015/Henan/China.
C:KX752057/China/2009/Beijing.
D:KX268709/2014/USA.
E: KM198530/2010/VNM.
− is the deletions in the alignment. + is the insertion in the alignment. / is the number can not obtained for C norovirus strains without the VP2.
The amino acids substitutions, deletions and insertions calculated by align two or more sequences by Blastp,the sequences in the rows were the query sequences.
[a] The number of the mutations, including the substitutions, insertions and the deletions.

Bruggink et al. reported that the GII.P7 sequences of the GII.P7/GII.6 and GII.P7/GII.7 isolates fall into two clusters, with one cluster corresponding to the GII.6 ORF2 genotype and the other cluster to the GII.7 ORF2 genotype [18]. In our study, the sequences of the GII.P7/GII.6 isolates also had these characteristics based on trees constructed from partial sequence of ORF1, the tree subdivided into three or two clusters, clustered with GII.6 (A cluster), GII.7 (B cluster) or separate (C cluster), while the tree based ORF2 subdivided into A and B clusters (Fig. 3). The sequences of the Australian outbreak strains reported by Bruggink et al. were included in two clusters on the ORF2 tree: All the GII.P7/GII.6 strains included sequences from Australia (KT970473) and the Fengtai outbreak strain together with GII.6 strains formed a cluster (Fig. 3b). All these data indicate that the evolutionary origin of the GII.P7/GII.6 noroviruses differ from that of the GII.P7 noroviruses described by Bruggink et al. [19].

There have been many studies of the norovirus nomenclature and genotyping [32,19], and many studies of the definition of the GII.4 variants within the genotypes [30,31]. Chan-It et al. showed that the major capsid protein sequences of the GII.6 noroviruses isolated in 2008–2009 in Japan formed three subclusters with other norovirus sequences from GenBank [32], but no specific criteria that can be applied to classify other norovirus strains within genotype. In our study, we subdivided the GII.P7/GII.6 noroviruses into different clusters according the GII.7 and GII.6 reference strains. More practical criteria with which to classify the noroviruses within a single genotype are urgently required to understand their rapid evolution. The constant generation of genetic and antigenic diversity among noroviruses may allow them to persist in human populations, with epidemic potential developing in different norovirus genotypes [32,33]. Amino-acid differences occurred predominantly in the VP1 P2 domain (10%–17.5% change), with 53 hotspots of variation across the P domain and 15–24 substitutions in the different groups [34]. Our data also show that there were several insertion/deletion sites and substitutions in the P1 and P2 domain of the strains from different areas compared with the Fengtai strains. These results also suggest that the recombinant strain is evolving and driving antigenic change over time to allow it to evade the host immune responses and to achieve long-term stability in the environment. If so, this strain has the potential ability to predominate in a specific region over a certain period, and may even give rise to outbreaks or a pandemic in the future.

Conclusion

In this study, we identified the first outbreak of acute gastroenteritis caused by human recombinant norovirus strain GII.P7/GII.6 in China. Norovirus strain GII.P7/GII.6 is evolving quickly, and more attention must be paid to this recombinant virus. The classification of the GII.P7/GII.6 virus must be rationalized. Currently, all publications use their own definitions, which is very confusing.

Authors' contributions

XGD, MQ, Z-EW, and HRF performed the experiments; XXY, QRW, XXW, and HY gathered epidemic and analyzed the data; JSL conceived the study, analyzed and checked all the data, and drafted the manuscript; and JL conceived the study. All authors reviewed the manuscript and approved its publication.

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had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests

All authors declare that they have no competing interests and that there are no other conflict interests.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jiph.2018.12.007.

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