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ORIGINAL ARTICLE

An outbreak of multiple norovirus strains on a cruise ship in China, 2014

X. Wang[1], W. Yong[1], L. Shi[1], M. Qiao[1], M. He[1], H. Zhang[1], B. Guo[1], G. Xie[1], M. Zhang[2], M. Jin[3] and J. Ding[1]

1 Nanjing Municipal Center for Disease Control and Prevention, Jiangsu, China
2 Gulou District Center for Disease Control and Prevention, Nanjing, Jiangsu, China
3 National Institute for Viral Disease Control and Prevention, China CDC, Beijing, China

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Correspondence
Jie Ding, Zizhulin 2, Nanjing 210003, China.
E-mail: yu2an2002@163.com

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Abstract

Aims: To determine the cause of an outbreak of acute gastroenteritis that occurred on a cruise ship sailing along the Yangzi River from Chongqing to Nanjing, China.

Methods and Results: Noroviruses were identified by reverse transcription-PCR (RT-PCR) in rectal swabs from 34 of 54 subjects tested (63·0%). Sequencing and genotyping showed that noroviruses of up to seven different genotypes circulated in this outbreak: noroviruses GI.1, GI.2, GI.3, GI.4, GI.8, GI.9 and an uncommon strain GII.17. Common genotypes were not identified in this event. None of the food or water samples were tested positive for noroviruses.

Conclusions: We suspected that it was a point-source infection due to contaminated water or food harvested from contaminated water, taking account of the co-existence of diverse norovirus genotypes.

Significance and Impact of the Study: In this study, we presented the molecular investigation of a norovirus outbreak on a cruise in China. We revealed that the outbreak was caused by several different norovirus genotypes and analysed the possible source of infection as well, thus facilitating the evaluation of epidemiological issues regarding noroviruses in this area.

Introduction

Noroviruses have become the most common cause of viral acute gastroenteritis worldwide, with a significant ability to cause epidemic outbreaks in such settings as homes, hospitals, child-related settings, catering establishments, and cruise ships (CDC 2002; Medici *et al.* 2009; Domenech-Sanchez 2011). In these semi-closed or closed communities, noroviruses are highly contagious and spread very easily among people through modes of transmission other than food, including water, person-to-person contact and environmental contamination. Aerosols arising from vomiting episodes are known to transmit noroviruses (Marks *et al.* 2000, 2003). Despite the strict hygiene protocols and improved sanitary practices, norovirus-associated acute gastroenteritis continues to be one of the most frequently encountered problems on cruise ships in recent years (Isakbaeva *et al.* 2005; CDC 2007; Neri *et al.* 2008; Heijne *et al.* 2009; Mouchtouri *et al.* 2010). According to the data from Vessel Sanitation Program, U.S. Centers for Disease Control and Prevention (CDC), noroviruses account for more than 90% of recent gastroenteritis outbreaks with a known aetiology (http://www.cdc.gov/nceh/vsp/surv/gilist.htm). On cruise ships, one issue related to the norovirus outbreaks is the close proximity of people due to shared living quarters, dining areas and facilities, which creates favourable conditions for the occurrence of new outbreaks. Food and drinks contaminated before loading or after unloading may become a common source of infection.

In April 2014, an outbreak of acute gastroenteritis occurred during consecutive voyages of a cruise ship sailing along the Yangtze River, calling at ports in

Wuhan, Jiujiang, Chizhou and Nanjing. The outbreak started on the early morning of 20 April 2014, with persons being affected by nausea, vomiting, abdominal cramps and diarrhoea. The number of subjects with similar gastrointestinal symptoms sharply increased between 8 pm April 20 and 4 am April 21 while the cruise was heading for Nanjing. The ongoing gastroenteritis outbreak was reported and an investigation was initiated to ascertain the causative agents and the possible sources of infection. The control measures included sealing up the food and conducting sanitation and disinfection procedures in the galley, public areas and the medical office. Symptomatic persons were transferred to a local hospital for treatment according to symptoms. The outbreak ceased during the afternoon hours on 21 April with no further cases following. Noroviruses became the suspected etiology on the basis of symptoms. Here, we present the molecular identification of the strains and discuss the possible cruise-ship-related source of infection in this event.

Materials and methods

Epidemiological investigation

From the time the outbreak was reported, Gulou District Center for Disease Prevention and Control inspected the ship docking at Wumadu wharf in the city of Nanjing. Probable cases were defined as those with an onset of three or more episodes of loose stools, or two or fewer episodes of loose stools plus one or more episodes of vomiting within a 24-h period. Epidemiological data were available from standardized case report forms. The reports included basic information of the patients such as name, sex, age, occupation, residency, symptoms, date of onset, duration of illness. Patients were also interviewed to find out food consumption data on the evening of 19 April assuming an incubation period of 6–48 h, and the connection with ill persons on the cruise ship.

Laboratory investigation

Although norovirus was implicated as the etiologic agent based on symptoms, all specimens were processed by routine culture to identify the presence of common foodborne enteric bacterial pathogens, including *Salmonella* spp, *Shigella* spp, *Vibrio* spp and enteric *Escherichia coli*. All bacterial tests were performed in accordance with standard isolation protocols. In addition to noroviruses, routine real-time PCR assays using commercial kits for the detection of rotaviruses, astroviruses, adenoviruses and sapoviruses were also performed.

Norovirus analysis

Rectal swabs

Fifty-four case-originating rectal swabs from different individuals that involved in the outbreak were tested for the presence of noroviruses. Of these, 23 samples were collected from symptomatic persons, including 22 passengers and 1 cleaner, and 31 samples were from asymptomatic crew members, including food handlers, cleaners, attendants, drivers, broadcasters, managers and engineers.

Potential source samples

Implicated food items were collected on 21 April from the cruise ship and sent to Nanjing Center for Disease Prevention and Control for noroviruses screening. All the food samples available were taken: fried eggplant with pickled vegetables, steamed pork with rice flour, garlic sprouts fried with shredded pork, braised bean curd, steamed rice and flavouring substances. Five surface swab samples collected from food utensils were also tested. Twenty-five gram portion of each food sample was analysed. If the amount of food was insufficient, all the remaining food available was processed. Food samples were diluted in the ratio 1 to 10 using 0·85% NaCl aqueous solution (saline) and homogenized and incubated for 10 min at room temperature, then were centrifuged for 5 min at 3273 ***g*** at 4°C as determined in China Entry-Exit Inspection and Quarantine Professional Standards SNT 2626-2010 (Detection of noroviruses at frontier port). The supernatant of each sample was then subjected to RNA extraction and PCR.

One litre tank water sample used for food processing was collected from the galley on April 22 and passed through a Mixed Cellulose Esters Membrane (pore size 0·45 μm; Millipore, Bedford, MA). The sample was then eluted from the filter with 3% beef extract- 50 mmol l^{-1} glycine (pH 9·5) and further concentrated with the addition of equal volume of PEG 6000(final concentration: 10%) as described before (Zhou *et al.* 2010). The pellets were resuspended in 1 ml of RNA-free water (Japan TaKaRa BIO, Dalian, China) and subjected to RNA extraction and PCR.

RNA extraction and TaqMan Real-time RT–PCR

Viral RNA was extracted from 140 μl of sample prepared as described above, using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA extracts were used directly for real- time reverse transcription (RT-PCR) or capsid RT-PCR or were frozen at −70°C until analysis. TaqMan real-time RT-PCR was performed using a commercially available real-time PCR detection kit targeting both GI and GII noroviruses (Bio Perfectus technologies, Taizhou,

China). Real-time PCR was performed in a 25 μl volume with the following cycling profile: 50°C for 30 min, 95°C for 5 min, followed by 45 cycles of amplification (95°C for 10 s, and 55°C for 40 s). The amplified products were analysed by the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

Capsid RT-PCR

Two pairs of primers, primers G1-SKF/G1-SKR and primers COG2F/G2-SKR (Kojima *et al.* 2002), were synthesized based on capsid N/S gene of GI and GII noroviruses and produced PCR fragments of 330-bp and 387-bp respectively. Primer-Script one step RT-PCR kit Ver.2 (Takara) was used to perform RT-PCR as described by Wang *et al.* (2014). Briefly, one step RT-PCR was performed in a 20 μl reaction mixture following the manufacturer's protocol. The cycling profile was as follows: 50°C for 30 min, 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 59°C for 35 s, and 72°C for 40 s, and a final step at 72°C for 7 min. All PCR products were examined by electrophoresis on 1·5% agarose gels. The norovirus-positive samples were selected for sequencing.

Sequencing analysis

The RT-PCR products for norovirus region were sequenced in GenScript Corporation (Nanjing, China) using the ABI3730 instruments (Applied Biosystems, Foster City, CA). The sequences were assembled by MEGA software ver. 5.2 (Molecular Evolutionary Genetics Analysis). Genotyping was performed by comparison with a set of cognate sequences of norovirus strains from the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST). Megalign (DNAstar, Inc., Madison, WT) was applied to establish the homology between the analysed sequences. The dendrograms were generated using MEGA software ver. 5.2 (Molecular Evolutionary Genetics Analysis) by neighbor-joining (NJ) method.

Nucleotide sequence accession numbers

The virus genome sequences of strains in this study are available in the GenBank nucleotide sequence database under the following accession numbers: KP753266-KP753288 respectively.

Results

Epidemiology

There were a total of 377 people on board the ship, 32 (8·5%) crew members and 345(91·5%) passengers. Of passengers and crew members, 95 (25·2%) experienced symptoms of sickness and diarrhoea during the cruise and 51 (13·5%) were identified as probable cases. Among the probable cases, the average age was 65 years (range: 30–83), and 66·3% were female. The index case was a 51-year-old female cleaner who developed symptoms of diarrhoea and stomachache only 7 h (at approximately 01:00 on April 20) after departure from the Jiujiang (18:00 on April 19). She went to the medical team for medical help and recovered on the morning of April 21. She was the only symptomatic crew member in this outbreak.

Virological and bacterial results

Fifty-four rectal swabs were received and analysed for the presence of suspect pathogens. 82·6% (19/23) from symptomatic individuals and 48·4% (15/31) among asymptomatic persons were identified as norovirus-positive, confirming noroviruses as the causative agent of the outbreak. Among the 34 norovirus-positive samples, 17 from passengers and 13 from crew members were GI positive, 3 from crew members were GII positive, and 1 from a 60-year-old male passenger presented both GI and GII positive. Single genotypes were present in 95·83% of the specimens. All the norovirus-positive samples were negative for *Shigella*, *Salmonella*, *Vibrio*, enteric *E. coli* and other gastroenteritis-causing viruses mentioned above. Food and water samples and other rectal swabs were tested negative for all the target pathogens included in the study.

Genetic analysis of norovirus strains

A phylogenetic tree was constructed comprising norovirus strains from the outbreak along with GI prototype strain Norwalk virus and 26 reference genotypes obtained from Genbank. The sapovirus representative strains Manchester was used as an outgroup (Fig. 1). Twenty-two GI positive samples and one GII positive sample were successfully sequenced and seven different genotypes were identified: GI.1 (n = 8, 34·8%), GI.2 (n = 5, 21·7%), GI.8 (n = 4, 17·4%), GI.9 (n = 2, 8·7%), GI.3 (n = 2, 8·7%), GI.4 (n = 1, 4·3%) and GII.17 (n = 1, 4·3%). For the sample containing GI and GII, GI.2 was detected but the GII was not successfully sequenced. No common genotypes were identified. Among the symptomatic individuals sampled for detection, 6 (37·5%) fell into GI.1, 4(25%) GI.8, 3(18·7%) GI.2, 2(12·5%) GI.9, 1 (6·2%) GI.3. Similar distribution of norovirus genotypes was found in asymptomatic individuals with GI.1 (28·6%) plus GI.2 (28·6%) comprising more than half of the strains. It was noted that the 4 GI.8 strains were only present among symptomatic persons.

Eight GI.1 sequences identified in this study showed high nucleotide similarity (97·6%~98·8%) when

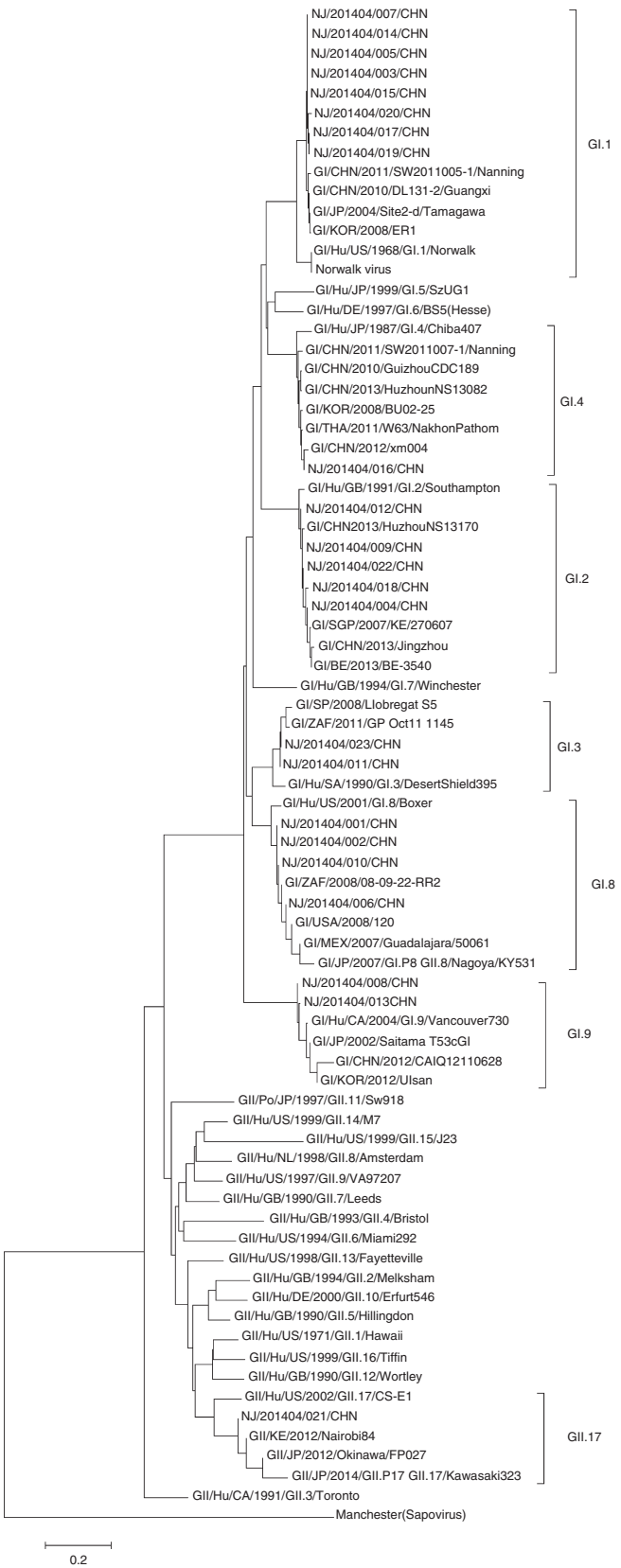

Figure 1 Dendogram of the capsid N/S sequences of norovirus strains detected in patient samples during the outbreak. Letters in italics designate the GI and GII reference strains. Outgroup values:1000 replicates. The tree was constructed by the neighborjoining method using the MEGA 5.2 software package, and the numbers on each branch indicate the bootstrap values.

compared to GI.1 reference sequence GI/JP/2004/Site2-d/Tamagawa (AB537002) which was identified from the concentrated Tamagawa River (Japan) water samples. Two GI.3 sequences had 96·9–99·3% similarity to two reference strains which were identified from wastewater in South Africa and Spain respectively. The two GI.9 sequences displayed 97–97·2% similarity to a GI.9 strain GI/CHN/2012/CAIQ12110628 (KF586507) from a foodborne outbreak on a cruise ship in China in 2012. The norovirus GII sequence NJ/201404/021/CHN exhibited 97·7% similarity in capsid protein region with a newly discovered GII/JP/2014/GII.P17_GII.17/Kawasaki323 (AB983218) which contained novel open reading frame 1. Of note, the polymerase genotype of NJ/201404/021/CHN was unknown as we failed to sequence the polymerase region.

Discussion

In this study, we conducted molecular diagnosis of a gastroenteritis outbreak on a Chinese cruise ship. Two largest noroviruses clades, GI and GII and 14 genotypes for GI and 17 genotypes for GII based on capsid N/S sequences have been described (Kageyama *et al.* 2004). Among these,the GII.4 strains have been recognized as the most frequent cause of norovirus-related outbreaks on cruise ships worldwide. (Verhoef *et al.* 2008; Vivancos *et al.* 2010; Wikswo *et al.* 2011; Greening *et al.* 2012). Here, we nevertheless described a distinctive feature of diversity of norovirus strains circulating in the outbreak, including noroviruses GI.1, GI.2, GI.3, GI.4, GI.8, GI.9 and GII.17 (Tables 1 and 2, and Fig. 1). The GII.4 genotype has been the predominant one in sporadic cases in Nanjing area in the last few years (Fu *et al.* 2013; Zhang *et al.* 2014) and was responsible for two outbreaks in student-related settings (Wang *et al.* 2014). However, this particular genotype was not present in the outbreak. Travellers afflicted with a variety of noroviruses may be the source of infection in Nanjing city, if they have been ashore for sight-seeing and recreational activities while shedding viruses.

The specific source of contamination with noroviruses remains unclear. It was unlikely that one ill person on board introduced the noroviruses as diverse genotypes were identified. In addition, epidemiological data indicated that all of the illnesses occurred within 2 days between 20 and 21 April with a short-term peak. We assumed that contaminated water or food was introduced to the cruise acting as a point source of infection, rather than a person-to-person transmission. Despite the lack of

Table 1 Real-time PCR detection of noroviruses in passenger samples and sequence-based genotyping of norovirus strains

Passengers	Age (year)/gender	Real-time PCR	C_T*	NoV genotype†	Sequence	Symptoms
P1	62/M	NEG	ND	ND	ND	Symptomatic
P2	63/M	Norovirus GI	38·05	ND	ND	Symptomatic
P3	62/M	Norovirus GI	32·38	GI.8	NJ/201404/001/CHN	Symptomatic
P4	68/M	Norovirus GI	28·85	GI.8	NJ/201404/002/CHN	Symptomatic
P5	80/M	Norovirus GI	34·10	GI.1	NJ/201404/003/CHN	Symptomatic
P6	60/M	Norovirus GI	29·23	GI.2	NJ/201404/004/CHN	Symptomatic
		Norovirus GII	28·07	ND	ND	Symptomatic
P7	67/M	Norovirus GI	32·31	GI.1	NJ/201404/005/CHN	Symptomatic
P8	69/M	Norovirus GI	25·92	GI.8	NJ/201404/006/CHN	Symptomatic
P9	52/F	Norovirus GI	23·95	ND	ND	Symptomatic
P10	80/F	Norovirus GI	31·39	GI.1	NJ/201404/007/CHN	Symptomatic
P11	63/F	Norovirus GI	23·96	GI.9	NJ/201404/008/CHN	Symptomatic
P12	60/F	Norovirus GI	20·63	GI.2	NJ/201404/009/CHN	Symptomatic
P13	58/F	Norovirus GI	28·03	GI.8	NJ/201404/010/CHN	Symptomatic
P14	76/F	Norovirus GI	31·41	ND	ND	Symptomatic
P15	64/F	Norovirus GI	21·50	GI.3	NJ/201404/011/CHN	Symptomatic
P16	66/F	Norovirus GI	24·94	GI.2	NJ/201404/012/CHN	Symptomatic
P17	67/F	NEG	ND	ND	ND	Symptomatic
P18	68 F	NEG	ND	ND	ND	Symptomatic
P19	68 F	NEG	ND	ND	ND	Symptomatic
P20	80/F	Norovirus GI	20·66	GI.9	NJ/201404/013/CHN	Symptomatic
P21	62/F	Norovirus GI	30·88	GI.1	NJ/201404/014/CHN	Symptomatic
P22	65/F	Norovirus GI	34·50	GI.1	NJ/201404/015/CHN	Symptomatic

ND, no data; NEG, negative.

*Cycle threshold value.

†Based on RNA-dependent RNA polymerase-N/S capsid sequenceing.

Table 2 Real-time PCR detection of noroviruses in samples from cruise members and sequence-based genotyping of norovirus strains

Cruise members	Gender	Job	Real-time PCR	C_T*	NoV genotype†	Sequence	Symptoms
C1	M	Food handler	Norovirus GI	36·53	GI.4	NJ/201404/016/CHN	Asymptomatic
C2	M	Driver	NEG	ND	ND	ND	Asymptomatic
C3	M	Driver	Norovirus GI	34·74	ND	ND	Asymptomatic
C4	M	Driver	Norovirus GI	33·48	ND	ND	Asymptomatic
C5	M	Driver	Norovirus GI	36·18	ND	ND	Asymptomatic
C6	M	Food handler	NEG	ND	ND	ND	Asymptomatic
C7	M	Driver	NEG	ND	ND	ND	Asymptomatic
C8	M	Driver	NEG	ND	ND	ND	Asymptomatic
C9	M	Food handler	Norovirus GII	26·40	ND	ND	Asymptomatic
C10	M	Driver	NEG	ND	ND	ND	Asymptomatic
C11	M	Engineer	Norovirus GI	33·50	GI.1	NJ/201404/017/CHN	Asymptomatic
C12	M	Driver	NEG	ND	ND	ND	Asymptomatic
C13	M	Engineer	Norovirus GI	27·50	GI.2	NJ/201404/018/CHN	Asymptomatic
C14	M	Driver	NEG	ND	ND	ND	Asymptomatic
C15	M	Engineer	Norovirus GI	33·06	ND	ND	Asymptomatic
C16	M	Engineer	Norovirus GI	30·25	ND	ND	Asymptomatic
C17	M	Food handler	Norovirus GI	28·71	GI.1	NJ/201404/019/CHN	Asymptomatic
C18	M	Engineer	NEG	ND	ND	ND	Asymptomatic
C19	F	Cleaner	Norovirus GI	33·17	GI.1	NJ/201404/020/CHN	Symptomatic
C20	F	Manager	NEG	ND	ND	ND	Asymptomatic
C21	F	Chief	NEG	ND	ND	ND	Asymptomatic
C22	F	Cleaner	Norovirus GI	37·19	ND	ND	Asymptomatic
C23	F	Broadcaster	Norovirus GII	27·51	GII.17	NJ/201404/021/CHN	Asymptomatic
C24	F	Cleaner	NEG	ND	ND	ND	Asymptomatic
C25	F	Cleaner	Norovirus GI	32·62	GI.2	NJ/201404/022/CHN	Asymptomatic
C26	F	Cleaner	Norovirus GII	30·12	ND	ND	Asymptomatic
C27	F	Food handler	NEG	ND	ND	ND	Asymptomatic
C28	F	Dishwasher	Norovirus GI	25·01	GI.3	NJ/201404/023/CHN	Asymptomatic
C29	F	Cleaner	NEG	ND	ND	ND	Asymptomatic
C30	F	Cleaner	NEG	ND	ND	ND	Asymptomatic
C31	F	Cleaner	NEG	ND	ND	ND	Asymptomatic
C32	F	Attendant	NEG	ND	ND	ND	Asymptomatic

ND, no data; NEG, negative.

*Cycle threshold value.

†Based on RNA-dependent RNA polymerase-N/S capsid sequenceing.

direct evidence based on molecular or epidemiological data, we gave an assumption regarding the cause of the outbreak. Seafood (shellfish), fresh produce (vegetables and fruits like berries) and prepared food (salads, sandwiches) were the foods most often implicated in norovirus outbreaks (Tuan Zainazor *et al.* 2010). Neither of the foods mentioned above was served up on the cruise. The foods on table were fried, steamed or braised, which were traditional Chinese cuisine. The possibility of food contamination thus was low. In addition, the abundance of multiple norovirus GI strains in patients provided an epidemiologic indicator of waterborne infection. Outbreaks of food-borne and waterborne gastroenteritis were often caused by multiple norovirus strains (Kageyama *et al.* 2004; Maunula *et al.* 2005). The role of surface water as an important reservoir for these viruses has been well documented (Lysen *et al.* 2009). Nearly half of the waterborne outbreaks were due to GI viruses (Maunula *et al.* 2005). Among the mixed genotypes, GI.3 was one of the most frequent GI genotypes in water samples (Maunula *et al.* 2005), having caused multiple waterborne norovirus outbreaks as reported in the United States (Parshionikar *et al.* 2003) and in the Netherlands (Hoebe *et al.* 2004). In this study, the two GI.3 strains identified from patient samples closely related to those from wastewater in South Africa and Spain. Therefore, waterborne outbreak could be a reasonable explanation in this study. However, drinking water was not likely to be responsible for the infection as only boiled tap water or commercial bottled water was served as drinking water on the ship. We generated a hypothesis that improper use of river water in food processing may be implicated in the infection. On the cruise ship, raw-water taken from Yangzi River served as the water supply for passengers

and underwent filtration and disinfection before using, but the measures were not convincing in removing noroviruses from river water, a possible source of contamination. Filtrated water was stored in a water tank in the kitchen for food processing in room temperature. The inappropriate storage increased the risk of increasing viral load. Soaked in water from the tank for at least several hours before cooking, bean curd was very likely to be linked to the infection.

A limitation of this study was that tanked water and bean curd were suspected but not confirmed as the source of contamination. The case–control study or retrospective cohort study on the basis of the supposed causal attribute was not conducted. The confirmation based on laboratory diagnosis was complicated. Such factors as low virus concentration, RT-PCR inhibitors in various food matrices, limited volumes of water or consumed food, a mixed distribution of viruses, generated practical problems and remained challenging for norovirus detection (Rutjes *et al.* 2006; Verhoef *et al.* 2011). It is possible that the low recovery efficiency of noroviruses from food items may result in false negatives. Quality control is therefore important in the pre-amplification analytical procedure. The use of a sample process control was reported for assessing the performance of extraction of target viruses from food products like strawberry, lettuce, and shellfish (Diez-Valcarce *et al.* 2011). In this study, we processed food items according to the China Entry-Exit Inspection and Quarantine Industry Standards SNT 2626-2010. However, standardized concentration techniques and quality control measures were not included in this standard. As the expected levels of noroviruses in food and water were very low, and many food matrices contained substances capable of inhibiting virus extraction, we suggested that the use of concentration methods and quality control measures should be included in inspection standards in China for noroviruses detection. Last but not least, the water samples were taken almost 3 days after the first case occurred, limiting the laboratory tests.

Thirty-one crew members were asymptomatic, and 15 of which were detected positive with norovirus strains comprising GI.1, GI.2, GI.3, GI.4 and GII.17. The proportion of asymptomatic subjects among crew members was higher compared to 32% in a study using volunteers (Graham *et al.* 1994). The search for viruses in affected staffs was performed. Given the importance of crew members in the prevention of virus spread, the identification of asymptomatic carriage of noroviruses among crew members was essential. Although the role of asymptomatic infection in the transmission of the virus remained unclear, all of the crew members with positive norovirus results were substituted during the following voyage.

In conclusion, this outbreak displayed 3 distinct characteristics: it included a range of norovirus genogroup types; it was suspected a point-source infection; there were a lot of asymptomatic cases among crew members.

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Conflict of Interest

No conflict of interest declared.

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