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Norovirus contamination in retail oysters from Beijing and Qingdao, China

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A B S T R A C T

The consumption of raw oyster has been linked to numerous foodborne gastroenteritis outbreaks caused by norovirus (NoV) in different countries of the world, including in China. This study investigated the prevalence of NoV and contamination levels in retail oysters taken from markets in Beijing and Qingdao in China. The oysters were collected monthly from seafood markets between September 2015 and September 2016 (13 months) in both cities. The digestive glands of these oysters were dissected and NoV particles were extracted. Viral RNA was detected using a TaqMan-based real time one-step reverse transcription-PCR (qRT-PCR) protocol. Of total 672 oyster samples assessed, 652 were considered to be valid for inclusion in the study. The prevalence of NoV was 20.71% (135/652), and this included 21.48% (29/135) of the samples that were positive for the GI strain alone, 62.96% (85/135) contained the GII strain alone and 15.56% (21/135) were mixed with GI and GII, respectively. A total of 68 NoV-positive samples were quantified by qRT-PCR and values obtained ranged from 3.55 x 10³ to 1.45 x 10⁶ genomic copies per g digestive tissue. The NoV contamination in retail oysters fluctuated with the sampling month and peaks of contamination occurred in February (49.12%) and March (55.36%) 2016, respectively. Considering some of the oysters were consumed as raw or half-cooked in China, as a risk reduction measure these oysters should be well heated prior to consumption. Furthermore, successive and region-extended monitoring in retail oysters for NoV as well as risk communication is recommended.

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1. Introduction

Norovirus (NoV) is known as the leading cause of human nonbacterial acute gastroenteritis that can infect individuals of all age groups worldwide (Hall, 2012). NoV is a non-enveloped virus with a single-stranded, positive-sense RNA genome. It is belonging to genus Norovirus, family Caliciviridae (Kroneman et al., 2013). NoV genus is currently divided into 6 distinct genogroups (denoted as GI through to GVI), each of which has been subdivided into multiple genotypes (Zheng et al., 2006; Kroneman et al., 2013). GI and GII, can be subdivided into 9 and 22 genotypes respectively, and both of these constitute the primary strains epidemiologically linked to human cases of acute gastroenteritis (Liao et al., 2016). Norovirus infections constitute a serious disease burden both in developed and developing countries. According to the U.S. Centers for Disease Control and Prevention (US CDC), NoV was responsible for 60% of acute gastroenteritis cases or 21 million cases in the United States each year (Patel et al., 2009). Children, the elderly and immunocompromised persons may experience more severe symptoms and/or extended duration of illness or even chronic diarrhea. There are an estimated 70,000 norovirus-associated deaths recorded among children <5 years annually worldwide (Lanata et al., 2013). In China, reports of acute gastroenteritis cases caused by NoV have been increasing and these now account for 60–96% of nonbacterial infectious diarrhea recorded since 2012 (Liao et al., 2016).

Norovirus is mainly transmitted via the fecal–oral route and...
contaminated food is one of the important vehicles for transmission. According to US CDC, of 348 NoV outbreaks reported from January 1996 to November 2000, the sources included food (39%), person-to-person contact (20%) and water (3%) (Thorton, Jennings-Conklin, & McCormick, 2004). Shellfish, especially oysters, are a recognized transmission vector for human NoV in the environment (Yu et al., 2015). These shellfish can filter large volumes of water as part of their filter-feeding activities and consequently accumulate and concentrate the virus in their digestive gland. Furthermore, because oysters are typically grown in coastal water that potentially is contaminated by human waste along with the fact that oysters are often consumed under-cooked or raw, this food matrix presents a high risk for viral infections, frequently being involved in NoV outbreaks (Webby et al., 2007). On the basis of data provided by European Food Alert System for Food and Feed (RASFF), all but 3 of the 36 outbreak notifications involving viruses reported during an 11-year period (2000–2011) were attributed to NoV. Among these, 22 were associated with oysters (Le Guyader, Atmar, & Le Pendu, 2012). In addition, secondary transmission from person-to-person may occur, and outbreaks may give rise to school and workplace closures, as well as the closure of oyster harvesting waters along with recalls. Although the adoption of regulations that specify acceptable levels of bacterial enteric pathogens in shellfish tissues or in shellfish-growing water has significantly decreased the impact of bacteria as causes of shellfish-associated disease outbreaks, these regulations have failed to prevent many outbreaks of viral origin (Le Guyader et al., 2009).

China is one of the largest countries in the world for consumption and production of oysters. Little information is available to describe oyster contamination with NoV. To protect consumer health in China, the NoV contamination in retail oysters sourced in Beijing and Qingdao cities were monitored over a 13-month period. Data obtained reported on the quantification of this viral genomic copies in the retail oysters collected. Further the temporal geographical distribution of oyster-related NoV was described in order to extend our knowledge of NoV prevalence in China.

2. Materials and methods

2.1. Oyster sampling

A total of 672 fresh oyster samples were randomly collected from one seafood wholesale market in Beijing and in addition six retail markets in Qingdao from September 2015 to September 2016 (covering a period of 13 months). This accounted for approximately 30–40 samples per month. The seafood wholesale market recruited to the study was the biggest establishment in Beijing and oysters from here were placed routinely for sale in many supermarkets or farmer’s markets, being dispatched from this location.

On each sampling day, no more than 4 retail outlets were selected for study. A maximum of 30 individual oysters were selected randomly for testing from each of these sampling sites. Samples were transported on ice directly to the laboratory and NoV extraction was carried out within 24 h.

2.2. Oyster sample processing

Since the weight of the oysters differed depending on the individual oyster or collection season, each sample was adjusted so that it contained 2–5 individual oysters and make sure each sample contains 2 g digestive gland of oyster. The extraction procedure of NoV from digestive gland of oyster was referred to Microbiology of the food chain–Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR (15216-1), but has some changes in number of oysters as one sample. Briefly the shell of each oyster was carefully washed with tap-water and the digestive glands dissected carefully from other tissues. All digestive glands for these 2–5 oyster samples were pooled and homogenized thoroughly. A portion of 2 g of homogenized digestive tissues was then transferred into a 50-ml centrifuge tube followed by the addition of 10 µl mengovirus VM0 (CeeramTools, France) as the process control and 2 µl protease K (Sigma, U.S.A.) solution with a final concentration of 0.1 mg/mL. This mixture was incubated in a shaking incubator at 280 rpm for 60 min at 37 °C and then for a further 15 min in a water bath at 60 °C. After centrifugation at 3000 rpm for 5 min at room temperature, the supernatant (approximately 3.0 mL) was collected into a sterile Eppendorf tube and stored at −80 °C until required.

2.3. NoV nucleic acid purification

Viral RNA was extracted and purified using a QIAamp Viral RNA Mini kit (QiAgen, Hilden, Germany) in a final volume of 50 µL, in accordance with the manufacturer's instructions. The extracted RNA were then used as the template for real time reverse transcription polymerase chain reaction (qRT-PCR) and maintained at −80 °C prior to analysis for not longer than one week.

2.4. Real-time RT-PCR amplification

Oligonucleotide primers and detection probes employed in this study to identify GI, GII and along with the mengovirus were shown in Table 1. The real-time RT-PCR was carried out using RNA UltraSense One-step Quantitative RT-PCR System (Invitrogen, U.S.A.). All samples extracted were screened for NoV GI, GII and mengovirus by tripleplex qRT-PCR, performed in a CFX96 (BIO-RAD, U.S.A.) real-time PCR platform. The GI probe was labelled with 5-hexachlorofluorescein (HEX) at 5′-end and black hole quenching1 (BHQ1) at 3′-end and be detected at 556 nm fluorescence channel. The GII probe was labelled with 6-carboxyfluorescein (FAM) at 5′-end and black hole quenching1 (BHQ1) at 3′-end and be detected at 515 nm fluorescence channel. The mengovirus probe was labelled with 5-N-hydroxysuccinimide ester (CYS) at 5′-end and black hole quenching2 (BHQ2) at 3′-end and be detected at 667 nm fluorescence channel. The three different gene fragments can be detected simultaneously through different fluorescence channel in each well. The real reaction volume of 20 µL (without nucleic acid template) consisted of 500 nM of each upstream primer; 900 nM of each downstream primer; 250 nM of each detection probe, along with buffer and enzymes at concentrations recommended by the manufacturer. 5 µL of purified nucleic acid or control was added per well, and the final volume in each well was 25 µL.

The thermal profile comprised 55 °C for 30 min and 95°C for 5 min, followed by 45 cycles of 95 °C for 15s, 60 °C for 1min and 65 °C for 1 min. Additionally, highly purified water was used as the blank qRT-PCR control. Phosphate-buffered saline (0.01 mol/L, pH7.2) was used as the negative process control and was run through all stages of the analytical process.

The cycle threshold (Ct) was set manually at 0.1, and it was always on the logarithmic portion of the amplification curve thereby being distinguishable from the background fluorescence. Those samples for which reactions yielded a Ct value below or equal to 35 for NoV GI or GII were considered as positive, and the Ct value above or equal to 38 for NoV GI or GII were considered as negative, and the Ct value between 35 and 38, qRT-PCR detection should be repeated.

2.5. Extraction efficiency

To control for false-negative signals due to extraction failure, a
process control (mengovirus) was included in each sample. The extraction efficiency of the spiked mengovirus from an individual sample was calculated using the following equation: extraction efficiency (\%) = (the number of recovered mengovirus genomic copies / the number of seeded mengovirus genomic copies) \times 100\%. Samples with the Ct value of GI or GII NoV above or equal to 38 and the extraction efficiency below 1\% should be discarded.

2.6. Sensitivity and standard curves of GI and GII NoV

The sensitivity and standard curves of GI and GII NoV were determined using a Norovirus GI Q and Norovirus GII Q Standard kit (CeeraMTooLS, France) with real-time RT-PCR. A standard curve of GI or GII was generated using a 10-fold series of dilutions ranging from $10^4$ to $10^1$ viral genomic copies per microliter and a 2-fold series of dilutions ranging from 10 to 1.25 viral genomic copies per microliter. Viral genomic copy numbers contained in each of the analyzed samples were determined by comparison with the standard curve. The Norovirus GI Q Standard and Norovirus GII Q Standard were also used as positive control.

3. Results

3.1. Sensitivity and standard curves of GI and GII NoV

The sensitivity of the method is 2.5 viral genomic copies per microliter (or 12.5 viral genomic copies per reaction) for GI and GII, and both of them corresponding Ct values were below 38. The limit of quantification was 10 genomic copies per microliter (or 50 viral genomic copies per reaction) for GI and GII. The standard curves of GI and GII were shown in Fig. 1.

![Fig. 1. Standard curves for real-time RT-PCR detection of GI and GII, respectively.](image)

3.2. NoV prevalence in retail oysters

Among 672 oyster samples analyzed, 20 were determined to have a Ct value above 38 for both GI and GII NoV and thus were excluded as their extraction efficiency was unacceptable (less than 1\%). Data obtained from 652 samples were considered to be valid. A total of 135 (20.70\%, 135/652) samples were contaminated with NoV, 55 positives were recorded from Beijing and 80 from Qingdao, respectively. Genogrouping data for these 135 NoV-positive samples revealed that 29 (21.48\%, 29/135) samples were identified as being contaminated with GI NoV alone, 85 (62.96\%, 85/135) by GII NoV alone and 21 (15.56\%, 21/135) were mixtures of GI and GII NoV.

The prevalence of NoV in retail oysters from different regions was shown in Table 2. In terms of NoV contamination in oysters from Beijing, 55 (16.03\%, 55/334) samples were positive for NoV, comprising 12 (21.82\%, 12/55) GI strains alone, 37 (67.27\%, 37/55) GII strains alone and 6 (10.91\%, 6/55) co-contaminated with both GI and GII strains. Comparatively, samples from Qingdao, a coastal city, were more heavily contaminated with norovirus (80/309, 25.89\%) than those from Beijing (p = 0.002). The prevalence of NoV genogroups identified in samples taken from Qingdao was 21.25\% (17/80) for GI alone, 60.00\% (48/80) for GII alone, and 18.75\% (15/80) for both, respectively. No significant difference in prevalence of any genogroups of NoV detected from oysters between the two cities was observed.

3.3. NoV seasonality

The prevalence of NoV in retail oysters varied with the sampling seasons. Contamination appeared to peak in February (49.12\%) and March (55.36\%); in 2016. A peak of oyster contamination by NoV in the cool and dry months of the year was observed in Beijing (February, 42.22\%) and Qingdao (March, 78.12\%), respectively. As Qingdao is a coastal city with a large oyster production industry together with high levels of consumption, NoV contamination were also frequently detected in the late autumn with 35.29\% of recorded positives occurring in October and 46.88\% for November, respectively. No NoV was detected in oysters collected in August 2016 in both cities. Table 3 showed the oyster contamination with NoV as it varied with sampling time.

3.4. Quantification of NoV in naturally contaminated oysters

The number of viral genomic copies in oysters was calculated by multiplying the value generated from the real-time RT-PCR by the dilution factors from the viral and RNA extraction. Accordingly, the number of viral genomic copies in oysters was calculated using the following equation: extraction efficiency (%) = (the number of recovered mengovirus genomic copies / the number of seeded mengovirus genomic copies) \times 100\%.

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Primers and Probes</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI</td>
<td>QNIF(FW) 5'-CCG TGG ATG CGN TTC CAT-3'</td>
<td>da Silva et al., 2007</td>
</tr>
<tr>
<td></td>
<td>NV1LCR (REV) 5'-CCT TAG ACG CCA TCA TCA TTAC-3'</td>
<td>Svraka et al., 2007</td>
</tr>
<tr>
<td></td>
<td>NVG19P(PROBE) 5'-HEX-TGG ACA GAY GCC RAT CT-BQ1-3'</td>
<td>Svraka et al., 2007</td>
</tr>
<tr>
<td>GII</td>
<td>QNIF(FW) 5'-ATG TTC AGT TGG ATG AGR TTC TCW GA-3'</td>
<td>Loisy et al., 2005</td>
</tr>
<tr>
<td></td>
<td>COG2R (REV) 5'-TCG ACG CCA TCT TCA TTC ACA-3'</td>
<td>Kageyama et al., 2004</td>
</tr>
<tr>
<td></td>
<td>QNIFS(PROBE) 5'-FAM-ACG ACC TGG CAG GGC CAT CG-BQ1-3'</td>
<td>Loisy et al., 2005</td>
</tr>
<tr>
<td>Mengo</td>
<td>Mengo110(FW) 5'-GGC GGT CCT GCC GAA AGT-3'</td>
<td>Pinto, Costaforada, &amp; Bosch, 2009</td>
</tr>
<tr>
<td>virus</td>
<td>Mengo209 (REV) 5'-GAA GTA ACA TAT AGA CAC CAC AC-3'</td>
<td>Pinto et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Mengo147(PROBE) 5'-CCT TAG ACG CCA TCA TCA TTT AC-3'</td>
<td>Pinto et al., 2009</td>
</tr>
</tbody>
</table>


Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Detection (%)</th>
<th>GI</th>
<th>GII</th>
<th>Both</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beijing</td>
<td>21.82%</td>
<td>21.82%</td>
<td>67.27%</td>
<td>10.91%</td>
</tr>
<tr>
<td>Qingdao</td>
<td>42.22%</td>
<td>35.29%</td>
<td>78.12%</td>
<td>25.89%</td>
</tr>
<tr>
<td>Total</td>
<td>20.70%</td>
<td>21.48%</td>
<td>62.96%</td>
<td>15.56%</td>
</tr>
</tbody>
</table>

3.4. Quantification of NoV in naturally contaminated oysters

The number of viral genomic copies in oysters was calculated by multiplying the value generated from the real-time RT-PCR by the dilution factors from the viral and RNA extraction. Accordingly, the number of viral genomic copies in oysters was calculated using the following equation: extraction efficiency (%) = (the number of recovered mengovirus genomic copies / the number of seeded mengovirus genomic copies) \times 100\%.

A total of 68 samples were therefore available for NoV detection.
quantification (LOQ) were more theoretical LOD and LOQ. The result may overestimate the sensitivity of the method and underestimated the content of NoV genomic copies in samples.

Beijing is an international metropolis. Foods produced all over the country and many part of the world can be consumed in Beijing. In contrast, Qingdao is a coastal city in northern China and oyster farming is one of the important economic activities. Both of these cities were considered as useful sampling pools for oysters potentially contaminated with NoV.

The results of this study showed that the average prevalence of NoV in retail oysters was 20.71% a figure that fluctuates with the sampling season. The contamination rate in winter was higher than that in summer. It was reported previously that oyster glycogen played an important role in NoV binding and it is present at high levels in the flesh of shellfish from November through March (Furuya et al., 2005; Le Guyader et al., 2006; Burkhardt and Calci, 2000), a period coinciding with seasonal outbreaks of NoV. The prevalence of NoV GII(62.96%) was higher than that of GI(21.48%) in the retail oysters and only 21 samples (15.56%) were co-infected by GI and GII. This result differed from observations published earlier wherein it was reported that GI NoV was detected more often than GII (Furuya et al., 2005; Le Guyader et al., 2006; Burkhardt and Calci, 2000).

Table 4
Quantification of NoV contamination in oysters.

<table>
<thead>
<tr>
<th>NoV genotype</th>
<th>No. of samples</th>
<th>Geometric concentration of GI NoV (copies/g DT)</th>
<th>Geometric concentration of GII NoV (copies/g DT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>maximum</td>
<td>minimum</td>
</tr>
<tr>
<td>GI</td>
<td>15</td>
<td>$1.70 \times 10^4$</td>
<td>$3.55 \times 10^3$</td>
</tr>
<tr>
<td>GII</td>
<td>51</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>GI+GII</td>
<td>2</td>
<td>$6.80 \times 10^3$</td>
<td>$3.79 \times 10^3$</td>
</tr>
</tbody>
</table>
was determined by genomic copies. The data in this study may overestimate the risk of oyster contamination and human infections, as the latter strategy does not relate to the infectious dose. Further, quantification of NoV is regarded as challenging as it may produce considerable variation in results generated by different researchers because of different PCR parameters, reagents, NoV standards (e.g., double-stranded DNA plasmids or synthesized RNA) and even different machines. Therefore, NoV prevalence data and the detection methods used in the specific study must be considered when evaluating results.

In summary, a high prevalence of NoV contamination was observed in oysters placed on the retail market for sale and the contamination levels of NoV may result in gastroenteritis symptoms in consumers. Considering some oysters were consumed as raw or lightly-cooked, from a public health viewpoint, it suggests that as a risk reduction measure, consumers should be encouraged to cook oysters. Results of this study will provide the basic information required for risk assessment on dietary exposure to NoV via oysters, and these data will assist policy-makers to develop strategies to limit NoV contamination of oysters and its transmission to humans. Surveillance of NoV contamination in marketed oysters is highly recommended, as a means of extending the epidemiological data available to describe this risk to human health. Additionally, public education and risk communication is the key to prevent NoV gastroenteritis, in the future.

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Conflicts of interest
All authors listed in the manuscript contributed to conception, acquisition, analysis and interpretation of data, design of the manuscript, critically revised the manuscript and approved the final submitted version. The authors have no conflict of interest to declare.

Appendix A. Supplementary data
Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2017.11.043.

References