Development of a reverse transcription (RT) polymerase chain reaction (PCR) method for the detection of human norovirus in bivalve molluscs

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ABSTRACT

Noroviruses are significant seafood-borne pathogens, commonly associated with the consumption of filter feeding bivalve molluscs. Here, we report the development of a reverse transcription polymerase chain reaction (RT-PCR) method using primers based on the RNA-dependent RNA polymerase gene of norovirus genogroup II (NoV GII). Samples of bivalves were processed for the concentration of virus and extraction of RNA, followed by reverse transcription PCR. A total of 50 molluscan shellfish samples were analyzed, of which 16 samples yielded positive amplifications of norovirus nucleic acid. The PCR method described here, involving a single set of primers, is useful for rapid screening of shellfish for NoV GII.

Key words | enteric virus, norovirus GII, RT-PCR, seafood, shellfish

HIGHLIGHTS

- Norovirus is detected by a single-step PCR after reverse transcription.
- The sensitivity of the single-step PCR is comparable with the nested PCR.
- The new protocol will help in risk assessment of norovirus in molluscan shellfish.

INTRODUCTION

Human enteric viruses belonging to the norovirus (NoV) group are important agents of illness through the consumption of contaminated shellfish that are not adequately cooked prior to consumption (Hassard et al. 2017). Contamination of coastal waters with human sewage results in the introduction of enteric viruses into the environment, which are accumulated by the filter-feeding bivalve molluscs (Maalouf et al. 2011). NoV belong to the Caliciviridae family of RNA viruses, comprising 5 genogroups, of which genogroup I (GI) and genogroup II (GII) are commonly associated with human infections (Campos & Lees 2014). NoV is able to survive longer outside the human host and, therefore, bacterial indicators of fecal contamination are not reliable tools for the detection of norovirus presence in shellfish (Romalde et al. 2002). In the absence of reliable cell culture-based techniques, the detection of NoV is dependent on molecular tools such as reverse transcription PCR (RT-PCR) (Bosch et al. 2016). Several PCR methods have been designed, both conventional and real time, for the sensitive detection of NoV in shellfish (Girones et al. 2010; Stals et al. 2012; Fusco et al. 2019). In this study, we designed specific primers to amplify a 113 bp region of the NoV GII RNA-dependent RNA polymerase gene in order to device a faster PCR method for screening of samples with norovirus. This method rules out false positive results obtained by commonly used nested PCR.
METHODS

Sample collection

A total of 50 samples of bivalve molluscan shellfish comprising clams, oysters and mussels were collected from retail markets, fish markets and the intertidal rocky shore of Mumbai, and analyzed for NoV. The samples were collected in sterile plastic bags, transported to the laboratory in ice and processed immediately. Live bivalve samples of clams, oysters and mussels were opened and the digestive glands were collected along with the intra-valvular fluid.

Concentration of norovirus from samples

The samples were subjected to a concentration process for viruses prior to the extraction of RNA (Sdiri et al. 2006). Briefly, the samples were homogenized in a stomacher and 50 g of the homogenates were mixed with equal volumes of glycine buffer (0.5 M glycine, 0.15 M NaCl, pH 9.5), agitated for 15 min at room temperature and centrifuged at 10,000 ×g for 10 min at 4 °C. The supernatant was mixed with an equal volume of 3% meat extract and the pH was adjusted to 7.2 and the mixture was incubated overnight at 4 °C, followed by centrifugation at 10,000 ×g for 45 min at 4 °C. The pellet was re-suspended in 5 mL PBS (pH 7), centrifuged at 10,000 ×g for 15 min at 4 °C. The supernatant was mixed with an equal volume of 5% PEG 6000 (1:4; v/v). The pH was adjusted to 3.5. The mixture was agitated for 30 min at room temperature and centrifuged at 10,000 ×g for 15 min at 4 °C. The pellet was re-suspended in 5 mL PBS (pH 7), aliquoted and stored at −20 °C.

Reverse transcription PCR (RT-PCR)

Total RNA was extracted from the viral concentrates using the SV Total RNA Isolation Kit (Promega, USA). cDNA was synthesized from total RNA using GoScript reverse transcription system (Promega, USA) and 3 μL of the cDNA was used as template for PCR. PCR amplifications were done using EmeraldAmp PCR master mix (TaKaRa, Japan) in a SimpliAmp thermal cycler (Thermo Fisher Scientific, USA). Primers NVRT-F2 (5′-TTTACGTGCCCAGACAAGAG-3′) and NVRT-R2 (5′-CTCGACGCATCTTACATCA-3′) were designed to amplify a 113 bp region of the NoV GII RNA-dependent RNA polymerase gene (GenBank accession no. KY471399.1) (Das et al. 2020). NVRT-F2 binds between the nucleotides 667–687, while NVRT-R2 binds between 760 and 779. The thermocycling conditions for the primers NVRT-F2 and NVRT-R2 consisted of an initial denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 96 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds. The PCR assays were carried out in 30 μL volumes that consisted of 15 μL of a 2X PCR master mix (EmeraldAmp® PCR Master Mix, TaKaRa, Japan), 30 picomoles of forward and reverse primers and 3 μL of cDNA. In the case of nested PCR, 2 μL of the first step PCR product served as the template for the nested amplification. The PCR products were electrophoresed on 1.5% agarose gels, followed by staining with ethidium bromide (0.5 μg/mL), and photographed using a gel documentation system (Bio-Rad, Hercules, USA). A 200 bp cDNA fragment prepared from norovirus GII RNA (ATCC® VR-3255SD™) and cloned in pUC57 was used as the positive control in all the PCR reactions, while sterile deionized water was used as the negative control.

Determination of sensitivity of PCR for detection of norovirus

The sensitivity of PCR for detection of norovirus using primers NVRT-F2 and NVRT-R2 was determined by serial dilution method using a 200 bp fragment of the target gene amplified from norovirus GII synthetic RNA (ATCC® VR-3255SD™) and cloned in pUC57 plasmid. The concentration of the extracted plasmid was measured using NanoDrop 2000 (Thermo Scientific, USA). The copy number (http://cels.uri.edu/gsc/cndna.html) was calculated based on the plasmid concentration and the size of vector and size of insert. The plasmid with a stock concentration of 50 ng/μL was serially diluted by mixing 2 μL with 18 μL nuclease-free water. Dilutions were made to get plasmid copies from 10⁹ to 10². Two-microliters of each of these serial dilutions were used as template for PCR using the primers NVRT-F2 and NVRT-R2 and the thermocycling conditions described above.

RESULTS AND DISCUSSION

The primers NVRT-F2 and NVRT-R2 amplifying a 113 bp region of the norovirus genome were designed using the RNA-dependent RNA polymerase (RdRP) gene sequence of norovirus (Das et al. 2020). The samples analyzed in this study and the PCR results with two PCR methods are shown in Table 1. Altogether, the NoV nucleic acids were
detected in 16 of 50 samples. Primers NVRT-F2 and NVRT-R2 detected NoV in 16 samples, while the nested PCR detected NoV in 12 samples, suggesting that the PCR assay developed in this study has good sensitivity for the detection of NoV genome in oysters. These primers did not produce any non-specific amplification products with any of the samples, suggesting that the primers were specific for norovirus RdRP sequence (Figure 1). Representative PCR products \((n = 10)\) from both the PCR assays were sequenced (Xcelris Inc., Ahmedabad, India) and their sequence identities were confirmed by BLAST analysis. Except in the case of black clam \((Villorita cyprinoides)\), our PCR performed better than the nested PCR assay (Table 1). Among different bivalve molluscan species tested in this study, the highest detection rate was observed in cupped oyster \((Crassostrea gryphoides)\), in which all 5 samples tested were positive for NoV genome by PCR.

Further, the sensitivity of the newly designed PCR assay was determined using a 200 bp fragment of the target gene amplified from norovirus GII synthetic RNA (ATCC, USA) and cloned in pUC57 plasmid. The PCR could detect 100 copies of the plasmid, equivalent to 0.1 femtogram of nucleic acid (Figure 2). Non-nested PCR assays are always advantageous in terms of the relatively short time required for the detection of the target organism and they do not suffer from the problem of contamination usually encountered in nested PCR assays. However, considering the higher sensitivity of PCR in nested format, most of the PCR assays for enteric viruses use nested primers. A few multiplex PCRs have also been described for enteric viruses. Formiga-Cruz and colleagues described a multiplex PCR for the adenovirus, enterovirus and hepatitis A virus in urban sewage and shellfish with a sensitivity of detection of 1 copy of adenovirus and 10 copies of enterovirus and hepatitis A virus (Formiga-Cruz et al. 2005). One of the earliest PCR methods employed primers specific for the detection of norwalk virus and poliovirus (Atmar et al. 1993). This method could detect 10 PFU of poliovirus in poliovirus-seeded oysters. A multicenter evaluation of the same PCR revealed the sensitivity and specificity of this PCR assay to be 87 and 100\%, respectively for the detection of norovirus in shellfish tissues (Atmar et al. 1996). Quantitative real time PCR is advantageous in terms of determining the copy numbers of NoV in shellfish (Woods et al. 2016; Lowther et al. 2017; Rupprom et al. 2017; Lee et al. 2018). De Medici et al. (2004) developed a RT-booster PCR using published primer sequences of Vinjé et al. (2004). This booster RT-PCR showed an increase in sensitivity of at least 2 log units for all the norovirus strains compared to the standard

### Table 1: Results of PCR analysis of shellfish samples for norovirus genome using a nested PCR (Nishida et al. 2003) and the single-step PCR method described in this study

<table>
<thead>
<tr>
<th>Sample</th>
<th>Scientific name</th>
<th>No. analyzed</th>
<th>No. positive by nested primers</th>
<th>No. positive by NVRT-F2 &amp; R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black clam</td>
<td>Villorita cyprinoides</td>
<td>25</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Asiatic hard clam</td>
<td>Meritrix meritrix</td>
<td>10</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Marsh clam</td>
<td>Polymesoda erosa</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rock oyster</td>
<td>Saccostrea cucullata</td>
<td>7</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Cupped oyster</td>
<td>Crassostrea gryphoides</td>
<td>5</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Green mussel</td>
<td>Perna viridis</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>50</td>
<td>12</td>
<td>16</td>
</tr>
</tbody>
</table>

### Figure 1: Detection of NoV nucleic acids in samples tested in this study using primers NVRT-F2 and NVRT-R2. Lane M: GeneRuler 100 bp ladder (Fermentas, USA), Lanes 1–6: clam samples, Lanes 7 & 8: oyster samples, Lane 9: positive control, Lane 10: negative control.

### Figure 2: Sensitivity of primers NVRT-F2 and NVRT-R2 designed in this study. A cloned 200 bp fragment of the target gene was serially diluted from \(10^9\) (lane 1) to \(10^2\) copies (lanes 8) and used as template for PCR. Lane M: GeneRuler 100 bp ladder (Fermentas, USA), Lane 9: negative control.
RT-PCR method (De Medici et al. 2004). Several real-time reverse transcription PCR methods with varying levels of sensitivity have been reported for the detection of noroviruses in shellfish and environmental samples (Le Guyader et al. 2009; Wolf et al. 2010; Fuentes et al. 2014; Lowther et al. 2017; Fusco et al. 2019). The PCR protocol described in the present study could be employed in real time PCR detection and quantification of norovirus in seafood samples. The sensitivity of detection is very critical in the case of enteric viruses owing to the possibility of them being in low numbers, as well as the presence of PCR inhibitory compounds in shellfish meat, which can considerably reduce the detectability of NoV nucleic acid by PCR (Atmar et al. 1993; Kingsley et al. 2002).

CONCLUSIONS

The reverse transcription PCR involving single stage amplification described here will considerably reduce the time required for screening samples for NoV and also minimize the chances of carry over contaminations encountered in nested PCR assays. Considering the probability of NoV being present in low numbers in seafood and the environment, unlike the clinical specimens such as the stool, sensitivity of the PCR assay is very critical especially in shellfish. Currently, a real time PCR protocol using primers NVRT-F2 and NVRT-R2 designed in this study is being optimized for the detection of NoV in fish, shellfish, coastal water and sediment samples.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES


PCR detection of norovirus


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