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Protocols

Improved detection and quantitation of norovirus from water

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A B S T R A C T

Norovirus is associated commonly with human sewage and is responsible for numerous cases of waterborne and foodborne gastroenteritis every year. Assays using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) have been developed for norovirus, however, accurate detection and quantitation remain problematic owing to a lack of suitable positive controls. To improve enumeration of norovirus genomes from water, a synthetic norovirus genogroup II quantitation standard and competitive internal positive control were developed. The quantitation standard demonstrates identical amplification efficiency as wildtype norovirus and can be used as a viral surrogate in labs with restricted access to norovirus. The internal control quantifies sample inhibition, allowing for accurate quantitation of norovirus from complex environmental samples. Seawater samples spiked with sewage or bird guano were evaluated using the norovirus assay as part of a methods comparison study. Inhibition was detected in nine of 36 (25%) samples, two of which proved to be positive upon re-analysis. Results support the specificity of this assay for human-source (sewage) fecal contamination. Overall, use of this quantitation standard and internal control signify a great advance over traditional positive controls and suggest that molecular techniques for viral analysis could become standardized for routine water quality monitoring.

1. Introduction

Accurate assessment of water used for drinking, agricultural and recreational purposes requires the ability to rapidly assess microbial contaminants. Traditional measures of microbial water quality are based on concentrations of fecal indicator bacteria such as fecal coliforms, *Escherichia coli* or enterococci. These indicator assays are slow, requiring an overnight incubation step to obtain results; and monitoring for these indicators is not always effective for determining when water is contaminated. Studies have demonstrated that indicator bacteria can take up residence in aquatic systems and sediments (Hardina and Fujioka, 1991; Whitman and Nevers, 2003; Yamahara et al., 2007). Studies have also shown that indicator bacteria are more readily inactivated than some waterborne pathogens during sunlight exposure (Nasser et al., 2007), and during wastewater disinfection (Blatchley et al., 2007). The lack of correlation between indicator bacteria and pathogens raises concern about the ability of traditional water quality monitoring to predict health risks accurately (Boehm et al., 2009). To help address these concerns, reliable methods are needed to directly detect common waterborne pathogens, including noroviruses.

Noroviruses are the most common cause of viral gastroenteritis worldwide and are implicated routinely in waterborne outbreaks (WHO, 2003; Kageyama et al., 2004; Nygard et al., 2004; Yoder et al., 2008; Soller et al., 2010). These viruses are members of the family *Caliciviridae*. They are non-enveloped viruses, 27–35 nm in diameter, and possess a single-stranded RNA genome of 7.5–7.7 kb (Atmar and Estes, 2001).

Highly heterogeneous, noroviruses are currently grouped into five different genogroups (GI-GV) with GII accounting for the majority of human infections (Donaldson et al., 2008). Rearrangements in the norovirus capsid appear to have contributed to their prevalence in human populations, evolving in response to immune-driven selection and antigenic drift (Lindesmith et al., 2008).

Noroviruses are primarily transmitted via the fecal-oral route, and they are highly infectious. Teunis et al. (2008) estimate an average probability of infection for a single prototypical Norwalk particle to be close to 0.5. Norovirus infections are self limiting to the epithelial cells of the small intestine, causing fever, diarrhea and explosive vomiting that typically lasts for 12–72 h. Noroviruses are also highly resistant to inactivation, and have been detected in treated wastewaters and surface waters (Ueki et al., 2005; Lodder

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Table 1
Primer and probe sequences.

Oligonucleotide	Sequence	Location[a]
JJV2F[b]	5′-**CAA GAG TCA ATG TTT AGG TGG ATG AG**-3′	4997–5022
COG2R[c]	5′-**TCG ACG CCA TCT TCA TTC ACA**-3′	5074–5094
RING2-TP[c]	5′-[FAM]-**TGG GAG GGC GAT CGC AAT CT**-[BHQ-1]-3′	5042–5061
CIPC-Probe[d]	5′-[Cy5]-*TGT GCT GCA AGG CGA TTA AGT TGG GT*-[BHQ-2]-3′	
NoV IC Mut Rev	5′-*ACC CAA CTT AAT CGC CTT GCA GCA CA***G TAC GTG CTC AAG TCA GAA**-3′	5026–5042
NoV IC Mut For	5′-*TGT GCT GCA AGG CGA TTA AGT TGG GTA* GC**T CTG GCT CCC AGT TTT G**-3′	5059–5075
JJV2F T7 Comp	5′-CAC GTA ATA CGA CTC ACT ATA GGG **CAA GAG TCA ATG TTT AGG TGG ATG AG**-3′	4997–5022

Nuceotides homologous to NoV GII are shown in bold, nucleotides homologous to the CIPC Probe (or the reverse compliment) are show italicized, and nucleotides representing the T7 RNA polymerase promoter region are underlined.
[a] Nucleotide position in reference to the Neustrelitz (accession no. AY772730) sequence.
[b] Sequence taken directly from Jothikumar et al. (2005).
[c] Sequence taken directly from Kageyama et al. (2003).
[d] Sequence taken directly from Kleiboeker (2003).

and de Roda Husman, 2005; da Silva et al., 2007; Astrom et al., 2009).

Due to a lack of suitable cell culture systems, noroviruses are detected by molecular methods (Bosch et al., 2008). Reverse transcriptase polymerase chain reaction (RT-PCR) has proven successful for the detection of norovirus in water (Karim et al., 2004; Lamothe et al., 2003; Huffman et al., 2003). However, accurate detection and quantitation is often complicated by a lack of quantitation standards, and by the presence of inhibitors in environmental samples. These substances, including polysaccharides, humic acids, tannic acids, fulvic acids and terpenoids, inhibit the activity of the polymerase responsible for amplification of nucleic acids or they bind to nucleic acids and prevent amplification. Presence of these inhibitory substances in water samples can inadvertently lead to underestimation of target concentrations or to false negative results (Wilson, 1997).

The goal of this research is to (1) develop a norovirus GII quantitation standard (NoV qSTD) that allows for accurate assessment of genome concentrations and (2) develop a competitive internal positive control (NoV CIPC) that can be used to calibrate reactions inhibited by environmental substances. The NoV qSTD is intended as a surrogate for cultured norovirus RNA of known concentrations. It can be used to generate standard curves or to compare cycle threshold (C_T) values for quantitative analysis (e.g. Haugland et al., 2005). The CIPC further facilitates accurate quantitation of norovirus by integrating correction factors for sample inhibition. Additionally, the control allows determination of whether a negative result is due to the absence of norovirus or a result of inhibitors. Construction of the NoV qSTD and NoV CIPC described are easily accomplished in a typical molecular biology laboratory, and their designs are widely adaptable to other molecular targets for environmental and clinical applications.

2. Materials and methods

2.1. Amplification approach

The NoV qSTD and NoV CIPC were developed based on the one-step norovirus genogroup II RT-qPCR assay introduced by Jothikumar et al. (2005). This assay targets the highly conserved ORF1-ORF2 junctions of the NoV GII genome. A one step RT-qPCR set-up, using the RNA UltraSense One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA) and TaqMan chemistry, was chosen over a two-step approach to reduce the risk of cross contamination and increase throughput.

2.2. RNA extraction

In order to initiate construction of the NoV GII qSTD and NoV CIPC, a norovirus GII positive stool sample was obtained courtesy of Dr. Jan Vinjé at the Centers for Disease Control and Prevention in Atlanta, GA. Fifty microliter of the stool sample was diluted in 150 μl of phosphate buffered saline (PBS), extracted using the method developed by Boom et al. (1990), eluted to a final volume of 100 μl in molecular grade RNase free water, and stored at −80 °C.

2.3. Quantitation standard synthesis

A NoV qSTD was developed to estimate accurately the number of norovirus genomes present in the RT-qPCR. The NoV qSTD was created by amplifying a 1:1000 dilution of norovirus GII RNA using the conditions established for the norovirus GII RT-qPCR assay introduced by Jothikumar et al. (2005), but the forward primer JJV2F was replaced with primer JJV2F-T7 comp (Table 1). The JJV2F-T7 comp primer added a T7 RNA polymerase promoter site to the norovirus RT-qPCR amplicon, allowing for subsequent transcription. Following amplification, the 122 bp product was confirmed visually by electrophoresis of the product in a 12% polyacrylamide-1× TAE gel, purified using the Qiagen Qiaex II Gel Extraction kit (Qiagen, Valencia, CA) using the protocol for desalting and concentrating DNA solutions, and quantified using a NanoDrop, ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE). Following purification, NoV qSTD transcripts were created through in vitro transcription using the MegaScript In Vitro T7 Transcription Kit (Applied Biosystems/Ambion, Austin, TX). The template DNA was adjusted to 2 pmol to ensure a robust yield of transcription product. Following transcription, the 101 nucleotide NoV qSTD was treated with TURBO DNase (Applied Biosystems/Ambion) and purified using a MEGAclear RNA purification kit (Ambion). Transcripts were stored in the presence of RNasin PLUS (Promega, Madison, WI), in 5 μl aliquots. Using a RNA StdSens analysis kit (BioRad, Hercules, CA), the NoV qSTD transcripts were analyzed on a BioRad Experion automated electrophoresis system to ensure the transcripts were of the correct size and integrity. The concentration of the transcripts was determined using UV spectrophotometry on a NanoDrop, ND-1000 spectrophotometer, then concentration was converted into moles of product, and a copy number was inferred using the Avogadro constant (6.022×10^{23} copies per mole). The transcripts were diluted down to 10^6 copies per μl and stored in 5 μl single use aliquots.

2.4. Competitive internal positive control synthesis

A CIPC was constructed to assess inhibition in the previously developed NoV RT-qPCR (Jothikumar et al., 2005). Norovirus GII genomic RNA was used as the template for synthesis of the CIPC. The CIPC was constructed through two separate RT-PCRs run in parallel, followed by two downstream PCRs. The first two RT-PCRs, referred to as reactions A and B, were mutagenic RT-PCRs used to incorporate a T7 RNA polymerase promoter site into the CIPC, dis-