Food Microbiology 59 (2016) 76–84

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Detection and molecular characterization of norovirus from oysters implicated in outbreaks in the US

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ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form
13 May 2016
Accepted 13 May 2016
Available online 15 May 2016

Keywords:
Norovirus
Outbreak
Molecular characterization
Oysters

ABSTRACT

Human noroviruses are the leading cause of non-bacterial shellfish associated gastroenteritis. Here we report on the detection and characterization of norovirus (NoV) in shellfish associated outbreaks. Requests were received from state and federal officials for technical assistance in the analysis of shellfish for NoV and male specific coliphage (MSC; an enteric virus surrogate) during the years 2009 thru 2014. In outbreaks where NoV was detected, genogroup II (GII) levels ranged from 2.4 to 82.0 RT-qPCR U/g of digestive diverticula (DD) while NoV genogroup I (GI) levels ranged from 1.5 to 29.8 RT-qPCR U/g of DD. Murine norovirus extraction efficiencies ranged between 50 and 85%. MSC levels ranged from <6 to 80 PFU/100 g. Phylogenetic analysis of the outbreak sequences revealed strains clustering with GI.8, GI.4, GII.3, GII.4, GII.7, and GII.21. There was 100% homology between the shellfish and clinical strains occurring in 2 of 8 outbreaks. Known shellfish consumption data demonstrated probable infectious particles ingested as low as 12. These investigations demonstrate effective detection, quantification, and characterization of NoV in shellfish associated with illness.

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1. Introduction

Enteric viruses are the leading cause of foodborne infectious diseases in the United States and of the 9.4 million cases of known foodborne illnesses estimated annually, 58% are attributed to human noroviruses (Scallan et al., 2011). Noroviruses (NoV) are single-stranded polyadenylated RNA viruses and they are separated into genogroups I–VI, which includes more than 30 genotypes based on the sequence comparison of the RNA polymerase and capsid regions (Ando et al., 1995, 2000; Caddy et al., 2013; Jiang et al., 1993; Zheng et al., 2006). Genogroups I, II, and IV are known to infect humans, with NoV GII.4 being the most common genotype identified within the past 8 years (Patel et al., 2009; Pringle et al., 2015).

Human norovirus is responsible for 48% of all shellfish associated outbreaks, which can result in secondary transmission where rates can be as high as 88% (Alfano-Sobsey et al., 2012; Baker et al., 2011; Becker et al., 2000). Virtually any food may be implicated in NoV transmission, but bivalved molluscan shellfish present a relatively high risk because of their ability to concentrate viruses from contaminated water. Shellfish, particularly oysters, are typically eaten raw or lightly cooked and may contain enteric viruses capable of causing illnesses (Baker et al., 2011; Le Guyader et al., 2008). Although the fecal indicator system has been in place for many years, it has been very well documented that this system does not adequately index for the presence of enteric viruses (Formiga-Cruz et al., 2002; Hernroth et al., 2002; Kingsley, 2007; Lees, 2000). Male specific coliphages (MSC) have been proposed as an indicator for viral contamination in shellfish as they are accumulated like fecal coliforms but MSC, like NoV, are not rapidly eliminated by the shellfish (Formiga-Cruz et al., 2003; Love et al., 2010).

Various methods have been employed for the concentration of enteric viruses from shellfish (Baert et al., 2007; Kingsley and Richards, 2001; Le Guyader et al., 2009; Shieh et al., 2003). The primary method for detection of enteric viruses involves molecular based real time qPCR or RT-qPCR assays. Because viruses may be present in very low concentrations in environmental and outbreak samples, the level of sensitivity of real time qPCR and RT-qPCR is advantageous for detection of low copy number. While the sensitivity of PCR is beneficial, the presence of inhibitory substances (e.g. polysaccharides), that are co-extracted during sample preparation is of concern (Atmar et al., 1993; Cannon and Vinje, 2008; Kingsley et al., 2002). PCR inhibition is especially relevant for the detection of enteric viruses in shellfish and other foods. The ability to detect viruses at low levels is critical in outbreak analysis because enteric

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http://dx.doi.org/10.1016/j.fm.2016.05.009
0740-0020/Published by Elsevier Ltd.

viral pathogens, unlike bacterial pathogens, cannot be enriched.

This study reports on the concentration, detection, and characterization of NoV in shellfish implicated in several outbreaks that occurred during the years 2009–2014. During this time, states' Departments of Health and/or FDA regional shellfish specialists requested technical assistance in the analysis of shellfish for possible norovirus contamination. Clinical samples from ill individuals in most of the outbreaks were analyzed by the states' Department of Health for NoV genogroup I (GI) and genogroup II (GII). Oysters were received at FDA's Gulf Coast Seafood Laboratory and analyzed for the presence of norovirus using the ultracentrifugation protocol and real-time RT-qPCR described in this study. Positive samples were also amplified by conventional RT-PCR using primers from the RNA dependent RNA polymerase (RdRp) regions and/or the capsid region of the norovirus genome.

During this study, NoV was detected and quantified from shellfish implicated in outbreaks. In addition, NoV was characterized and the probable infectious particles ingested were determined based NoV levels and oyster consumption.

2. Material and methods

2.1. Clinical samples

Clinical specimens collected from ill individuals associated with each outbreak were analyzed for norovirus GI and GII by the states' Department of Health using RT-qPCR. Most samples were sent to CDC for further analysis, while clinical samples from two outbreaks were analyzed in-house after analysis of shellfish samples. In house clinical samples were diluted with tissue culture phosphate buffered saline (t.c PBS) (8.0 g NaCl, 0.2 g KCl, 0.12 g KH_2PO_4, 0.91 g Na_2PO_4 per liter), extracted using QIAamp viral RNA kit (Qiagen, Valencia, CA), and detected by conventional RT-PCR using primers targeting the capsid region or the RdRp region on the Cepheid Smart Cycler II®(Beuret et al., 2002) (Kojima et al., 2002; Williams-Woods et al., 2011).

2.2. Outbreak details for shellfish samples

Between 2009 and 2014 the shellfish associated norovirus outbreaks were in the states of TN and MS in 2009 (outbreak 1), AK in 2009 (outbreak 2), MS in 2010 (outbreak 3), WA and PA in 2011 (outbreak 4), LA in 2013 (outbreak 5), MA in 2013 (outbreak 6) LA in 2014 (outbreak 7), and WA in 2014 (outbreak 8). Corresponding parties reported symptoms of nausea, vomiting, and/or diarrhea within 12–48 h after consumption of the implicated oysters—consistent with norovirus infection. Oysters (*Crassostrea virginica*) implicated in the TN and MS outbreak in 2009 were harvested in MS and repacked by dealers in AL and MS. Oysters were then shipped to restaurants in LA, MS, FL, and TN. Shellfish from the AK outbreak were harvested from a local aquafarm. After harvesting from the farm, oysters (*Crassostrea gigas*) were transported to a harbor dock and held for dock-side sale. Samples from the implicated lots were received from two local retail establishments. In the MS 2010 outbreak, the oysters (*Crassostrea virginica*) were harvested from a growing area in LA. Oysters were then shipped to dealers in MS where the shellfish were purchased and served at a conference. Oysters from the outbreaks in WA and PA were individually quick frozen imported shellstock (*Crassostrea gigas*) harvested from aquafarms, while the outbreaks occurring in TN, AK, LA, MA or MS were domestic fresh whole or shuck oysters. All samples were shipped under frozen or refrigerated conditions and immediately analyzed or stored at −20 °C until analysis. In all outbreaks, shellfish was consumed raw, with the exception of one where the shellfish was partially cooked and served as Oyster Rockefeller. The actual internal cooking temperature of the Oyster Rockefeller was unknown.

2.3. Shellfish analysis

For virus concentration and extraction, 6 to 10 whole oysters were shucked and the digestive diverticula (DD) were removed to obtain a 4 g sample (Fig. 1). In some instances 12 to 20 oysters were dissected and portioned into 4 g samples. A 100 μl aliquot at a final concentration of 10^3 PFU per gram of a non-human Calicivirus, San-Miguel Sea Lion Virus (provided by Alvin Smith University of Washington) serogroup 17 (SMSV-17) or murine norovirus (propagated in house from stocks provided by David Kingsley) (MNV-1) was added directly to DD prior to homogenization of the DD with 40 ml of sterile Milli-Q water (Wobus et al., 2004). Viruses were adsorbed onto the oyster homogenate by adjusting the pH to 4.0–5.0 using 3N HCl. After the adsorption step, samples were then centrifuged for 15 min at 2000x *g* at 4 °C. Following centrifugation, the supernatant was discarded. Viruses were eluted from the pellet by adding 40 ml of 0.75 M glycine-0.15 M NaCl (pH 7.6) and the pH adjusted to 7.5–7.8 with 5 M NaOH. The samples were centrifuged for 15 min at 5000x *g* at 4 °C followed by an additional elution with 20 ml of 0.5 M threonine-0.15 M NaCl (pH 7.5) and centrifuged as previously described. The glycine and threonine eluates, around 60–65 ml, were combined, balanced, and ultracentrifuged at 170,000x g (Sorvall WX90, Thermofisher Scientific) for 1 h at 4 °C. The pellet was resuspended in 5-ml of tcPBS (8.0 g NaCl, 0.2 g KCl, 0.12 g KH_2PO_4, 0.91 g Na_2PO_4 per liter) and transferred to a 50 ml conical tube. Samples were then extracted first with 5 ml of chloroform, by vortexing for 1 min and then centrifuged at 1700x *g* for 15 min at 4 °C. The upper aqueous phase was transferred to a clean ultracentrifuge tube. The remaining portion was extracted with 5-ml of 0.5 M threonine-0.15 M NaCl (pH 7.5), vortexed for 1 min, and centrifuged as previously described. Both aqueous phases were combined into the ultracentrifuge tube and 50 ml of tcPBS was added to each sample, balanced, and ultracentrifuged at 170,000x *g* for 1 h at 4 °C. After ultracentrifugation, the pellet was resuspended with 800 μl of tcPBS and evenly distributed into 4 DNase/RNase free 1.5 ml microcentrifuge tubes. One concentrate was selected and extracted for RNA with RNeasy Mini Kit (Qiagen, Valencia, CA) as described in (Williams-Woods et al., 2011). The remaining three concentrates were stored at −70 °C. Extracted RNA was tested by real-time RT-qPCR and conventional RT-PCR as described below.

Male-specific coliphage (MSC) densities in the oysters were determined by using a modified double-agar-overlay method described by Cabelli (Cabelli, 1988) where the *Escherichia coli* strain HS(pFamp)R (ATCC # 700891) was utilized as the suitable bacterial host strain (Cabelli, 1988; Debartolomeis and Cabelli, 1991; Environmental Protection Agency, 2001).

2.4. Real-time RT-qPCR

2.4.1. Norovirus

Real-time RT-qPCR for NoV GI and NoV GII was completed on two clinical samples and all shellfish samples. Positive controls used for NoV GI and GII were *in vitro* RNA transcripts of sequences cloned from positive clinical samples previously identified as NoV (Burkhardt et al., 2006; Kageyama et al., 2003). Primers and probes for NoV GI and GII targeted the most conserved region of the ORF1-ORF2 junction. The real-time RT-qPCR included an RNA internal amplification control (IAC) and was performed in a 25-μl reaction using a One-Step RT-PCR Kit (Qiagen, Valencia, CA) as previously described (DePaola et al., 2010). The inclusion of the IAC was used

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