Negative Correlation between the Prevalence of Norovirus and High Bacterial Loads of *Escherichia coli* in Oysters *Crassostrea gigas*

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Abstract

Seasonal variation in the prevalence of norovirus in oysters *Crassostrea gigas* was investigated and compared to levels of *Escherichia coli,* a fecal indicator in oysters. Oysters were collected from Iwon-myeon, Taean-gun, Korea, a primary production area for European Union export of oysters between 2013 and 2014. We observed seasonality in the prevalence of norovirus in oysters, with a higher prevalence and viral load detected during winter months. Oysters taken from production areas that complied with the European Union standard for raw consumption (< 230 MPN/100 g of *E. coli*) had 22.1% of the samples test positive for norovirus (15/68 samples). However, norovirus was not detected in any of the samples (0%, 0/4 samples) that were collected from production sites that exceeded the standard fecal contamination level for raw consumption (> 230 MPN/100 g of *E. coli*). These results indicated that there is a negative correlation between the prevalence of norovirus and high levels of *E. coli* in oysters. Therefore, our results suggest that current food safety guidelines using only a bacterial fecal contamination indicator, *E. coli*, may not adequately assess shellfish production areas for viral and bacterial contamination.

Key words: Bivalve molluscs, Oyster, Norovirus, Fecal indicator, *Escherichia coli*

Introduction

Shellfish (bivalve molluscs) are a food consumed worldwide. However, areas of shellfish production are often located in estuaries or coastal waters, which are heavily influenced by human-mediated factors (Carlos et al., 2013). Shellfish filter suspended particles and accumulate microorganisms from the surrounding waters. They are passive carriers of pathogens, making them vehicles in the transmission of several bacterial and viral enteric diseases. This is especially prevalent and of concern in areas that partake in raw consumption (Bagordo and Grassi, 2013).

Indicator organisms including fecal coliform (U.S. National Shellfish Sanitation Program) and *Escherichia coli* (EC Regulation 854/2004) are microbial pathogens used to assess the safety of shellfish and the waters in which they inhabit (Carlos et al., 2013). Several reports indicate that these bacterial standards do not adequately reflect the risk of viral transmission from shellfish, including viruses such as adenovirus, rotavirus, astrovirus, norovirus, enterovirus, and hepatitis A virus (Lees, 2000; Jiang et al., 2001; Gerba et al., 2002; Fong and Lipp, 2005; Haramoto et al., 2006; Diez-Valcarce et al., 2012; Donia et al., 2012; Shin et al., 2012).

The purpose of this study was to investigate the seasonal
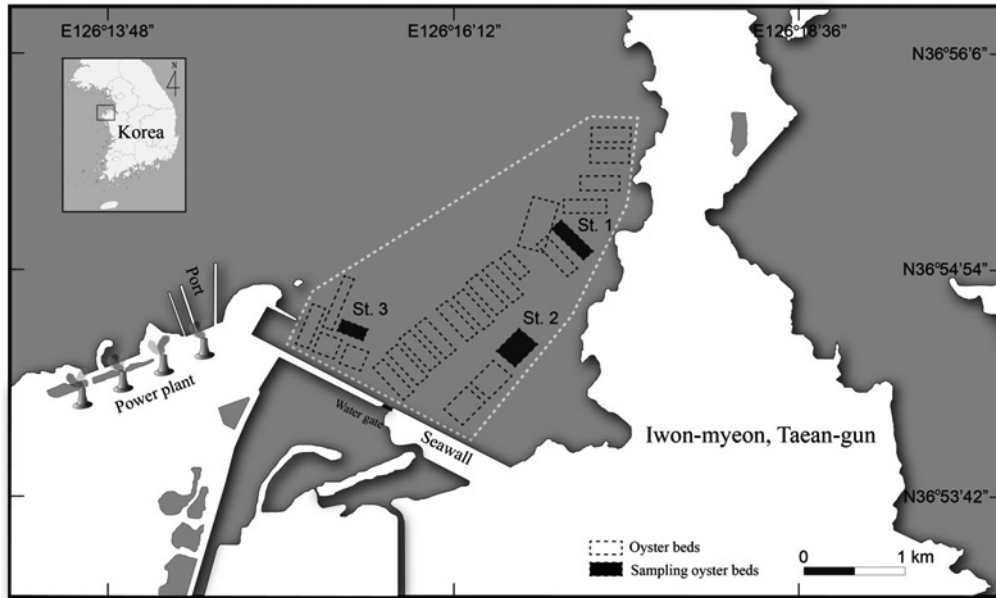
Fig. 1. Sampling locations for detection of norovirus and *Escherichia coli* from oyster *Crassostrea gigas* in Iwon-myeon, Taean-gun, Korea.

prevalence of norovirus in shellfish and to compare it to the level of *E. coli*, a fecal indicator, in shellfish. Our results indicated that shellfish may be a vehicle for norovirus transmission even when *E. coli* levels are below the regulatory limits considered void of bacterial risk. Thus, we do not believe that the use of a single fecal indicator for classification of shellfish production areas is adequate to evaluate the public health risk of viral transmission from shellfish.

Materials and Methods

Sample collection

In Iwon-myeon, Taean-gun, Korea, a primary area for European Union export, three selected beds were chosen from an oyster production area. These beds were closed off to any potential inland pollution. From each bed, one sample of 20 or more oysters *Crassostrea gigas* was collected per month between January 2013 and December 2014 (Fig. 1). In total, 72 oysters were transported to the laboratory under temperature-controlled conditions and tested.

Preparation of sample homogenate for norovirus detection

Digestive glands from 20 shellfish were excised and finely chopped using a razor blade. A 2-g subsample of each chopped gland was transferred to a clean tube and 10 μL of feline calicivirus (FCV) tissue culture supernatant was added as an extraction process control for viral RNA. Homogenates were treated with 2 mL of 100-μg/mL proteinase K solution (30 U/mg; Promega) (Dancer et al., 2010). Next, the sample was incubated at 37°C while shaking at 320 *g* for 1 h and subsequently incubated at 60°C for 15 min. Finally, the sample was centrifuged at 3,000 *g* for 5 min. The soluble portion was retained and stored at 4°C prior to further analysis (Lowther et al., 2012).

RNA extraction

Total RNA was extracted from 500 μL of shellfish homogenate using Qiagen-Viral RNA Mini Kits (Qiagen, Venlo, Germany) according to the manufacturer's instructions. Negative (water only) and positive (homogenate-derived from bioaccumulated oysters) controls were also prepared and tested in parallel with each set of samples. Eluted RNA was stored at -20°C until further analysis. The positive control was used as a within-batch RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) control in addition to a FCV process control.

Duplex quantitative reverse transcription polymerase chain reaction (qRT-PCR)

For G1, primers COG1F/COG1R and probe RING1(a)-TP (Kageyama et al., 2003) were used. For G2, primers BPO-13/BPO-13N/BPO-14 and probe BPO-18 (KFDA, 2014) were used. We used FCV primers and probes as described previously (Lee et al., 2009). For both norovirus genogroup-specific assays, three 5-μL aliquots of RNA from samples and the control were tested in a 25-μL total volume reaction mixture

containing the following components: 12 μL (2X) RT-PCR buffer, 0.5 μL (25X) enzyme mix, 1.5 μL enhancer, 1 μL (10 pmol) of each primer, and 0.5 μL (10 pmol) of each probe. Amplification was performed using a Dice TP600 thermocycler (Takara, Japan) at the following cycling parameters: 45°C for 30 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 56°C for 60 s. Wells containing nuclease-free H_2O and the above Duplex qRT-PCR mixes were also included on each plate as negative controls.

qRT-PCR quantification

All samples were assessed for extraction efficiency by comparing the sample cycle threshold (CT) values for FCV to a standard curve generated from the process control material, as described previously (Lee et al., 2009). Quantification of viral load was determined by the methods outlined by the Joint CEN-ISO Coordinating Group (Lees, 2010). For each qRT-PCR replicate, the quantity (copies/μL) was calculated using the corresponding standard curve (Lowther et al., 2012). Average quantities from three replicates were calculated and used as the representative quantity of virus per sample.

Quantification of *Escherichia coli*

Homogenates were prepared from the flesh and intravalvular fluid of shellfish and assayed according to the ISO/TS 16649-3 most probable number (MPN) technique using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (International Organization for Standardization, 2005). Results were recorded as MPN/100 g of shellfish flesh and intravalvular fluid.

Results

Norovirus prevalence

In total, 15 samples (20.8%) tested positive for norovirus; three (20.0%) were positive for both genogroups, five (33.3%) were positive for G1 only, and seven (46.7%) were positive for G2 only. Norovirus prevalence had high seasonal variability, with 66.7% of the samples positive (10/15 samples) during winter months (Dec.-Feb.) and 0% positive (0/15) during summer months (Jun to Aug) from 2013 to 2014 (Table 1).

Quantitative data indicated that levels of norovirus varied greatly among individual oysters. We observed 66 copies/g of norovirus G1 and 939 copies/g of G2. However, a large proportion of the positive results (40%) were below the method's limit of detection (10 copies/g). Levels of norovirus G2 were on average higher than those observed for G1.
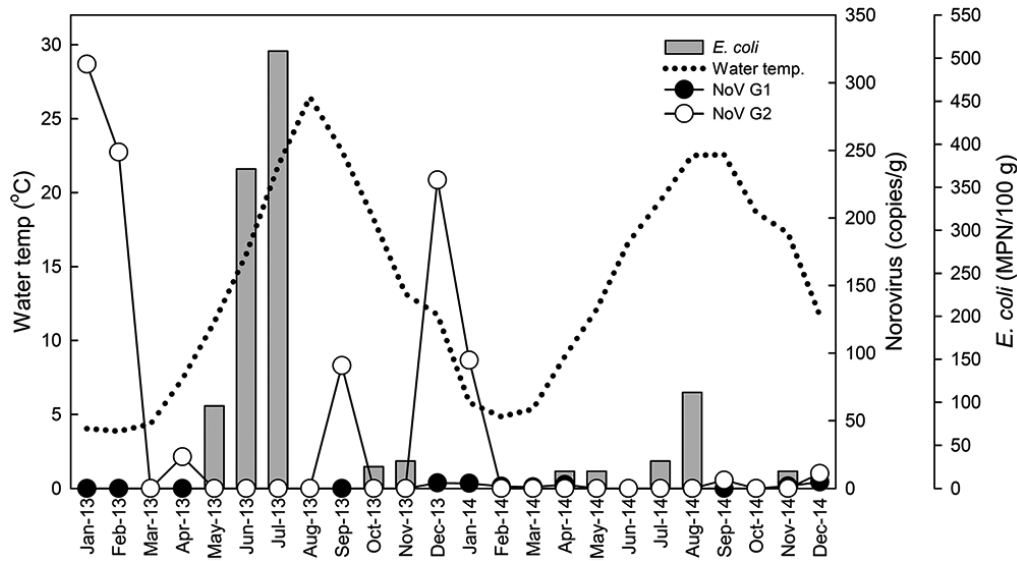
Comparison with *Escherichia coli*

In total, 68 of the 72 samples contained bacterial fecal levels compliant with the standards required by the European Union Food Regulations for raw consumption of shellfish (< 230 MPN/100 g) (European Community, 2004). The highest level recorded was 3,500 MPN/100 g in July 2013 (Table 1). There was seasonal variation of *E. coli* during the summer months of 2013 and 2014, especially during the period from June to July. All measured environmental parameters were examined. We observed that the amount of accumulated rainfall prior to sampling was a key factor associated with elevated levels of *E. coli* in oysters. In 2013, a copious rainfall occurred

Table 1. Monthly levels of norovirus and *Escherichia coli* level in oysters *Crassostrea gigas* collected from Iwon-myeon, Taean-gun, Korea from 2013 to 2014

Month	2013			2014		
	E. coli (MPN/100 g)	NoV G1 (copies/g)	NoV G2 (copies/g)	*E. coli* (MPN/100 g)	NoV G1 (copies/g)	NoV G2 (copies/g)
Jan	<20[a] ~ 20	0	0 ~ 939	<20 ~ <20	0 ~ 53	127 ~ 156
Feb	<20 ~ <20	0	0 ~ 744	<20 ~ <20	0 ~ 3	0
Mar	<20 ~ <20	0	0	<20 ~ <20	0	0
Apr	<20 ~ <20	0	0 ~ 68	<20 ~ <20	0 ~ 22	0
May	50 ~ 160	0	0	<20 ~ 20	0	0
Jun	220 ~ 750	0	0	<20 ~ <20	0	0
Jul	50 ~ 3,500	0	0	20 ~ 40	0	0
Aug	<20 ~ <20	0	0	70 ~ 220	0	0
Sep	<20 ~ <20	0	0 ~ 270	<20 ~ <20	0	0 ~ 16
Oct	<20 ~ 40	0	0	<20 ~ <20	0	0
Nov	<20 ~ 40	0	0	<20 ~ 20	0 ~ 5	0
Dec	<20 ~ <20	0 ~ 66	0 ~ 682	<20 ~ <20	2 ~ 10	6 ~ 26
Range	<20 ~ 3,500	0 ~ 66	0 ~ 682	<20 ~ 220	0 ~ 53	0 ~ 156

[a]Quantification limit.

Fig. 2. Comparison of within-site geometric mean levels of norovirus and *Escherichia coli* in oysters *Crassostrea gigas* with water temperatures at shellfish production area in Iwon-myeon, Taean-gun, Korea from 2013 to 2014.

between June and July (131 mm), but in 2014 during these same months only 13.5 mm rainfall was recorded.

In samples with < 230 MPN/100 g of *E. coli*, 22.1% of the samples tested positive for norovirus (15/68 samples). In samples with *E. coli* levels > 230 MPN/100 g (too high for raw consumption) 0% of the samples were positive for norovirus (0/4 samples). Comparing the presence of norovirus and *E. coli* in individual oysters indicated a negative correlation between norovirus prevalence and high levels of *E. coli* contamination.

In total, 60 samples were collected from the oyster production area in Iwon-myeon, Korea with a category A classification status and 12 from a category B classification. We observed a difference in the average amount of norovirus contamination between oysters collected from categories A and B (Table 2). For each classification, the percentage of samples positive for norovirus (G1 and/or G2) was different. Samples from category A, a status that indicates that oysters can be consumed raw without any post-harvest treatment under the relevant regulation of the European Union (European Community, 2004), had 25.0% of the samples test positive for nor-

ovirus (15/60). However, norovirus was not detected in any of the samples taken from category B areas, areas in which shellfish can only be consumed after purification by relaying or depuration.

Comparison with water temperatures

In Fig. 2, water temperatures during the study period are shown in conjunction with norovirus and *E. coli* levels in oyster at St. 1. These microorganisms had a strong inverse relationship relative to seasonality. Norovirus levels were highest during periods of low water temperatures. Alternatively, *E. coli* levels were highest during periods of high water temperatures. The maximum norovirus viral load detected in oysters was in January 2013, correlated with a low water temperature (4.0°C). The maximum *E. coli* bacterial load in oysters was observed in July 2013, correlated with a high water temperature (21.8°C). There was a negative correlation between water temperature and norovirus levels as well as between *E. coli* and norovirus loads (Fig. 2).

Table 2. Norovirus positive and not-detected samples taken from shellfish production areas complied with EU classification category A and B

Category	No. (%) of sample with norovirus result of	
	Positive	Not detected
Class A (Jan to May & Aug to Dec)	15 (25.0)	45 (75.0)
Class B (Jun to Jul)	0 (0.0)	12 (100.0)

Discussion

Similar to previous studies (Le Guyader et al., 2000; Formiga-Cruz et al., 2002; Lowther et al., 2008; Flannery et al., 2009), we observed norovirus seasonality in shellfish, with a higher prevalence and viral load detected during the winter months of the study period. Possible explanations include a greater persistence of the virus in low solar irradiation, low temperature, higher turbidity, and higher norovirus prevalence in the human population during winter months (Blanton et al., 2006; Lowther et al., 2012; Allwood et al., 2005; Cannon et al., 2006). It is thought that oysters are more effective at viral purging during summer months due to heightened metabolism in warmer waters (Doré et al., 1998; Hernroth and Allard, 2007), Furthermore, shellfish production areas are located in transitional and coastal waters which are influenced by many environmental factors that are present at the boundaries of land, freshwater, and seawater environments (Clements et al., 2015). These factors are more stressful on bacterial organisms including *E. coli* and other gastro-intestinal tract bacteria in warm-blooded animals (Troussellier et al. 1998).

Routine bacterial monitoring of shellfish production areas using fecal indicators is a common global practice designed to prevent human consumption of contaminated shellfish (Clements et al., 2015). European Union food regulation 854/2004 (European Community, 2004) also outlines microbiological criteria required for shellfish production areas in EU countries as well as third-world countries, including Korea, that participate in the international shellfish trade. Shellfish from category A production areas meet the requirements necessary to be consumed raw. Shellfish from these areas must contain < 230 *E. coli* per 100 g. Shellfish from category B production areas (< 4,600 *E. coli* per 100 g of flesh for 90% of samples and < 46,000 *E. coli* per 100 g of flesh for all samples) must be purified by relaying or depuration prior to consumption. Shellfish from category C classification production areas (all samples with < 46,000 *E. coli* per 100 g of flesh) must be subjected to long-term relaying (> 2 months) or appropriate heat treatment prior to consumption (Lowther et al., 2012). Current monitoring procedures focus solely on fecal indicator quantification. However, several norovirus outbreaks occur from shellfish taken from areas that comply with the bacterial fecal indicator criteria required for raw consumption (Chalmers and McMillan, 1995; Ang, 1998). As shown in this study, on an individual sample basis, *E. coli* was poorly predictive of norovirus risk.

This study supports the assumption that using bacterial parameters (fecal indicator) alone is not sufficient in identifying the presence of enteric viruses in shellfish (Greening and McCoubrey, 2010). Previously, routine assessment of pathogenic viruses was difficult because of viral issues regarding small dimensions, variety of species, lack of replication in the environment, low concentrations, and our inability to cultivate these species (Bagordo and Grassi, 2013). However, recently technological advances have overcome the difficulties associated with detecting viruses in the environment. Several methods are now available to evaluate viral contamination in shellfish (Legeay et al. 2000; Manso and Romalde, 2013). For example, validated methods to detect norovirus and hepatitis A virus have been developed (Greening and Hewitt, 2008; Lees, 2010).

Similar to other studies, this study observed the presence of norovirus in oysters that had levels of *E. coli* below the regulatory limits. These findings suggest the potential need to revise the safety criteria for shellfish production areas. The inclusion of a viral indicator could have important implications for both public health and economic prosperity of the shellfish industry.

Acknowledgments

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