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 variation in the prevalence of norovirus in oysters Crassostrea gigas and compare it to the levels of Escherichia coli, a fecal indicator in oysters.
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 homog
enate using Qiagen-Viral RNA Mini Kits (Qiagen, Venlo, Ger-
many) according to the manufacturer’s instructions. Negative
(water only) and positive (homogenate-derived from bioac-
cumulated 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 tran-
scription-polymerase chain reaction (qRT-PCR) control in ad-
insection to a FCV process control.

**Duplex quantitative reverse transcription poly-
merase 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 previ-
ously (Lee et al., 2009). For both norovirus genogroup-spe-
cific assays, three 5-µL aliquots of RNA from samples and the
control were tested in a 25-µL total volume reaction mixture

![Fig. 1. Sampling locations for detection of norovirus and Escherichia coli from oyster Crassostrea gigas in Iwon-myeon, Taean-gun, Korea.](image-url)
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 H₂O 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.

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

<table>
<thead>
<tr>
<th>Month</th>
<th>2013</th>
<th>2014</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.coli (MPN/100 g)</td>
<td>NoV G1 (copies/g)</td>
</tr>
<tr>
<td>Jan</td>
<td>&lt;20 ~ 20</td>
<td>0</td>
</tr>
<tr>
<td>Feb</td>
<td>&lt;20 ~ &lt;20</td>
<td>0</td>
</tr>
<tr>
<td>Mar</td>
<td>&lt;20 ~ &lt;20</td>
<td>0</td>
</tr>
<tr>
<td>Apr</td>
<td>&lt;20 ~ &lt;20</td>
<td>0</td>
</tr>
<tr>
<td>May</td>
<td>50 ~ 160</td>
<td>0</td>
</tr>
<tr>
<td>Jun</td>
<td>220 ~ 750</td>
<td>0</td>
</tr>
<tr>
<td>Jul</td>
<td>50 ~ 3,500</td>
<td>0</td>
</tr>
<tr>
<td>Aug</td>
<td>&lt;20 ~ &lt;20</td>
<td>0</td>
</tr>
<tr>
<td>Sep</td>
<td>&lt;20 ~ &lt;20</td>
<td>0</td>
</tr>
<tr>
<td>Oct</td>
<td>&lt;20 ~ 40</td>
<td>0</td>
</tr>
<tr>
<td>Nov</td>
<td>&lt;20 ~ 40</td>
<td>0</td>
</tr>
<tr>
<td>Dec</td>
<td>&lt;20 ~ &lt;20</td>
<td>0 ~ 66</td>
</tr>
<tr>
<td>Range</td>
<td>&lt;20 ~ 3,500</td>
<td>0 ~ 66</td>
</tr>
</tbody>
</table>

aQuantification limit.
Norovirus (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 oysters Crassostrea gigas with water temperatures at shellfish production area in Iwon-myeon, Taean-gun, Korea from 2013 to 2014.

![Graph showing water temperature and microbial levels](image)

**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.

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 norovirus (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 loads 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

<table>
<thead>
<tr>
<th>Category</th>
<th>No. (%) of sample with norovirus result of</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Class A (Jan to May &amp; Aug to Dec)</td>
<td>15 (25.0)</td>
</tr>
<tr>
<td>Class B (Jun to Jul)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

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238
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; Hernandez 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 (Legeny 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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