Molecular Surveillance of Enterovirus and Norwalk-Like Virus in Oysters Relocated to a Municipal-Sewage-Impacted Gulf Estuary

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An 18-month survey was conducted to examine the prevalence of enteric viruses and their relationship to indicators in environmentally polluted shellfish. Groups of oysters, one group per 4 weeks, were relocated to a coastal water area in the Gulf of Mexico that is impacted by municipal sewage and were analyzed for enteroviruses, Norwalk-like viruses (NLV), and indicator microorganisms (fecal coliform, Escherichia coli, and male-specific coliphages). The levels of indicator microorganisms were consistent with the expected continuous pollution of the area. Fourteen of the 18 oyster samples were found by reverse transcription (RT)-PCR to harbor NLV and/or enterovirus sequences. Of the four virus-negative oysters, three had exposure to water temperatures of >29°C. Concomitant with these findings, two of these four oysters also accumulated the lowest levels of coliphages. PCR primers targeting pan-enteroviruses and the NLV 95/96-US common subset were utilized; NLV sequences were detected more frequently than those of enteroviruses. Within the 12-month sampling period, NLV and enterovirus sequences were detected in 58 and 42%, respectively, of the oysters (67% of the oysters tested were positive for at least one virus) from a prohibited shellfish-growing area approximately 30 m away from a sewage discharge site. Eight (4.6%) of the 175 NLV capsid nucleotide sequences were from the contaminated oysters throughout all seasons except hot summer, with a higher prevalence of NLV than enterovirus. Although a high percentage of the oysters harbored enteric viruses, the virus levels were usually less than or equal to 2 logs of RT-PCR-detectable units per gram of oyster meat.

Virus infection accounts for two-thirds of the 13.8 million food-borne illnesses each year in the United States for which the pathogen is known (25). Major food vehicles associated with viral gastrointestinal diseases are shellfish (12, 21, 30, 31), fresh produce and produce products (17, 28, 32), and ready-to-eat deli food (1). Shellfish and fresh produce have been feocally contaminated in the field before or during harvest. Illegal overboard sewage discharges into shellfish harvesting waters was the most probable cause for recent major U.S. outbreaks (4, 37). Norwalk-like virus (NLV), recently renamed norovirus, was largely responsible for U.S. shellfish-borne viral gastroenteritis in the 1990s (8, 19, 22, 35) and has been classified into different genogroups (16, 39). Subgrouping of NLV strains has been carried out based upon (i) amino acid sequences of the open-reading frame 2 capsid region (2), resulting in 15 genetic clusters for genogroups I and II, (ii) the NLV capsid N/S domain (18), etc. Importantly, NLV genogroup II (among four genogroups) was identified predominantly in the 1990s outbreaks (10, 11, 13, 20, 24, 41), and the 95/96-US subset of genotype II (cluster 4) was responsible for many outbreaks in the U.S. and elsewhere (15, 29, 40, 41).

Successful molecular detection of the virus in naturally contaminated shellfish by using reverse transcription (RT)-PCR relies upon (i) efficient virus recovery in the sample concentration process, (ii) sufficient sample concentration factor, (iii) effective removal of inhibitors, and (iv) efficient PCR primers for the targeted viruses, specifically genome-diverse NLV. The above factors become particularly critical for food and environmental samples contaminated with low levels of virus. Current U.S. regulatory guidelines do not require testing for enteric viruses but utilize sanitary surveys augmented with testing for bacterial indicators to classify shellfish growing areas. We carried out a survey to examine the prevalence of human enterovirus and NLV in environmentally polluted shellfish to assess shellfish safety with the focus on viral contamination and to evaluate their relationship to microbial indicators, including alternative male-specific coliphages (MSC).

MATERIALS AND METHODS

Determination of virus recovery. Cytopathic hepatitis A virus (HAV) HM175 was seeded into oyster homogenate, eluted, and assayed in fetal rhesus kidney 4 cells for the recovery examination. Serial dilutions of each sample were inoculated onto confluent fetal rhesus kidney 4 cell dishes (60-mm-diameter) and were overlayed with Eagle’s minimum essential medium mixed with 2% fetal calf serum and 0.75% agar (27). A second agar medium overlay with neutral red was added to the dishes after a week’s incubation. HAV plaques were recorded after a total of 11 to 12 days’ incubation.

Oyster relocation and environmental parameters. Oysters were harvested from a conditionally approved shellfish harvesting area in open status in the Mississippi Sound near Mobile Bay, Alabama. Oysters were placed into a deputation flume of the wet lab at the Food and Drug Administration Gulf Coast Seafood Laboratory for a minimum of 2 weeks. A group of 30 depurated oysters in a rack was then suspended in the Mobile River approximately 30 m downstream of the effluent discharge point of a municipal sewage treatment plant. The permit for the tertiary treatment plant allowed discharge of 106 million liters (28 million gallons) per day, and the plant discharged on average 80 million liters (21 million gallons) per day. The oyster rack remained 4.5 m above the river bottom and 1.5 to 2.4 m below the water surface, depending on the tide, for 2 weeks. Thirty oysters were then removed from the rack and transported on ice in a cooler to the laboratory for immediate shocking. An average of four sample portions (each containing 25 to 30 g derived from three to four shucked oysters

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layer of the chloroform-sample mixture was reextracted with half the volume of 0.5 M threonine, and both supernatants from steps 5a and b were combined. For step 6, the viruses were precipitated again at a final concentration of 8% PEG–0.3 M NaCl at 4°C for 2 to 4 h (the pellets were collected after centrifugation at 14,000 × g for 15 min). For step 7, RNAs from PEG precipitates were extracted by using a silica gel membrane device (RNacx kit; QIAGEN Inc., Valencia, Calif.). In the early phase of the study, glycin solutions (0.05 to 0.75 M) and threonine (0.5 M) were compared to a common eluent of 0.05 M glycine (frequently described by researchers) to determine which was the most effective at recovering HAV at elution step 3a.

Twenty-five grams of oyster meat was processed by this procedure to attain an approximate volume of 250 μl of RNA. The viruses from two oyster samples (25 g each) were concentrated. RNAs of the concentrates, in which each microtube contained approximately 50 μl of RNA from the original 5 g of oyster meat, were derived. For viral analysis, a minimum of two tubes containing the RNA concentrates from approximately 10 g of oyster meat were analyzed using RT-PCR.

**RT-PCR.** Two virus targets and oyster actin mRNA as the RNA control were examined by using RT-PCR in the RNA concentrates derived from the oysters. Two-step RT-PCR was performed as previously described (27, 34), with RT being carried out at 42°C for 1 h, followed by the PCR profiles listed in Table 1. Comparable results were obtained by using a one-step RT-PCR kit (QIAGEN) and were performed on a second set of sample concentrates. One-step RT-PCR allowed the continuous reactions of RT at 45°C (15 min), Taq enzyme activation at 95°C (15 min), and the first PCR (Table 1) to occur in a single microtube. Then the second round of PCR was performed with one-tenth the volume of the first PCR amplicon serving as a template under the identical PCR profile as the first round (Table 1). The presence of virus in shellfish was further confirmed by Southern hybridization, sequencing, or fluorometric scanning (36) of the second PCR amplicon.

**Gel electrophoresis and Southern analysis.** As previously described (27, 34), PCR amplicons were analyzed by 1.8% agarose gel electrophoresis and transferred onto positively charged nylon membranes. DNA-embedded membranes were placed in a 50-ml conical centrifuge tube and prehybridized with 10 ml of Express Hyb solution (Clontech, Palo Alto, Calif.) for 1 h at 52°C for enterovirus and 58°C for NLV and actin mRNA. The membranes were then hybridized under the same conditions with another 10 ml of Express Hyb solution containing 2 to 10 pmol of digoxigenin-labeled probe/ml. Colorometric detection conditions recommended by the manufacturer (Roche Applied Science, Indianapolis, Ind.) were followed.

**Cloning and sequencing.** NLV amplicons were subcloned into the vector pPCR-XL-Topo (Invitrogen, Carlsbad, Calif.). Two to three clones for each sam-
Occasionally, two sets of oysters were relocated during the same month, one early (E) and the other late (L) in the month.

The ratios were calculated by dividing the indicator levels in the final relocated oysters.

**RESULTS**

**Virus recoveries from oysters by various eluents.** When the eluents of PBS, glycine, and threonine were tested for recovery of HAV from seeded oysters at elution step 3a, higher concentrations of glycine (0.75 M) or threonine (0.5 M) (Table 2) were found to recover higher levels of HAV. Therefore, in the present protocol, 0.75 M glycine rather than 0.05 M glycine was used at the elution step immediately after acid adsorption of the virus. A concentration of 0.75 M glycine was expected to result in recovery of 2.3-fold more viruses than would result with a concentration of 0.05 M glycine (60 versus 26%).

**Parameters of oyster-surrounding water: temperature, salinity, and indicator microorganisms.** At each oyster relocation, we measured the water for temperature, salinity, and level of indicator microorganisms (E. coli, fecal coliform, and MSC). The average for each parameter was derived from three data points collected at the beginning, middle, and end of the 2-week relocation period (Table 3). The mean water temperature ranged from 12.6°C in February to 30.4°C in September of 2000. The mean salinity range was 2 to 22.3 ppt during the study. The estuarine water of the oyster relocation site was constantly impacted by sewage effluents, in which the densities of indicator E. coli, fecal coliform, and MSC usually fluctuated between 1 and 2 logs per 100 ml of water. The levels of two bacterial indicators were similar (always within the same log) at each sampling but were frequently different from the corresponding MSC level. The densities of MSC in two-thirds of the samples were higher than those of the bacteria. The levels of bacterial indicators (fecal coliform and E. coli) clearly illustrated that the estuarine water was being polluted.

**Fecal coliform, E. coli, and MSC in oysters.** The levels of microorganisms in shellfish were generally higher than those for the surrounding water because of the bio-accumulation characteristics of molluscan shellfish. Due to differential depuration (accumulation) rates between bacteria and viruses (including MSC) (7, 33), we calculated the accumulation ratios in the study by using one data point (the final bacterial level in accumulation mean ± SD)

### TABLE 2. Recovery of HAV from oyster homogenates by using different eluents

<table>
<thead>
<tr>
<th>Trial</th>
<th>Recovery (%) by:</th>
<th>Glycine</th>
<th>Threonine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>0.05 M</td>
<td>0.25 M</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>29</td>
<td>40</td>
</tr>
<tr>
<td>Mean</td>
<td>21.0</td>
<td>26.5</td>
<td>36.0</td>
</tr>
</tbody>
</table>

* Recovery was determined by plaque assays of the HAV seeded oyster homogenates and the extract after elution.

### TABLE 3. Indicator microorganisms in shellfish and the surrounding water during relocation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Salinity (ppt)</th>
<th>Temp (°C)</th>
<th>Log/100 ml of water FC*</th>
<th>Log/100 g of shellfish FC*</th>
<th>Accumulation ratio of: (no. in shellfish/no. in water)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. coli*</td>
<td>E. coli*</td>
<td>E. coli*</td>
</tr>
<tr>
<td>1199</td>
<td>18.0 ± 2.2</td>
<td>20.3 ± 0.2</td>
<td>2.29</td>
<td>2.30</td>
<td>2.17 ± 0.6</td>
</tr>
<tr>
<td>1299</td>
<td>19.2 ± 3.7</td>
<td>19.0 ± 1.3</td>
<td>1.78</td>
<td>1.77</td>
<td>2.54 ± 1.2</td>
</tr>
<tr>
<td>0100</td>
<td>13.2 ± 9.3</td>
<td>15.1 ± 0.2</td>
<td>1.78</td>
<td>1.78</td>
<td>2.20 ± 0.4</td>
</tr>
<tr>
<td>0200</td>
<td>19.1 ± 7.1</td>
<td>12.6 ± 2.0</td>
<td>1.28</td>
<td>1.26</td>
<td>1.98 ± 0.2</td>
</tr>
<tr>
<td>0300</td>
<td>11.7 ± 2.0</td>
<td>13.8 ± 2.5</td>
<td>1.08</td>
<td>1.06</td>
<td>2.20 ± 0.3</td>
</tr>
<tr>
<td>0400</td>
<td>3.5 ± 2.1</td>
<td>19.0 ± 1.9</td>
<td>1.85</td>
<td>1.88</td>
<td>2.26 ± 0.3</td>
</tr>
<tr>
<td>0500</td>
<td>2.0 ± 1.7</td>
<td>20.8 ± 1.7</td>
<td>1.54</td>
<td>1.52</td>
<td>2.72 ± 1.8</td>
</tr>
<tr>
<td>0600</td>
<td>7.5 ± 1.3</td>
<td>25.4 ± 1.3</td>
<td>1.94</td>
<td>1.94</td>
<td>1.64 ± 0.5</td>
</tr>
<tr>
<td>0700</td>
<td>16.7 ± 1.1</td>
<td>29.8 ± 1.2</td>
<td>1.15</td>
<td>1.11</td>
<td>2.00 ± 0.6</td>
</tr>
<tr>
<td>0800</td>
<td>16.3 ± 0.9</td>
<td>29.3 ± 0.9</td>
<td>1.41</td>
<td>1.35</td>
<td>2.28</td>
</tr>
<tr>
<td>0900</td>
<td>16.5 ± 0.3</td>
<td>30.4 ± 0.3</td>
<td>1.38</td>
<td>1.37</td>
<td>1.48</td>
</tr>
<tr>
<td>1000</td>
<td>22.3 ± 1.2</td>
<td>22.8 ± 1.2</td>
<td>1.76</td>
<td>1.75</td>
<td>1.40</td>
</tr>
<tr>
<td>1100</td>
<td>13.8 ± 3.2</td>
<td>17.5 ± 3.2</td>
<td>2.10</td>
<td>2.10</td>
<td>1.73 ± 0.7</td>
</tr>
<tr>
<td>1200</td>
<td>17.9 ± 0.9</td>
<td>14.6 ± 0.9</td>
<td>1.70</td>
<td>1.65</td>
<td>1.74 ± 0.1</td>
</tr>
<tr>
<td>0801</td>
<td>7.0</td>
<td>28.7</td>
<td>2.02</td>
<td>2.01</td>
<td>1.36 ± 0.1</td>
</tr>
<tr>
<td>0901</td>
<td>6.9 ± 1.2</td>
<td>28.4 ± 1.2</td>
<td>1.86</td>
<td>1.80</td>
<td>2.34</td>
</tr>
<tr>
<td>E1201</td>
<td>7.5 ± 1.9</td>
<td>20.3 ± 1.9</td>
<td>1.30</td>
<td>1.30</td>
<td>1.45 ± 0.6</td>
</tr>
<tr>
<td>L1201</td>
<td>6.4 ± 0.6</td>
<td>17.7 ± 0.6</td>
<td>1.96</td>
<td>1.93</td>
<td>ND†</td>
</tr>
</tbody>
</table>

* Four-digit numbers represent the month (first two digits) and the year (second two digits) that the oyster sample was returned to the lab after a 2-week relocation. Occasionally, two sets of oysters were relocated during the same month, one early (E) and the other late (L) in the month.

b Mean ± SD for each parameter in water or for two data points with no SD.

c Log10 most probable number of bacteria in the third (final) water sample.

d Mean ± SD of three log10 plaque-forming units of MSC in water samples. No SDs are given for means derived from fewer than three points of data.

e Log10 bacteria or MSC in the final relocated oysters.

f The ratios were calculated by dividing the indicator levels in the final samples of oyster meat by the final FC, final E. coli, or mean MSC levels, respectively, in water.

**log**

d Log10 most probable number of bacteria in the third (final) water sample.

d Mean ± SD of three log10 plaque-forming units of MSC in water samples. No SDs are given for means derived from fewer than three points of data.

e Log10 bacteria or MSC in the final relocated oysters.

f The ratios were calculated by dividing the indicator levels in the final samples of oyster meat by the final FC, final E. coli, or mean MSC levels, respectively, in water.

ND, not determined.
water) for bacterial indicators coupled with the average of three data points for MSC. Throughout the years, the oysters accumulated fecal coliform, *E. coli*, and MSC from the surrounding water at an average of 87-, 28-, and 52-fold, respectively (Table 3). Levels of MSC in shellfish ranged from 1 to 4 logs, and the accumulation ratios ranged between 0.2 and 222. The depurated oysters were always found to contain less than the minimum detectable levels of indicators prior to relocation (Table 3). Statistics demonstrated that the correlation coefficient range was $<0.33$ for all pairings between environmental parameters and microorganisms in shellfish (data not shown).

**Enteric viruses detected in oysters exposed to municipal sewage effluents.** To examine enterovirus and NLV in the relocated oyster samples, 25-g portions of oyster meat were processed. RNA integrity was evaluated by RT-PCR amplification of oyster actin mRNAs in each oyster sample along with selected depurated oysters. Each of the samples used in the enteric virus analysis was shown to contain intact mRNA, as detected by the presence of RT-PCR amplicon (data not shown). To analyze viruses in the sample concentrates, RT-PCR was performed with two different primer sets: (i) pan-enterovirus primers targeting polio, echo, and coxsackie viruses, and (ii) NLV primers targeting the 95/96-US common subset strains as well as other strains of the Lorsdale cluster. Fourteen of the 18 oyster samples exposed to municipal sewage effluents for 2 weeks tested positive for enteric viruses (Fig. 1). Seven of the 14 accumulated both enteroviruses and NLVs, while the other 7 were positive for only one of the two viruses. NLVs were detected more frequently than enteroviruses.

When a subset of the data was examined (from the period of November 1999 to October 2000, encompassing a full year), 67% of the samples were found to be virus positive. The four virus-negative samples occurred in the consecutive warm-weather months of July, August, September, and October (Fig. 1). The 12 samples were 58 and 42% positive for NLV and enterovirus, respectively. Of the four oyster samples negative for both viruses, two oyster samples (0700 and 0800) presented the lowest levels of MSC and lowest accumulating ratios of MSC. Three logs of MSC were observed in the remaining two oyster samples (0900 and 1000), with accumulation ratios of 73 and 72 (Table 3). Throughout the study, the levels and accumulation ratios of MSC and bacterial indicators in the oyster samples were distributed differently among seasons. However, the mean water temperatures during the four virus-negative oyster relocations ranged from 22.8 to 30.4°C, with three of the four above 29°C.

**Characteristics of enteric viruses accumulated by oysters.** The majority of the virus-positive samples were positive in the 10-μl RNA concentrates but negative in the 10-fold-diluted RNAs equivalent to ≤0.1 g of oyster meat. Therefore, we calculated that most of the virus levels measured were close to the threshold of detection. The results of Southern analysis indicated that the maximum concentrations of viruses in these oysters reached approximately 2 logs per gram of oyster. This calculation incorporated a factor of 48 to account for the loss of approximately 1.5 to 1.6 logs of viruses during the concentration procedure.

Sequencing was carried out on two to three clones of the NLV amplicon derived from oysters for each relocation in the early part of the study. Clones derived from each oyster sample usually had one to three different bases among 175 nucleotides sequenced (Fig. 2). One of two clones from samples 1199 (1199-1) and L0200 (L0200-1) shared 100% homogeneity (Fig. 2). The rest of the clones listed in Fig. 2 had at least one nucleotide different from each other. A total of eight bases among 175 nucleotides varied among clones (4.6% heteroge-
neity in the NLV partial capsid gene) derived from environmentally contaminated oysters. The sequencing results proved the identity of NLVs in the early part of the study; therefore, NLV amplicons derived from later collections were confirmed only by Southern analysis.

**DISCUSSION**

U.S. sanitary guidelines that prohibit the harvest of shellfish from growing areas with unsanitary conditions have eliminated shellfish-associated typhoid and reduced many other diseases commonly associated with shellfish consumption in the 1900s (31). However, viral gastroenteritis has emerged in the past decade as a major illness associated with shellfish consumption (4, 8, 19, 22, 35). The bacteriological standards to indicate fecal pollution that are implemented in the sanitary guidelines may not be adequate to indicate viral contamination of shellfish (7, 38). The prevalence of human enteric viruses in naturally contaminated shellfish, therefore, was explored through the present study.

In this study, current bacterial indicators and a shoreline survey of the selected relocation area clearly marked the area as closed for shellfish harvesting. MSC had been proposed as a viral indicator for market-ready oysters in Europe (7) and was compared to enteric viruses in our study. One to 2 logs of MSC in 100 ml of water and 1 to 4 logs of MSC in 100 g of oysters were observed during 18 relocations. We did not observe a seasonal trend for the levels of MSC in oysters, although seasonal trends for depurated market-ready oysters had been reported by Dore et al. (7). We suspect that the seasonally affected depuration rate may account for the difference. Additionally, the present study showed timing for maximum MSC accumulation by oysters similar to that observed in a previous study (3) in which microbial inputs were controlled in laboratory experiments, but the accumulation ratio was greater (maximum, 222 versus 99).

Since the volume of template in each RT-PCR was limited to 10 μl or less and a maximum of 2 logs of viruses per gram of oyster meat was found in the study, maintaining efficient virus recovery during the sample concentration procedure (keeping loss to a minimum) is vital for accurate virus detection. The virus detection limit by the present method was estimated to be 48 RT-PCR units seeded initially and detected in a 10-μl template. This calculation was derived from the detection limit averaged at 111 amplification units/reaction from our previous study (27) combined with a 2.3-fold increase in recovery (Table 2) from the present study. With the concentration factor close to 100-fold (10 μl derived from 1 g of oyster meat) in the present study, the viral signal should be detected when approximately 48 RT-PCR units of virus are present in 1 g of oyster meat. Our detection limit was possibly <48 units/gram, because we used a double round of PCR to overcome the problem of residual inhibitors and thus to lower the detection limit.

Because potential noninfectious enteroviruses were taken into account by the molecular assay, we compared our data with that from two previous studies that used enterovirus cell culture infectivity assays as endpoints. Forty oyster samples collected from open or closed areas in Galveston Bay, Texas,

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**FIG. 2.** Capsid nucleotide sequences of NLV strains of the 95/96-US common subset derived from environmentally polluted oysters. Samples 0300 and 0999 (not listed in Fig. 1 and Table 3) were collected in March of 2000 and September of 1999. Sample 0300 was collected from the same relocation site without information on indicator microorganisms. Sample 0999 was relocated further away from the original site (>30 m) but was contaminated by the same pollution source.
were screened for enteroviruses by plaque assays of BGKM cells (14). Twelve of the 30 samples from closed areas were positive for infectious enteroviruses. In another study carried out on the North Carolina coast during 1991 and 1992, an average of 39% (12 of 31) of the oysters sampled from closed areas were positive for cytopathic effect in AGMK or BGKM cells (5). The North Carolina oysters were collected from two stations located 1 and 2 km away from a sewage outfall (not described if the locations were right in the path of the outfall). Although the molecular assay was utilized in the present study and the infectivity assay was utilized in the previous two studies, all three studies coincidently presented similar percentages (42% for the present study) of survey oysters positive for enteroviruses.

NLVs in naturally contaminated oysters were surveyed in recent years (23) by molecular techniques. In southern France, NLV and enteroviruses were found in 23 and 19%, respectively, of the oysters collected from open and occasionally contaminated beds during a 3-year survey. Compared to those for the French study, we reported higher percentages of oysters as virus positive (58% for NLV and 42% for enterovirus). This result was expected because our oysters were relocated to a prohibited shellfish-growing area close to a municipal waste discharge. Note that we used PCR primers specific for the NLV 95/96-US common subset strains and other Lorsdal-related strains (GI/IV/IV). Compared to the degenerate and general primers corresponding to the NLV polymerase region, we found that capsid primers such as Mon 381/382/383 with semi-nested PCR efficiently recognize NLV in complex food matrices such as shellfish. When a sample was positive by Southern analysis, invariably we were able to clone amplicons and derive NLV-specific nucleotide sequences efficiently. The 95/96-US common subset was known to be responsible for many outbreaks in the United States and worldwide, and we believed that the NLV common subset likely existed in municipal sewage. On the other hand, strains of NLV other than the 95/96-US common subset might exist in samples E0500 and 1200, which were negative for the NLV common subset. Thus, we believe that the ratio of NLV to enterovirus in naturally contaminated oysters was potentially greater than 1.38 (58 versus 42%) and may be ±1.59 (67 versus 42%).

In order to verify the results for the four virus-negative samples, NLV general primers (e.g., B primers provided by the Centers for Disease Control and Prevention) were used to examine the samples, and the results were all negative. Virus-negative oyster samples 0700 and 0800 did possess the following features: low MSC levels (<1.76 logs/100 g) and negative accumulation ratios, the lowest fecal coliform and *E. coli* counts for samples from the same waters, and residence in extremely warm water temperatures (>29°C) for 2 weeks. Another virus-negative sample (0900) had only residence in high-temperature (30°C) waters in common with the above samples. No apparent reason was found to explain why the fourth sample was virus negative. The following might have contributed to the absence of enteric virus in shellfish during the 4 consecutive months: (i) the instability of viruses in warm water and (ii) the inefficient concentration of viruses by shellfish. The observation that virus was absent in shellfish (or in water) during hot months may explain in part why NLV outbreaks and sporadic cases worldwide are greatly reduced by the hot seasons of the year (26).

In summary, the present study illustrated that viruses were present in oysters exposed to effluents from a tertiary municipal sewage treatment plant. NLVs were detected more frequently than enteroviruses in oysters (58 versus 42% for NLV versus enterovirus). Enteric viruses were not detected in oysters exposed during July to October 2000, when water temperatures often exceeded 29°C. The levels of virus in oyster meat were limited to 2 logs of RT-PCR units per gram.

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