Non-Detection of Enteroviruses in Shellfish Collected from Legal Shellfish Beds in Massachusetts

KHALIFA I. KHALIFA1-2*, BARBARA WERNER3 and RALPH TIMPERI JR.3

Microbiology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt and Center for Laboratories and Communicable Disease Control, Massachusetts Department of Public Health, Jamilaca Plain, Massachusetts 02130

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ABSTRACT

Eighty-five samples of shellfish (50 soft shell clams, 21 hard shell clams and 14 oysters) were examined for the presence of human enteric viruses. In addition, bacterial contamination levels, both fecal coliform and standard plate count, were determined. Seventy-five samples were harvested from open shellfish areas and 10 samples from restricted shellfish areas during seasonal opening. Enteroviruses were not detected in any of the samples tested. In contrast, 33 (30 from open beds and 3 from restricted areas) of 82 shellfish samples had levels of bacterial contamination that exceed current regulatory limits for shellfish.

Several workers have demonstrated that fecal coliform levels do not correlate with the presence of enteroviruses (9,19,20,21,22,30).

Enteroviruses have been detected in shellfish using several methods of extraction for viruses from ground shellfish meat. The method of Sobsey et al. (28) uses differential centrifugations, precipitation and concentration procedures. Kostenbader and Cliver (14,15) developed a polyelectrolyte flocculation technique for extraction of enteroviruses from foods. Richards et al. (27) used a modified method for viral extraction and concentration from oyster tissues using meat extract and cat-floc. Sullivan et al. (29) described a method that uses commonly available laboratory equipment and materials to detect low numbers of poliovirus 1 in oysters, and found the percent recovery was 55.4 ± 2.1 (95% C.I.).

Hepatitis A virus (HAV), now classified as enterovirus type 72 (18), has been detected in stool by immune electron microscopy (6), radioimmunoassay (12,15) and enzyme-linked immunoassay (17). Chaudhary (3) described a modified commercial radioimmunoassay for detection of hepatitis A virus in tissue culture lysate, marmoset liver homogenate, and simulated stool samples. The purpose of this research is to examine samples of shellfish harvested from legal shellfish beds in Massachusetts for the presence of human enteric viruses.

MATERIALS AND METHODS

Shellfish samples

Eighty-five samples of shellfish (620 animals) were harvested from legal shellfish beds [75 samples came from open areas and 10 samples from restricted areas (during seasonal opening) located in coastal Massachusetts during the period June through August 1984]. These included 50 samples of soft shell clams (Mya arenaria) (426 shellfish), 40 samples were harvested from open beds and 10 samples from restricted beds, 21 samples of hard shell clams (Mercenaria mercenaria) (108 shellfish) and 14 samples of oysters (Crassostrea virginica) (86 shellfish). The samples were brought directly on wet ice to the State Laboratory Institute where they were stored in shell at 5°C. No water samples were taken. All samples were processed within 24 h.
of harvest. Sufficient shellfish meats were homogenized to provide a 100-g sample of ground tissue which was then processed according to the method described by Sullivan et al. (29) using Eagle’s medium, non-fat dry milk, Freon TF, Mg Cl₂·6H₂O, Na₂HPO₄ and Cat-Floc.

Tissue culture
BGM African green monkey kidney cells (BGM) were used in passages 170 through 190 (4) and divided twice weekly in 25-cm² and 150-cm² polystyrene tissue culture flasks (Corning Glass Works, Corning, NY). Primary rhesus monkey kidney (RMK) cells used were obtained from Whitetaker M. A. Bioproducts, Inc., Walkersville, MD. Both cell types were used because RMK cells are more sensitive to the cytopathic effect of the control virus while BGM cells are more resistant to the cytotoxic effect of shellfish extracts (27).

Virus detection
Poliovirus 1 (CHAT strain, ATCC VR-192) was used as a positive control, propagated in monolayers of BGM cells and harvested after 4+ cytopathic effect was observed. TCID₅₀ of the stock virus was estimated in RMK monolayer tubes using the Reed and Muench method (26). Plaque assays were made using agar-medium overlay prepared according to the method described by Sullivan et al. (29), except that the first overlay (29), described by Sullivan et al. (29), of agar-medium (without neutral red) was applied after inoculation of the cells and the second layer (containing neutral red) after 5 d of incubation at 37°C. Plaques were counted and recorded on day 7.

Enteroviruses were detected using 0.2 ml, 1 ml and 5 ml of the extracted shellfish meat inoculated onto each of two RMK monolayer tubes, one 25-cm² BGM tissue culture flask, and one 150-cm² BGM tissue culture flask, respectively. After incubation at 37°C for 2 h, the inoculum was aspirated and discarded. Growth media were added to RMK tubes and the 25-cm² tissue culture flask. The 150-cm² BGM tissue culture flasks were overlaid with agar-medium for plaque assay. RMK tubes and 25-cm² BGM flasks were incubated at 37°C and examined for CPE on days 1, 5, 7 and 14. One TCID₅₀ of poliovirus 1 (CHAT) was included as a positive control with both cell types and growth medium only was used as a negative control.

HAV was assayed by radioimmunoassay using a modification of the HAVAB-M test (Abbott Laboratories, N. Chicago, IL) described by Chaudhary (3), using two negative, two positive and two material controls with two periods of incubation, at room temperature for 2 h and 20 h. The beads were given another incubation at 45°C (Waterbath) for 4 h.

The effect of the extracted shellfish material on virus infectivity was determined. Serial dilutions (10⁻¹ to 10⁻⁶) of control virus were prepared both in PBS pH 7.2 and in shellfish extract. Two-tenths ml of each dilution of the virus was inoculated onto RMK cells and incubated at 37°C for 1 h. The inoculum was aspirated and discarded and growth medium added. Tubes were examined at days 1, 2, 5, 6, 7, 12 and 14 for cytopathic effect.

Bacterial analysis
Measurement of the bacterial contamination level of shellfish was done (82 samples were tested) using the most probable number of fecal coliform (MPN) per 100 g of shellfish meat and standard plate count (SPC) per 1 g of shellfish by the standard method described by the American Public Health Association (1).

RESULTS AND DISCUSSION
Eighty-five samples of shellfish were examined. Enteroviruses were not detected by culture methods in the samples tested using the method of Sullivan et al. (29). The method is more current than other methods (14, 15, 27, 28) and uses commonly available laboratory equipment and materials to detect lower numbers of poliovirus. All of the positive control tubes and flasks developed typical cytopathic effects and typical plaque forming units (PFU) with poliovirus type 1 (CHAT). No cytopathic effects were detected in the negative controls. A limited inhibitory effect of shellfish extract was noted on the growth of stock virus on cell lines. When the cytopathic effects of poliovirus type 1 strain on RMK cells were determined in the presence of negative shellfish extract, the cytopathic effect was consistently on dilution (one log) lower than the CPE seen in the absence of shellfish extracts.

Hepatitis A virus is considered as one of the genus enterovirus (18), for that reason the method used for extraction and concentration of hepatitis A virus from shellfish is the same as that for extraction and concentration of enteroviruses (29). We were unable to detect hepatitis A virus in the samples tested using the modified radioimmunoassay. There has been great difficulty in growing HAV on tissue culture and no standard method exists for detection in environmental or food samples such as shellfish. The method used in this study, although successful in detecting HAV in clinical samples (tissue culture lysate, marmoset liver homogenate and simulated stool samples) (3), may not be sufficiently sensitive for detection of hepatitis A virus in environmental or food samples.

The oyster samples in this study showed the highest frequency of bacterial contamination for both fecal coliform and standard plate count (Table 1). This distribution of contamination frequency is unusual for Massachusetts shellfish as soft shell clams typically exhibit higher rates of contamination.

We were unable to detect enteroviruses in shellfish although 33/82 samples (30 samples from open area and 3 samples from restricted areas) had fecal coliforms exceeding the violation level. Further work will be required to determine if the non-detection of enterovirus in these samples was due to methodologic limitations, or actual low levels of enteroviral contamination.

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TABLE 1. Number of fecal coliforms and standard plate counts in different types of shellfish.

<table>
<thead>
<tr>
<th>Type of shellfish</th>
<th>Bacterial numbers</th>
<th>Soft shell clams</th>
<th>Hard shell clams</th>
<th>Oysters</th>
<th>Number of shellfish samples tested</th>
<th>% of total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal coliform from per 100 g shellfish meat</td>
<td>Less than 230/100 g</td>
<td>30</td>
<td>18</td>
<td>1</td>
<td>49</td>
<td>49/82 59%</td>
</tr>
<tr>
<td></td>
<td>More than 230/100 g</td>
<td>17</td>
<td>3</td>
<td>13</td>
<td>33</td>
<td>33/82 41%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>47</td>
<td>21</td>
<td>14</td>
<td>82</td>
<td>82/82 100%</td>
</tr>
<tr>
<td>Standard plate count per g</td>
<td>≤500.000</td>
<td>45</td>
<td>21</td>
<td>7</td>
<td>73</td>
<td>73/82 99%</td>
</tr>
<tr>
<td></td>
<td>&gt;500.000</td>
<td>2</td>
<td>0</td>
<td>7</td>
<td>9</td>
<td>9/82 11%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>47</td>
<td>21</td>
<td>14</td>
<td>82</td>
<td>82/82 100%</td>
</tr>
</tbody>
</table>

REFERENCES


