A COMPARISON OF METHODS FOR THE ISOLATION OF A WIDE RANGE OF VIRUSES FROM SHELLFISH

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ABSTRACT

Four methods were compared for the isolation of a wide range of enteric viruses from shellfish. The comparison was aimed at finding a practical and sensitive method to be used routinely. Three of the methods (A, B and C) were based on viral adsorption and desorption using buffers at different pH values. A fourth method (D) consisted of a single homogenization step with no pH adjustments. High recoveries for polio I were shown with the first three methods A–C, namely 81%, 79% and 77% respectively. Recoveries for reo I (Lang strain) and the simian rotavirus SAll were not satisfactory using methods A–C. By contrast, method D gave high recoveries for reo and SAll virus (31% and 68%, respectively). A modified method D generally yielded higher recoveries as indicated by a 91%, 33% and 35% recovery for polio I, reo and SAll viruses respectively. Comparison of method A and modified method D for the detection of viruses from environmental shellfish samples indicated that the two methods were of equal sensitivity.

KEYWORDS

Shellfish, Polio I, Reo, SAll, pH, Primary vervet kidney cells, Recovery methods.

INTRODUCTION

Bivalve molluscs are filter-feeding animals. Their feeding process is carried out by filtering food particles from water; an oyster may filter as much as 1500 l of water per day (Anon., 1976). Feeding rates depend upon variable factors such as salinity, temperature, particulate matter, availability of suitable food and shellfish size (Gerba and Goyal, 1978). When shellfish feed in polluted water containing pathogenic bacteria and viruses, such microorganisms may become entrapped on the mucous membranes of the shellfish and are transferred to their digestive tract. Shellfish are usually consumed whole without thorough cooking, and may thus act as passive carriers of concentrated human pathogens (Gerba and Goyal, 1978). Many types of virus are present in human faeces and may find their way into domestic sewage systems (Gerba et al., 1975). Upon discharge of sewage into the marine environment, these viruses may remain infectious for weeks or even months in the seawater, during which time contact with, and infection of, shellfish may occur. These viruses may include enteric (polio, coxsackie and echo), reo, hepatitis A, rota and adenoviruses. Viruses may cause infections and illnesses such as fever, paralysis, meningitis, respiratory diseases and gastroenteritis (Dienstag et al., 1976; Gerba and Goyal, 1978; Sobsey et al., 1978).

The analysis of mussels and oysters for the presence of pathogenic bacteria and viruses is therefore of great importance. Different methods have been described for the detection of viruses from shellfish (Metcalf and Stiles, 1965; Vaughn and Metcalf, 1975; Sobsey et al., 1978; Richards et al., 1982). Principles used in virus isolation from
shellfish include: elution-precipitation and adsorption-elution-precipitation. Most of the results described in the literature are limited to recoveries of polio viruses; only a few deal with other viruses such as reo and adeno (Sobsey et al., 1978). A need exists for practical methods to detect a variety of viruses from shellfish.

The aim of this study was to obtain a method which would yield a high recovery of a wide range of viruses from polluted shellfish and which is practical for application on environmental shellfish samples. Four methods (A, B, C and D) are evaluated and compared for their recovery of polio 1, reo (Lang strain) and SAl1 viruses from shellfish.

**MATERIALS AND METHODS**

**Cell culture:** Primary vervet monkey kidney cells (National Institute of Virology, South Africa) were used to cultivate and assay enteric viruses. Cells were grown in modified Eagle's minimum essential medium (EMEM) with Earle's salts (Autopow), supplemented with 5% foetal calf serum (FCS) and antibiotics. The cells were maintained in EMEM with 3% heat-inactivated FCS at 37°C.

**Mussels:** Seeding experiments: Commercially available black mussels (Mytilus galloprovincialis) (Brown, 1987) were pooled to give 50 g samples. These samples were seeded with 0.5 mU of viral stock suspension of polio, reo, and SAl1.

Environmental samples: Black mussels were harvested from rocks in Algoa Bay, in the vicinity of the Paapenkuil discharge which contains domestic and industrial effluent. The mussels were transported at 2-10°C and analysed within 8-18 h. They were pooled to give 50 g samples.

**Virus strains and virus assays:** Three virus strains were used, viz. poliovirus type 1, reovirus (Lang strain) and the simian rotavirus SAl1. Viral stock suspensions were stored in a freezing medium (50% FCS, 10% glycerol and 40% MEM) at -70°C. A virus stock titration was carried out for each experiment. Viruses were assayed by using the Tissue Culture Infective Dose (TCID50) method (Reed and Muench, 1938). All experiments were carried out in triplicate and cultures were observed daily for cytopathogenic effects (CPE).

**Method A:** (Sobsey et al., 1978). The infected mussel samples were homogenized in sterile water (pH 4.5), centrifuged (10 000 rpm, 45 min) and the supernatant was discarded. The sedimented virus-containing mussel meat was washed by resuspending in glycine-saline (pH 7.5). Before low-speed centrifugation (5 000 rpm, 30 min), 0.2% Na3P04 was added. The virus-containing supernatant was adjusted to pH 4.5 followed by further centrifugation (10 000 rpm, 45 min). The sedimented mussel meat containing the virus was dissolved in 0.1M Na2HPO4 at pH 7.2-7.4. Chloroform was added for 2 h in order to inactivate unwanted microorganisms, followed by final centrifugation (10 000 rpm, 30 min). This virus-containing supernatant was used for viral analysis.

**Method B:** (Richards et al., 1982). Infected mussel meat was homogenized in glycine-saline (pH 9.5). Cat-floc was added (1%) and stirred to allow floc formation before centrifugation (10 000 rpm, 15 min). Meat extract powder (30 g) was added to the virus-containing supernatant, and the pH adjusted to 3.5. This was followed by centrifugation (10 000 rpm, 15 min). The sedimented virus-containing mussel meat was resuspended in 0.1M Na2HPO4 and the pH adjusted to 7.5. The final stages of chloroform treatment and viral analyses were the same as those described for method A.

**Method C:** (Prozesky, unpublished data). Infected mussel meat was homogenized in 3% beef extract with the addition of an anti-foaming agent (antifoam emulsion B). The sample was sonicated (100 watts, 2 min) and then centrifuged (10 000 rpm, 30 min). The pH of the virus-containing supernatant was adjusted to 3.5 with constant stirring. This was followed by centrifugation (10 000 rpm, 30 min). The sedimented virus-containing mussel meat was resuspended in 0.15M Na2HPO4. Dithizone/chloroform (1 mg diphenylthiocarbazole/100 ml) was added in order to inactivate unwanted microorganisms. The sample was allowed to stand for 2 h, followed by final centrifugation (10 000 rpm, 30 min). Calcium chloride (0.1%), 15 X PBS, penicillin (100 units/ml), streptomycin (100 µg/ml) and fungizone (0.25 µg/ml) were added to the virus-containing supernatant under sterile conditions. Viral analyses on the final supernatant were similar to those described in method A.
Isolation of a wide range of viruses from shellfish

Method D: (Vaughn and Metcalf, 1975). This method was described for coliphage enumeration from water and shellfish. Infected mussel meat was homogenized in sterile saline, followed by centrifugation (5,000 rpm, 10 min). The virus-containing supernatant was added to equal volumes of chloroform (500 ml) and left for 18 h at 4°C. Viral analyses on the final supernatant were similar to those described in method A.

Modified to Method D. (Mod D): Infected mussel meat was homogenized in sterile saline at pH 8.0, followed by centrifugation (5,000 rpm, 10 min). The pH of the virus-containing supernatant was adjusted to 7.2 and chloroform (50 ml) added for 2 h. The virus-containing supernatant was used for virus analysis.

RESULTS

Average percent virus recoveries obtained using methods A, B, C and D are summarized in Table 1.

TABLE 1: Average Percent Virus Recoveries on Seeded Mussels

<table>
<thead>
<tr>
<th>Virus Method</th>
<th>Polio 1 %</th>
<th>Reo %</th>
<th>SAl1 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>81</td>
<td>6</td>
<td>0.6</td>
</tr>
<tr>
<td>B</td>
<td>79</td>
<td>4</td>
<td>11.0</td>
</tr>
<tr>
<td>C</td>
<td>77</td>
<td>12</td>
<td>0.4</td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>31</td>
<td>68.0</td>
</tr>
<tr>
<td>MOD D</td>
<td>91</td>
<td>33</td>
<td>35.0</td>
</tr>
</tbody>
</table>

TCID50 averages

Results indicate high recoveries for poliovirus 1 using the virus adsorption-elution methods A-C. Very low recoveries were obtained for reo and SAl1 viruses using these methods. Recoveries for reo and SAl1 using method D showed a marked increase compared to the first three methods, while the recovery of polio 1 decreased drastically. Modified method D yielded recoveries of 91%, 33% and 35% for polio 1, reo and SAl1 respectively.

The results of method A and modified method D on environmental black mussel samples yielded the results shown in Table 2.

TABLE 2: Comparison of Method A and Modified D on Environmental Mussel Samples for the Presence of Enteric Viruses

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mod D</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Four of the six samples shown in Table 2, yielded the same result. This indicates that method A and modified method D are equally sensitive for analysis of environmental shellfish samples.

Comparison of the five methods using experimental laboratory-infected mussels showed modified method D to be the method of choice, yielding higher recoveries for polio 1 and reoviruses. The recovery of SAl1 was lower than that obtained using the unmodified method.
D, but still acceptable. From these results, it can be seen that modified method D yielded higher recoveries for mussels which contained high numbers of virus. When the numbers of virus present in the mussels dropped, the sensitivity of method D also decreased. However, results may have been influenced by inoculation volumes since the sample volume used for cell culture inoculation in method A was 12 ml whereas the volume in modified method D was 500 ml.

DISCUSSION

The high recoveries for poliovirus 1 using the virus adsorption-elution methods A, B and C indicate polio 1 to be resistant to pH fluctuations (Specter and Lancz, 1986). The low recoveries obtained using methods A, B and C for the recovery of reo and SA1 viruses may be due to the inability of these viruses to tolerate pH fluctuations, or to difficulties in desorption from the particulate matter. The low recovery for poliovirus 1 using method D could be due to low desorption at pH 7.2. This stresses the need for a desorption-elution step for poliovirus. The higher recoveries for reo and SA1 in method D could be due to a reduced effect of pH on adsorption and desorption of these viruses compared to poliovirus, especially at low conductivity. This has been shown by Sobsey et al. (1978) using reovirus. When low conductivity (<2 g NaCl/l) was blended to small pH fluctuations (7.2 - 8.0), acceptable average recoveries were obtained. An additional concentration step could increase the recovery and detection capabilities of the modified method even further. In conclusion, modified method D is a very short and practical method which could be applied with ease to recover a high percentage of a wide range of viruses.

REFERENCES


