Evaluation of Four Methods for Enumeration of *Vibrio parahaemolyticus*

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Two membrane filter (MF) and two most-probable-number methods for enumerating *Vibrio parahaemolyticus* were compared. The MF methods used elevated-temperature incubations (41 and 42°C) and were more specific than the most-probable-number methods (conducted at 35°C). The MF method with a hydrophobic grid and a repair step was most effective.

Since its discovery in Japan in 1950, the enteric pathogen *Vibrio parahaemolyticus* has been shown to be indigenous to estuaries and seaweeds throughout the world (3, 4, 7, 11). *V. parahaemolyticus* infections in the United States and abroad have been associated with the consumption of raw shellfish (2, 12).

The method of examination described by the Food and Drug Administration (FDA) in the *Bacteriological Analytical Manual* (BAM) (9) recommends an overnight selective enrichment step in glucose-salt-Teepol broth followed by isolation on thiosulfate-citrate bile salts-sucrose agar. For the quantitation of *V. parahaemolyticus*, this method is adapted to a three-tube most-probable-number (MPN) procedure (BAM-MPN). Ray et al. (12) developed a repair MPN (R-MPN) method based on preenrichment in tryptic soy broth followed by selective enrichment in glucose-salt-Teepol broth.

Watkins et al. (13) developed a membrane filtration (MF) method for the direct enumeration of *V. parahaemolyticus* (mVP) in seawater. This method features overnight incubation at 41°C on the selective agar, mVP, followed by in situ testing for the fermentation of galactose and sucrose and the production of oxidase. Entis and Boleszczuk (8) modified the mVP method by using hydrophobic grid MFs (HGMFs) and a 4- to 5-h repair step on nonselective agar at 35°C followed by overnight incubation at 42°C on selective *V. parahaemolyticus* sucrose agar (8).

The present study compared the four methods described above for recovery and enumeration of and specificity for *V. parahaemolyticus* in naturally contaminated environmental seawater and shellfish collected from a wide range of locations under various seasonal and hydrographic conditions.

**Sample collection.** Water and oysters were collected seasonally from 18 locations, both National Shellfish Sanitation Program-approved and prohibited areas, in Rhode Island, Virginia, South Carolina, Florida, Alabama, Louisiana, Texas, and Washington. Two NSSP-approved areas were sampled in California. Oysters were *Crassostrea virginica* from the Atlantic and Gulf coasts and *Crassostrea gigas* from the Pacific coast.

Water was collected in sterile 1-liter wide-mouth containers (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) by the method of the American Public Health Association (1). Oysters were hand picked or collected with oyster tongs or dredges. They were held in insulated containers with ice packs, and analysis was initiated within 24 to 48 h (usually 24 h) after collection. Surface water temperature and salinity were determined at each site by using a mercury thermometer and a hand refractometer (American Optical Corp., Buffalo, N.Y.), respectively.

*V. parahaemolyticus* analyses. *V. parahaemolyticus* densities in seawater were estimated by all four methods. The HGMF method used the ISO-GRID MF system (0.45-μm pore size; QA Laboratories Ltd., Toronto, Ontario, Canada). The mVP procedure used GN-6 Metrel filters (0.45-μm pore size, 47-mm diameter; Gelman Sciences, Inc., Ann Arbor, Mich.). The HGMF method was modified to include an in situ test for acid production from galactose. The following portions of seawater were used with each method: BAM-MPN and R-MPN, 10, 1.0, 0.1, and 0.01 ml; and mVP and HGMF, 100, 10, and 1.0 ml. Oysters were analyzed similarly, except that the mVP method was not used because oyster homogenate clogged the MFs. The following portions of oyster homogenate were used with each method: BAM-MPN and R-MPN, 0.1, 0.01, 0.001, and 0.0001 g; and HGMF, 0.1, 0.01, and 0.001 g.

Suspect colonies from each isolation agar were considered *V. parahaemolyticus* for enumeration purposes if they met the following criteria: positive growth at 42°C, positive for oxidase and galactose, no growth on gelatin medium with no NaCl, negative for *p*-nitrophenyl-β-D-galactopyranoside, and no acid from sucrose. Representative isolates (approximately 50 randomly chosen suspect isolates from each method) were tested by the API 20E system (Analytab Products, Plainview, N.Y.) for confirmation of *V. parahaemolyticus*.

Geometric means were determined for each sample type and each method. Samples with nondetectable colonies were assigned the minimum detectable density on the basis of the volumes examined. The Kruskal-Wallis test, a nonparametric rank test, was used with quantitative data to determine and locate any significant differences among the methods (10). Differences in qualitative data and suspect confirmation proportions (specificities) were determined by the normal deviate Z (5).

Data comparing the four *V. parahaemolyticus* isolation methods by the qualitative and quantitative recoveries of *V. parahaemolyticus* appear in Table 1. *V. parahaemolyticus* was found most frequently in water and oysters by the R-MPN and HGMF methods, with no significant differences between the methods with either substrate. The HGMF method yielded the highest *V. parahaemolyticus* counts in...
TABLE 1. Qualitative and quantitative recoveries of *V. parahaemolyticus* from seawater and oysters

<table>
<thead>
<tr>
<th>Method</th>
<th>% Positive for <em>V. parahaemolyticus</em></th>
<th>Mean <em>V. parahaemolyticus</em> density/100 g or 100 ml&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Oysters</td>
</tr>
<tr>
<td>R-MPN</td>
<td>68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HGMF</td>
<td>69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BAM-MPN</td>
<td>56&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>mVP</td>
<td>49&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> n = 65 each for seawater and oysters; means are geometric means.<br> <sup>b</sup> Values followed by the same letter within a column are not significantly different (P < 0.05) by normal deviate Z.<br> <sup>c</sup> ND, Not determined.

*V. parahaemolyticus* was recovered from both water and oysters, followed by the R-MPN and BAM-MPN methods. There were no significant differences among these methods for water, and all were significantly different from the mVP method (P < 0.05). For oysters, no significant difference was found between the HGMF and R-MPN methods, but both methods were significantly better than the BAM-MPN method (P < 0.05). The *V. parahaemolyticus* counts in oysters were generally two logs higher than those in water.

The specificity of each method was estimated by randomly selecting approximately 50 suspect isolates per method and confirming them as *V. parahaemolyticus* by the API 20E system. The selection of these isolates was based on typical colony morphology on each isolation medium; no additional biochemical data (i.e., α-nitrophenyl-β-D-galactopyranoside or oxidase) were used. The MF methods were significantly more specific (P < 0.05) than the MPN methods; 100% of the mVP and 85% of the HGMF suspects were confirmed as *V. parahaemolyticus*, whereas the specificities of the R-MPN and BAM-MPN methods were 65 and 58%, respectively.

The high qualitative and quantitative levels of *V. parahaemolyticus* recovered by the R-MPN and HGMF methods may have resulted from an enhanced recovery of thermally injured cells. A repair step used by both methods in a nonselective medium before selective plating increases *V. parahaemolyticus* recovery in chilled or frozen seafood (12). Differences in inoculum sizes may also have influenced these recoveries. For water analyses, 111.0 ml was used for the MF methods but only 33.3 ml was used for the MPN procedures. Because oyster homogenates clogged MFs, their size was limited to 0.111 g for the HGMF method; the mVP method could not be used with oysters because of severe clogging.

The methods also varied in the number of *V. parahaemolyticus* suspects selected. A maximum of five suspects per homogenate was selected with the MF methods. The threetube, four-dilution MPN methods produced up to 12 isolation plates per homogenate and often resulted in increased selection of suspects; however, the labor and materials also increased. MPN computations for the BAM-MPN and R-MPN methods were complicated by the occurrence of fewer verified positive tubes at lower dilutions than at higher dilutions (skips) in about half of the homogenates containing *V. parahaemolyticus*. As a result, bacterial density may have been underestimated.

The MF methods were generally more specific for *V. parahaemolyticus* than the MPN methods. The use of elevated temperature, which inhibits other competing estuarine microflora (6), may have been a major factor. However, the high (100%) specificity of the mVP method may have been at the cost of lower recovery of *V. parahaemolyticus*, since this method had the lowest qualitative and quantitative recoveries of *V. parahaemolyticus*.

Other factors, such as the rapidity and efficiency of the methods for enumerating *V. parahaemolyticus*, should also be considered. The MF methods gave presumptive results overnight and required only one-fourth of the materials and labor required by the MPN analyses. The overall performance of the HGMF method was superior to that of other test methods because it gave rapid results with minimal labor and supplies, allowed simple evaluation of results, was useful with both water and food, and provided the highest density estimates. In addition, its high specificity compared favorably with those of the other methods.

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**LITERATURE CITED**


