Comparison of Three International Methods with
APHA Method for Enumeration of Escherichia coli
in Estuarine Waters and Shellfish

MILES L. MOTES, JR.*, ROLAND M. McPHEARSON, JR. and ANGELO DePAOLA, JR.

Fishery Research Branch, Food and Drug Administration, Dauphin Island, Alabama 36528

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ABSTRACT

Three international methods were evaluated for enumerating Escherichia coli in estuarine waters, oysters (Crassostrea virginica), mussels (Mytilus edulis) and clams (Mercenaria mercenaria). Results of the French most probable number (MPN) method, a modification of the MacKenzie, Taylor and Gilbert (1948) method, were obtained within 48 h and compared favorably with those obtained by the standard American Public Health Association (APHA) MPN procedure in all sample types. Results of the Australian Anderson and Baird-Parker plate count method, obtained within 24 h, were significantly lower than those obtained with the standard APHA procedure for all sample types. Results of the British roll tube method, a 24-h direct count method, compared favorably with the standard APHA procedure only for mussels and waters.

Because of their ability to concentrate organisms from overlying waters in areas polluted with human sewage, shellfish are associated with outbreaks of enteric disease and food poisoning (7). Current standards used in the sanitary control of shellfish are based on detection and enumeration of indicator organisms such as Escherichia coli in shellfish-growing waters since testing for each bacterial or viral pathogen is impractical (4). The methods used for enumerating E. coli are based on most probable number (MPN) or direct count determinations.

MPN procedures are usually laborious and expensive, requiring many steps, multiple tubes of various media and a minimum of 72-96 h for confirmation. Direct count methods are more convenient and inexpensive, usually requiring the inoculation of only one set of media, followed by confirmation in 24 h. A reduction in confirmation time is critical in the regulation of shellfish-growing waters, as documented by the development of 24-h MPN methods (3,13).

Current microbiological standards require methods with sensitivity in the 2-5 cells/g range. Although direct count methods are quite accurate when bacterial populations are high, they lack sensitivity at this critical range. Fresh and fresh frozen shellfish require continuous surveillance to protect the public health. In the United States, the National Shellfish Sanitation Program (NSSP) has established uniform sanitation requirements and a fecal coliform MPN wholesale market standard of 230/100 g (2.3/g) for shellfish meats. The NSSP-certified American Public Health Association (APHA) method (1) evaluates the microbiological quality of shellfish as determined by this standard. Foreign countries exporting shellfish to the United States must also meet these standards before certification by NSSP.

Some foreign governments interested in shellfish exportation use microbiological methods which have not been approved by the NSSP. A systematic study of these various international methods is needed for comparison with the APHA method (1). Studies which have compared proposed or revised methods with the standard MPN method for various sample types include the minerals modified glutamate medium method in waters (6), the rapid agar plate method for sewage (12), the Anderson and Baird-Parker method for raw meats (14), the A-1 method for waters and shellfish (9,10) and the modified membrane filter method for marine waters (8). Results have shown no significant differences between methods and have not demonstrated better recovery with the proposed methods.

The present study compares the Anderson and Baird-Parker direct plating (DP) method of Australia (2), the direct count roll tube (RT) method of the United Kingdom (15) and the brilliant green lactose bile broth (BGLB) MPN method of France (11) with the APHA standard method (1) for both estuarine water and shellfish samples.

MATERIALS AND METHODS

Sample preparation
A total of 100 samples each of estuarine waters, oysters (Crassostrea virginica), clams (Mercenaria mercenaria) and mussels (Mytilus edulis) were collected from January to December 1982 from a wide range of naturally polluted shellfish-growing waters. Gulf Coast oyster and water samples were collected during the winter quarter. Atlantic Coast mussel and clam samples were collected during the summer and spring quarters, respectively. Clams were also collected during the fall quarter. Of the clams collected, 50 were artificially contaminated with various
concentrations of raw sewage in a closed system under controlled conditions. After shellfish were scrubbed with a clean brush under running tap water to remove surface mud and extraneous growth, they were allowed to air-dry and were then opened under aseptic conditions. The meats were transferred to sterile Waring blenders containing an equal volume by weight of 0.1% peptone water; the mixture was blended for 60 s at 14,000 rpm. The shell liquor was excluded from the sample in the direct count methods but was incorporated with the sample in the MPN methods. Water samples required no preparation and were inoculated directly. All dilutions were made with 0.1% peptone water instead of 0.5% peptone water as recommended by the APHA method to facilitate sample preparation, since the other methods recommend 0.1% peptone water. The exclusion or inclusion of shellfish liquor was as dictated by the countries employing the various methods.

Sample analysis

Standard MPN method (1). A series of five-tube ten-fold dilutions for each sample was inoculated into lauryl sulfate tryptone broth (Difco Laboratories, Detroit, MI) and incubated 24 and 48 ± 2 h at 35 ± 0.5°C in a warm air incubator. At 24 and 48 h, gas-positive cultures in the lauryl sulfate tryptone tubes were transferred to EC medium (Difco) and incubated in a water bath at 44.5 ± 0.2°C for 24 ± 2 h. All cultures from gas-positive tubes of EC medium were streaked to Levine eosin methylene blue agar (Difco) and incubated at 35 ± 0.5°C for 24 h. Representative colonies of each type were picked and inoculated into lactose broth (Difco) and incubated at 35°C for 24 and 48 h. Cultures from gas-positive cultures were inoculated into 1% tryptone, methyl red/Voges-Proskauer medium and Koser citrate medium at 35 ± 0.5°C. IMViC determinations were made, and only those colonies yielding type I (+ + - -) or type II (- - + +) were used to determine the MPN of E. coli.

French MPN method (11). Five-tube decimal dilutions for each sample were inoculated into BGLB (Difco) and incubated 24 ± 2 h at 37 ± 0.5°C in a warm air incubator. Cultures from gas-positive BGLB tubes were transferred to BGLB and peptone water (10 g of peptone and 5 g of NaCl in 1 L of distilled water, pH 7.4-7.5) and incubated in a water bath at 44.5 ± 0.2°C. After 24 ± 2 h, the BGLB tubes were examined for acid and gas, and the peptone water was tested for indole with Kovac’s reagent. A positive result from each test determined the MPN for E. coli type I.

Direct count plating method (2). Sterile Metricel™ membrane filters of 0.45-μm pore size and 90 mm in diameter (Gelman Sciences, Ann Arbor, MI) were transferred aseptically to the surface of tryptone bile agar (Difco) dispersed in 100-mm glass petri dishes (Corning). The membrane was flattened to make uniform contact with the surface of the agar and then inoculated with duplicate 1.0-ml volumes of the 1:1 and 1:10 dilutions. The inoculum was spread evenly over the entire surface of the membrane with a sterile glass spreader. After absorption of the inocula (3.5 min), plates were placed in warm Whirl-Pak bags and incubated in an upright position in a water bath at 44.5 ± 0.2°C for 24 ± 2 h. After incubation, 2 ml of indole reagent (20 g of p-dimethylaminobenzaldehyde in 40 ml of concentrated HCl diluted to 400 ml with distilled water) was pipetted onto the inner surface of the petri dish lid. The membrane was then carefully transferred from the TBA plate to the indole reagent in the lid. Indole-positive colonies on the surface of the membrane stained reddish-pink within 5 min, and the number of colonies of this type was recorded as the number of E. coli cells.

British roll tube direct count method (15). The roll tube medium was prepared by adding 4 ml of MacConkey agar (Oxoid No. 3 roll tube) to 1-ounce McCartney bottles and autoclaving for 15 min. The bottles were then maintained at 46.0°C in a water bath. Duplicate 1-ml volumes of the 1:1 and 1:10 dilutions were inoculated into roll tubes, shaken gently to mix the contents and placed on a roll tube apparatus (400 rpm) for 2 min or until the medium set. The roll tubes were then incubated in a water bath at 44.5 ± 0.2°C for 24 ± 2 h. After incubation, the tubes were placed on a counting chamber and all red colonies ≥0.5 mm were counted as the number of E. coli cells per sample.

Statistical analysis

Analysis of variance was determined for each method from the results of ten replicate E. coli analyses. For each commodity the same sample was used for all methods. From these data the coefficient of variation (CV) was calculated. Data were converted to logarithms (base 10) for geometric mean determinations and for fulfilling the assumptions in the regression analyses. Direct count values of 0.0 were assigned a value of 1.0, corresponding to the lower limit of measurement for these methods. Accordingly, all other values for all methods were increased one unit before logarithm transformations. At completion of analyses, values were decreased one unit after transformation from logarithm to equate original data. Statistical differences between the means and the correlation coefficients for the international methods and those for the APHA reference method were compared using a t-test (P≤0.05).

RESULTS AND DISCUSSION

The CVs for each method by sample type are presented in Table 1. Low CV values indicate good reproducibility for the method. A high CV value and a low mean count in the DP method for clams resulted from inconsistent recovery; consistent recovery in the RT method accounted for the lowest CV values. Generally, results of the MPN methods varied more than those of the direct count methods. Variations attributable to experimental design and/or methodology were not evident at the levels tested for each sample type.

The geometric means for each method by sample type and the differences in recovery capability among the methods for all sample types are indicated by the P values (Table 1). Generally, the APHA and the BGLB methods gave the best recoveries; lowest recovery was from the DP method. Recovery from mussels and clams by the RT method compared favorably with the APHA method. Stained colonies in the roll tubes from mussel samples were clearly distinguishable from particles of the homogenate. Colonies from other shellfish were more difficult to identify. For oysters, the BGLB method yielded 88%, the DP method 57% and the RT method 39% of the APHA method. Superior recoveries with the APHA method were confirmed by conventional IMViC procedures. The specificity for E. coli of the international methods tested is questionable since recovery, particularly by direct count, was less than expected.

Regression correlations are illustrated for all sample types in Fig. 1-4. P-values for the correlation coefficients derived from the regression analyses indicate a positive linear correlation between the APHA and the international methods. Most observations for the direct count methods fall below the line of equality, indicating consistently better recovery by the APHA method. Correlation coefficients were best for the BGLB method and worst for the DP method for all sample types. Correlation is, at best, an indicator of a predictable relationship. The only direct relationships to the reference APHA method, as exemplified by the regression coefficient (slope) were with the BGLB method.

The NSSP has established a market standard for fresh or fresh frozen shellfish not to exceed a fecal coliform MPN of 2.30/g (230/100 g) of sample. The values for the methods of comparison that correspond to this standard (Table 2) were calculated directly from the regres-
TABLE 1. Comparison of methods for reproducibility$^a$ and recovery of E. coli.

<table>
<thead>
<tr>
<th>Value</th>
<th>Water</th>
<th>Oyster</th>
<th>Clam</th>
<th>Mussel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean count (MPN/g or count/g or ml)</td>
<td>APHA</td>
<td>BGLB</td>
<td>DP</td>
<td>RT</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>22.7</td>
<td>85.8</td>
<td>23.5</td>
<td>59.4</td>
</tr>
<tr>
<td>Coefficient of variation (CV, %)$^b$</td>
<td>17.0</td>
<td>55.7</td>
<td>7.11</td>
<td>5.50</td>
</tr>
<tr>
<td>Geometric mean$^b$</td>
<td>0.80</td>
<td>0.75</td>
<td>0.31</td>
<td>0.80</td>
</tr>
<tr>
<td>P value</td>
<td>0.4573</td>
<td>0.0001$^c$</td>
<td>0.9191</td>
<td>0.2161</td>
</tr>
</tbody>
</table>

$^a$Based on 10 replicates per sample type.
$^b$Based on 100 samples; expressed per g or ml.
$^c$P<0.05; the log geometric mean is significantly smaller than the APHA mean as determined by the t-test.
Figure 3. Correlation between logs of international methods and APHA method in clam samples tested. BGLB method, slope 0.7992, correlation coefficient 0.8829, P = 0.0001; DP method, slope 0.2993, correlation coefficient 0.6250, P = 0.0001; RT method, slope 0.5243, correlation coefficient 0.6925, P = 0.0001. Dashed line represents line of equality.

Table 3 compares the number of samples for each range of values by method and sample type to the number of samples unacceptable by the NSSP/APHA standard. Again, the direct count methods lacked the ability to measure the lower levels critical to product acceptability. Inability to accommodate an adequate sample volume may account for this poor performance at low population levels. Mussels analyzed by the DP method were unacceptable regardless of the count since over 50% of the samples in the 0 to <1 cells/g range were above the APHA standard. Similar observations were noted with the RT method for oysters.

In conclusion, approval of these direct count methods by the NSSP is not recommended. Although the French

Table 2. Values of methods corresponding to APHA market standard {2.3/100 g} for each shellfish type.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>BGLB (MPN/g)</th>
<th>DP (count/g)</th>
<th>RT (count/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oyster</td>
<td></td>
<td>2.19</td>
<td>1.38</td>
<td>0.89</td>
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<tr>
<td>Clam</td>
<td></td>
<td>3.25</td>
<td>0.52</td>
<td>1.76</td>
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<tr>
<td>Mussel</td>
<td></td>
<td>2.87</td>
<td>0.70</td>
<td>3.50</td>
</tr>
</tbody>
</table>

*Market standard usually expressed as 230/100 g.

Wholesale market standard usually expressed as 230/100 g.

The number of samples ≥2.3 g by APHA method.

In a recent comparison of the DP method with the A-1 MPN method and the Standards Association of Australia MPN method in Sydney rock oysters (16), the DP method compared favorably with the MPN methods in the critical range of 2-5 E. coli cells/g of sample. Likewise, the A-1 MPN method was comparable to the APHA method for the American oyster (5); however, in this study, neither the Australian DP method nor the British RT method compared favorably with the APHA method for all sample types.

In conclusion, approval of these direct count methods by the NSSP is not recommended. Although the French
BGLB method compared favorably with the APHA method for all shellfish types, isolates were not confirmed as *E. coli*. Additional collaborative studies are recommended before NSSP approval.

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