Comparison of a Rapid Plate Count and MPN Methods for Enumeration of Fecal Coliforms and Escherichia coli in Soft-Shell Clams

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ABSTRACT

A direct elevated temperature plate count method utilizing modified fecal coliform agar with rosolic acid (ETPC/mFC) was compared to 5-tube and 3-tube most probable number (MPN) procedures for its accuracy in enumerating fecal coliforms and Escherichia coli in naturally and artificially contaminated soft-shell clams (Mya arenaria). The results indicated that the extent of overall recovery of fecal coliforms was similar in the two methods tested. Therefore, the ETPC/mFC method may be considered as a rapid procedure for fecal coliform screening during depuration of soft-shell clams.

Key words: Fecal coliforms, clams, enumeration, depuration

The National Shellfish Sanitation Program (NSSP) is a cooperative effort among the states, the Food and Drug Administration (FDA), and industry, which approves certification for interstate shipping of shellfish, according to procedures described in its Manual of Operations, parts 1 and 2 (5, 6). The program establishes tolerance levels allowed per 100 grams of shellfish meat, after controlled purification (depuration).

This process, which is under the supervision of the State Shellfish Control Agency (SSCA), is applied to reduce the number of pathogenic organisms that may be present in shellfish harvested from moderately polluted (restricted) waters to such levels that the shellfish will be acceptable for human consumption without further depuration.

To assure compliance, the NSSP Manual of Operations indicates that sample analysis shall be conducted by a laboratory approved by the SSCA according to specified requirements. The Manual also specifies use of the American Public Health Association (APHA) recommended procedures for the examination of seawater and shellfish for the collection, transportation, and bacteriological examination of shellfish samples (5, 6).

In addition to being detected by officially accepted most probable number (MPN) methods, fecal coliforms have been detected by direct plating on solid agar media such as modified fecal coliform (mFC) agar, which has been used in the hydrophobic grid membrane filter method to enumerate coliforms in foods (3, 4, 7). An elevated temperature of incubation can further enhance selectivity; therefore, the elevated temperature plate count using mFC agar with addition of rosolic acid (ETPC/mFC) was introduced for use in soft-shell clam analysis in 1983 by the Massachusetts Division of Marine Fisheries Shellfish Purification Plant in Plum Island, Newburyport, Massachusetts. The original pour plate method, developed in 1966 by Heffernan and Cabelli (8, 10, 11), used MacConkey agar. In 1977, Varga et al. (13) compared coliform densities recovered with MacConkey agar to those recovered by the APHA 5-tube MPN method and reported that plate counts were lower by 10% in comparison to standard MPN counts, while counts recovered on nutrient agar plates were lower by 20%. An unpublished study from Newburyport, Massachusetts, by Jack Delaney and Mr. Rosario, used ETPC/mFC agar plates with rosolic acid, instead of MacConkey agar plates, to detect E. coli in soft-shell clams (personal communication, D. T. Regan of the State Division of Marine Fisheries Shellfish Purification Plant, Plum Island, Newburyport, Massachusetts). The objective of the study reported here was to compare recoveries of fecal coliforms from soft-shell clams (Mya arenaria) using standard 3- and 5-tube MPN methods or the simpler and more rapid ETPC/mFC agar plating method.

MATERIALS AND METHODS

Clam sampling

All clam samples were shipped to the laboratory from the collection sites in plastic bags covered with ice in styrofoam containers, by overnight air carrier, and analyzed within 24 h of collection.

The study involved two parts: one compared recovery of coliforms with the ETPC/mFC agar plating method and a 5-tube MPN procedure (1), and the second compared recoveries with ETPC/mFC agar plates and a 3-tube MPN procedure (2). In the first part of the study (5-tube MPN) the analysis involved 12 samples of clams analyzed in 5 subsamples each. The soft-shell clams were...
collected from eleven commercial sites (twice from one site); 8 samples were from Massachusetts and 4 from the state of Washington. In addition, detection of fecal coliforms by these two methods was compared in clam samples from Massachusetts, which were inoculated in the laboratory with three levels ($10^4$, $10^5$, and $10^6$ colony-forming units (CFU)/100 g of tissue) of an *Escherichia coli* strain recovered from one of the samples.

In the second part of the study (3-tube MPN), 24 clam samples were collected from three different areas within each of two states (Massachusetts and Washington) during different seasons of the year, and from one site in Maryland. One sample of clams was collected from each of three sites in Massachusetts during the months of March, May, August, and November. These 12 samples were each divided into 5 subsamples for analysis. Thus, the total number of analytical units from Massachusetts was 60. Clams samples from the State of Washington were collected from two of three areas during January and from all three areas during March and June for a total of 36 subsamples analyzed. To test for recovery of injured cells, clam samples collected from one area in Maryland were inoculated with *E. coli* (wild strain recovered from clams) at levels of $10^4$, $10^5$, and $10^6$ CFU/100 g and tested before (6 subsamples) and after the cells were stressed by freezing and thawing twice (first at -13°C for one week, then at 0°C for one week, then back at -13°C for one week, and finally a 24-h thawing at 0°C). The total number of clam subsamples tested from Maryland was 98 (35 uninoculated, 18 tested before stress, and 45 stressed). The study also involved comparison of ETPC/mFC agar plating and 3-tube MPN detection of *E. coli* cells inoculated in sterile phosphate buffer.

**Clam inoculation**

A culture of *E. coli* (isolated from soft-shell clams) was used to inoculate the clam samples and the phosphate buffer, as indicated above. The culture was propagated for 18 to 24 h in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 35°C. Before inoculation of clams, the culture was diluted to yield a low ($10^4$), medium ($10^5$), and high ($10^6$) concentration of cells per 100 g of clams with sterile Butterfield’s phosphate buffer (pH 7.2) diluent (4) containing 0.2 g/l of magnesium chloride (BPBMg) for the 3-tube MPN study, and with Butterfield’s phosphate buffer diluent without magnesium chloride (BBP), as directed in a personal communication by Diane Regan, for the 5-tube MPN study. The number of cells inoculated onto the clams was estimated by absorbance readings at 530 nm and verified by plating on Levine’s eosin-methylene blue (L-EMB) agar and incubating at 35°C for 48 h.

**Analyses**

For the ETPC/mFC agar plating method mFC agar (Difco) was prepared in double strength, mixed, heated to boiling, and allowed to drain for up to 30 min at room temperature. The culture was propagated for 18 to 24 h in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 35°C. Rosolic acid (Difco) was then added (1%) to the cooled agar, which was mixed again and held in a 50 to 55°C water bath for use within 2 h. Five (or 4 in some instances) subsamples of clams per sample, consisting of 12 to 20 clams, or enough to yield a minimum volume of 250 g of shocked clams, were placed in a sanitized draining pan, washed with a sterile scrub brush under cold running tap water, and allowed to drain for up to 30 min at room temperature. Shells were opened with a sterile shocking knife and the meat was put into a sterile blender jar.

A portion of 250 g of shellfish was then blended for 90 s in an Osterizer blender (Oster Corp., Milwaukee, WI) with an equal amount of sterile buffer diluent. A 12-g portion of the blended shellfish was then added into a sterile disposable plastic cup containing 54 ml of sterile buffer and the mixture was swirled gently for even mixing. The sample cup contained 66 ml of diluted product (54 ml + 12 g of clam slurry). Then a volume of 60 ml of tempered mFC agar was added to the sample cup, which was inverted slowly once. Immediately the contents were distributed as evenly as possible into six sterile petri plates (15 by 100 mm). After the agar solidified, the plates were inverted and placed into a 45.5°C incubator. The colonies on all six plates were counted after 24 h. To obtain the fecal coliform concentration per 100 g of shellfish meat, the total number of colonies (from 6 plates per subsample) was multiplied by 16.6 (100 g/6 g of meat), rounding the dilution factor to 17. When developing on mFC agar, fecal coliform colonies exhibit a lighter blue color with a light blue halo against the grey background of the agar medium.

Two control samples inoculated with *E. coli* ATCC 25922 were analyzed with each sample, as positive controls, and a negative control of deionized water used to prepare media was also tested. Verification was performed by transferring counted colonies to lactose broth, *E. coli* (EC) broth, Levine’s eosin-methylene blue agar (L-EMB) agar, and to Indole, Methy red, Voges-Proskaven, and Citrate (IMViC) biochemicals. Colonies were picked from the agar plates using a sterile disposable pipette.

The 5-tube MPN procedure was performed as described by the APHA (1). Equal parts of shellfish meat (200 g) and sterile BPB (200 ml) were blended for 60 s and then diluted and inoculated into MPN tubes containing 10 ml of single-strength LST broth. The inoculated (lauryl tryptose) LST tubes were incubated at 35°C for 24-48 h. Then EC tubes (Difco) were inoculated from positive LST tubes, showing turbidity and gas production, and incubated at 45.5°C for 24 h. Fecal coliforms were again verified by using L-EMB agar, EC broth, and IMViC testing.

The 3-tube MPN method (2) involved inoculation of tubes containing 10 ml of LST broth with 1 ml of blended clam meat and buffer, and incubation at 35°C for 24 h. Growth from

**TABLE 1. Comparison of fecal coliform counts detected in soft-shell clams using the ETPC/mFC and the 5-tube MPN procedure**

<table>
<thead>
<tr>
<th>Site</th>
<th>State</th>
<th>Month</th>
<th>ETPC/mFC (log CFU/100 g)</th>
<th>5-Tube MPN (log MPN/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MA</td>
<td>Jan</td>
<td></td>
<td>2.61 ± 0.65</td>
<td>2.06 ± 0.18</td>
</tr>
<tr>
<td>2 MA</td>
<td>Jan</td>
<td></td>
<td>2.60 ± 0.07</td>
<td>2.20 ± 0.19</td>
</tr>
<tr>
<td>3 MA</td>
<td>Jan</td>
<td></td>
<td>2.19 ± 0.12</td>
<td>2.66 ± 0.38</td>
</tr>
<tr>
<td>4 WA</td>
<td>Feb</td>
<td></td>
<td>1.60 ± 0.32</td>
<td>1.55 ± 0.23</td>
</tr>
<tr>
<td>5 WA</td>
<td>Mar</td>
<td></td>
<td>2.48 ± 0.06</td>
<td>2.45 ± 0.19</td>
</tr>
<tr>
<td>6 MA</td>
<td>Apr</td>
<td></td>
<td>2.25 ± 0.12</td>
<td>2.44 ± 0.25</td>
</tr>
<tr>
<td>7 MA</td>
<td>Apr</td>
<td></td>
<td>2.23 ± 0.19</td>
<td>2.08 ± 0.24</td>
</tr>
<tr>
<td>8 MA</td>
<td>Apr</td>
<td></td>
<td>2.64 ± 0.08</td>
<td>2.63 ± 0.23</td>
</tr>
<tr>
<td>9 MA</td>
<td>Apr</td>
<td></td>
<td>2.42 ± 0.10</td>
<td>2.40 ± 0.11</td>
</tr>
<tr>
<td>10 MA</td>
<td>Apr</td>
<td></td>
<td>3.20 ± 0.07</td>
<td>3.30 ± 0.07</td>
</tr>
<tr>
<td>11 WA</td>
<td>May</td>
<td></td>
<td>2.13 ± 0.12</td>
<td>2.25 ± 0.17</td>
</tr>
<tr>
<td>12 WA</td>
<td>May</td>
<td></td>
<td>2.63 ± 0.06</td>
<td>2.70 ± 0.12</td>
</tr>
</tbody>
</table>

Mean ± SD

- SD, standard deviation; no significant differences (P > 0.05) were detected in pairs of the same row. Five observations per site.
- Samples taken from the same area.
- Total observations: 60.
positive tubes was transferred to EC broth and incubated at 44.5 ± 0.1°C for 24 h. All positive tubes were confirmed for the presence of *E. coli* by IMViC testing. Variances of all data were pooled and *t*-tests were computed to examine differences in recovery between the agar plating and MPN methods (9).

RESULTS AND DISCUSSION

5-Tube MPN comparison

The mean level of fecal coliforms detected by the 5-tube MPN procedure was not significantly different (*P > 0.05*) from the number of colonies recovered with the ETPC/mFC agar direct plating method (Table 1). In addition, analysis of the clams inoculated in the laboratory with three levels of *E. coli* showed that mean recovery by the ETPC/mFC agar plating method was not significantly different (*P > 0.05*) from mean recovery with the 5-tube MPN method for all levels of inoculation (10², 10³, and 10⁴ CFU/100 g) (Table 2).

TABLE 2. Comparison of *Escherichia coli* recovery by the ETPC/mFC agar and the 5-tube MPN methods from soft-shell clam samples inoculated at three levels

<table>
<thead>
<tr>
<th>Inoculum level</th>
<th>ETPC/mFC (log CFU/100 g)</th>
<th>5-Tube MPN (log MPN/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10² CFU/100 g</td>
<td>2.00 ± 0.43</td>
<td>1.64 ± 0.35</td>
</tr>
<tr>
<td>10³ CFU/100 g</td>
<td>2.86 ± 0.13</td>
<td>2.69 ± 0.16</td>
</tr>
<tr>
<td>10⁴ CFU/100 g</td>
<td>3.59 ± 0.11</td>
<td>3.65 ± 0.18</td>
</tr>
</tbody>
</table>

* Each mean based on 15 observations (3 replicates, 5 samples).

* MPN values of <17 or <18 were set at 17 or 18.

* Pairs of the same row significantly different at *α* = 0.05.

From a total of 164 colonies picked from ETPC/mFC agar plates that had been inoculated with naturally contaminated clams, and tested by IMViC, 119 (72.5%) were confirmed positive for *E. coli*. For clams inoculated with *E. coli*, 99 (79.8%) of 124 MPN agar colonies tested were found *E. coli*-positive by IMViC. A 100% verification was not achieved because background fermenting coliforms were probably selected accidentally for verification.

3-Tube MPN comparison

Statistical analysis of all the results in the second part of the study indicated that the 3-tube MPN and ETPC/mFC methods yielded significantly similar recoveries (*P > 0.05*) from uninoculated clams from all three states (Table 3). However, mean recovery of fecal coliforms by the 3-tube MPN procedure was significantly higher (*P < 0.05*) from clam samples collected in the state of Maryland, which were then inoculated with 10⁴ CFU/100 g and stressed by freezing and thawing. Also MPN recoveries were significantly higher (*P < 0.05*) than ETPC/mFC recoveries from sterile phosphate buffer solution inoculated with *E. coli* at levels of 10³ and 10⁴ CFU/100 g (Table 3). Overall, the freeze-thaw treatment of clam samples resulted in somewhat reduced numbers of fecal coliforms recovered by the MPN and ETPC/mFC agar plating methods compared to results with the unstressed bacteria. However, it appears that the ETPC/mFC agar plating method could be useful in enumerating fecal coliforms in frozen and thawed clams. There was no difference in recovery by the two methods attributed to the season of the year during which the clams were collected for analysis (Table 1).

The overall (all data from states in Table 3 combined) geometric mean of fecal coliforms recovered with the 3-tube MPN procedure was 690 cells per 100 g of sample, which was...
significant higher ($P < 0.05$) than that from the ETPC/mFC agar plating procedure (530 cells per 100 g of clams). It has been reported previously that the MPN procedure overestimates the coliform population compared to direct plating by an average of 29% (12). Therefore, it is likely that this bias in estimating bacterial numbers with the MPN procedure may account for the differences noted here. Previous research (13) also evaluated the ETPC method with MacConkey agar to measure the sanitary quality of soft-shell clams and found that it underestimated the density of fecal coliforms by about 10% compared to the standard 5-tube MPN procedure. The authors suggested that the fecal coliform standard used in the assessment of the sanitary quality of clams would need to be modified if the analysis were to be done by the ETPC procedure with MacConkey agar.

Of 613 total colonies picked from mFC agar plates and examined in EC broth for the second part (3-tube MPN) of the study, 427, or 69%, were verified as fecal coliforms (Table 4). From the 427 positive EC broth tubes, 224 cultures were transferred onto L-EMB agar and then onto plate count agar (PCA) slants, and 212, or 94.6%, were confirmed to be typical 

E. coli

by the IMViC test. The low percentage (69%) of verification may be due to the difficulty of picking colonies with a pipette from inside the agar and successfully transferring them to a broth. This is confirmed by the low (61.6%) verification for colonies picked from plates inoculated with buffer which contained only 

E. coli

organisms (Table 4). Thus, there is a need for improvement in picking typical colonies from inside the agar of inoculated mFC plates.

The recovery of 

E. coli

from sterile buffer samples inoculated with a pure culture was not comparable to the recovery from natural, inoculated, and frozen-thawed shellfish meat. Cultures from positive EC broth MPN tubes were streaked onto L-EMB and tested by using IMViC for confirmation. From a total of 164 positive EC broth test tubes was tested, 161, or 98.2%, were confirmed as 

E. coli

(Table 4). This high percentage reflects the ease of recovering organisms from an enrichment broth (EC) where cell numbers may be high.

Overall, the results indicated that the rapid (24-h) ETPC/mFC direct plating procedure was at least as effective in recovering fecal coliforms from soft-shell clams as the approved 5-tube APHA MPN procedure and a 3-tube MPN method. Before adoption, however, each laboratory should evaluate the ETPC/mFC agar plating method to determine its applicability in an individual operation.

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Escherichia coli