Effect of Stress and Resuscitation on Recovery of Indicator Bacteria from Foods Using Hydrophobic Grid-Membrane Filtration

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ABSTRACT

The effects of stress and resuscitation on selective enumeration of coliforms, *Escherichia coli* and enterococci in mechanically deboned raw poultry meat and in dried foods were studied using a hydrophobic grid membrane filter (HGMF) technique. The effects of four different stresses, sublethal heating, freezing, acid pH and drying, were examined on 25 to 30 samples per indicator organism for each type of stress. Counts obtained with resuscitation were compared statistically to direct selective counts for each series of samples. Also, both the direct and resuscitative HGMF results were compared to a 5-tube most probable number method for coliforms and *E. coli* and to a spread plate method for the enterococci. The use of appropriate resuscitation procedures not only produced a significant increase in counts over the direct HGMF procedure, but also yielded HGMF results that were statistically equivalent to those obtained by conventional methods.

The application of membrane filters (MF) to bacteriological enumeration has recently expanded into the realm of food microbiology (19). The development of the hydrophobic grid-membrane filter (HGMF) (15,21) and, particularly, the introduction of the ISO-GRID HGMF system (2,3,6) have made the MF technique an attractive alternative to conventional analytical microbiology for foods.

Water microbiologists have observed and accepted a reduced recovery of microorganisms by MF relative to most probable number (MPN) or spread and pour plate techniques. Even with fresh inocula, 80 to 90% recovery of the bacterial population by an MF technique has become the benchmark of MF acceptability (1,7). Maxcy (14) and Warsec (22) suggested that organisms from seemingly unstressed environments may, in fact, be subject to unknown stresses. Thus, the use of a single-step MF approach to the selective enumeration of such organisms may result in an underestimate of the true population (5).

Food microbiologists have expressed serious concern about the significance of stressed microorganisms in foods (20). Failure to recover and enumerate injured cells could have serious consequences to the interpretation of food quality and safety, since stressed cells have been shown to recover and multiply in processed foods under appropriate environmental conditions (4,10,20).

The recovery of stressed microorganisms has been the subject of a number of published investigations which have been reviewed extensively (4,10,11,17). All of these reports showed that after exposure to some sublethal treatment, bacteria lost their characteristic ability to multiply under conditions that were satisfactory for untreated cells (4). These studies clearly demonstrated the need to employ resuscitation procedures for selective enumeration of injured indicator organisms. They also suggested that the parameters for optimum resuscitation could vary depending upon the nature of the organisms being enumerated, the menstruum in which the organisms were suspended and the selectivity of the subsequent incubation conditions (16,18).

Since the application of membrane filtration techniques to food microbiology is relatively recent, the effect of stress on the recovery of microorganisms from foods by membrane filtration has not been extensively studied. Goff et al. (8) demonstrated that a raw milk flora, after sublethal heating in tryptic soy broth (TSB), failed to develop colonies on membrane filters incubated overnight on TSB containing 7.5% NaCl. If the membranes were subsequently transferred to TSB and incubated further, the injured cells repaired and formed colonies. They concluded that membrane filtration could be used to distinguish between injured and uninjured bacteria. Following a study of the enumeration of heat-, freeze-, acid- and dry-stressed *Escherichia coli* by the membrane spread technique of Anderson and Baird-Parker, Holbrook et al. (12) recommended routine inclusion in the method of a 4-h resuscitation period at 35°C to improve recovery of injured *E. coli*.

In a recent publication we showed that the HGMF technique could be applied to the selective enumeration of indicator organisms in foods (3). In that study with coliforms...
and *E. coli*, a 2-h resuscitation period at 35°C on a non-selective medium was used. HGMFs were then transferred to the appropriate selective medium and temperature. While this technique appeared adequate for enumerating the organisms from frozen foods, HGMF results for *E. coli* that had been exposed to an acid stress in salads were significantly lower than those obtained by the conventional method. These observations suggested that requirements for resuscitation might vary depending on the type of stress undergone by the organisms.

To explore this possibility further, we studied the effects of various parameters of resuscitation on the selective enumeration of stressed coliforms, *E. coli* and enterococci by the HGMF technique. This paper reports the results of our investigations.

**MATERIALS AND METHODS**

**HGMFs, filtration units and Sample Processor**

ISO-GRID HGMFs, filtration units and Sample Processor were manufactured and supplied by QA Laboratories Limited (Toronto, Canada) as previously described (2).

**Sample preparation and stress induction**

All work relating to heat, frozen and acid studies was performed with inoculated, mechanically deboned raw poultry. Dry stress studies were done on a variety of inoculated dry foods, including black pepper, parsley leaves, buttermilk pancake mix, seafood batter mix, cake mix, blueberry muffin mix, non-fat dry milk, vanilla pudding mix, non-dairy coffee whitener and onion flakes. All samples were pre-weighed in Stomacher bags in 10-g amounts. Each bag was inoculated with 0.1 ml of the appropriate mixed culture suspension prepared as described previously (3), using a mixture of 5 strains in each case. The bags were sealed and the contents mixed by massaging. Contents were then flattened inside the bag to minimize heat transfer delay for heat and freeze stressing. Preliminary experiments were done to determine the conditions for each type of stress that would produce an injured population consistently. These stressing parameters were then used in all of the succeeding experiments.

**Heat stress**

Freshly inoculated samples were heated in a dry air oven at 55°C for 20 min. Samples were analyzed immediately after removal from the oven.

**Freeze stress**

Inoculated samples were frozen at -18°C for a minimum of 4 d, then thawed after thawing at room temperature for approx. 30 min.

**Acid stress**

Freshly inoculated samples were macerated with 90 ml of acetate buffer, pH 3.5, and held at room temperature for 10 min. Samples were then further diluted in 0.1% peptone-1.0% Tween 80 diluent (6) and analyzed immediately.

**Dry stress**

Inoculated dried foods were held at room temperature for 3 d (coliforms and *E. coli*) or 7 d (enterococci).

**Determination of optimum resuscitation parameters**

This study was done separately with each type of stress for coliforms, *E. coli* and enterococci. Ten individually inoculated food samples were exposed to conditions previously determined to induce injury. Following stress, each sample was homogenized in 0.1% peptone-1.0% Tween 80 diluent using a Colworth Model 400 Stomacher. Further dilutions were prepared in the same diluent, as required, and a series of replicate filtrations were made from each sample. One HGMF was placed directly on the appropriate selective medium and incubated as described below, and the other HGMFs were placed on tryptic soy agar (Gibco Diagnostics Laboratories) supplemented with 0.15% magnesium sulfate (TSA/Mg). Five filters were incubated at 35°C and the remainder at 25°C. At hourly intervals, one filter from each temperature was transferred to the appropriate selective medium and incubation temperature for the remainder of the incubation period.

The HGMF counts were determined by the Sample Processor and the most probable numbers (MPN) were calculated as described previously (2). The percent differences between direct and resuscitative HGMF-MPNs were graphically displayed against resuscitation time for both temperatures of resuscitation. Those parameters of resuscitation (time and temperature) which resulted in the greatest increase in bacterial recovery relative to the controls were selected for further evaluation.

**Evaluation of effectiveness of resuscitation**

This study was done on 25 to 30 inoculated samples for each type of stress and group of indicator organisms. Ten replicate filtrations were done for each sample. Five filters were placed directly on the selective medium and incubated at the appropriate temperature for the specified time. The other five filters were first incubated on TSA/Mg for the time and at the temperature shown in Table 1 and then transferred to the appropriate selective medium and incubation temperature for the remainder of the incubation period. A conventional analysis was also done on each sample as described below.

**Enumeration procedures**

**HGMF.** Coliforms were enumerated by incubating the filters for 24 ± 2h at 35°C on m-FC agar base (Difco Laboratories) without rosolic acid. *E. coli* counts were obtained by incubating the HGMFs on tryptone bile agar (12) at 44.5°C for 24 ± 2h. The indole reaction was developed.
as described previously (3). m-Enterococcus agar (Difco) was used to enumerate enterococci. Incubation was done at 35°C for 48 ± 2h.

Conventional. Coliform and E. coli counts were done using a 5-tube MPN technique (acceptable method MFA-19, Health Protection Branch, Health and Welfare Canada). Enterococci were enumerated by surface plating five replicate 0.1-ml volumes on m-enterococcus agar and incubating at 35°C for 48 ± 2h.

Statistical evaluations.

Direct and resuscitative HGMF results were compared using analysis of variance (ANOVA). A t-test for paired data was used to compare the direct and resuscitative HGMF recoveries with the 5-tube MPN or spread plate counts (9).

Figure 1 illustrates the effects of time and incubation temperature on resuscitation of injured bacteria. Symbols: -- 35°C; --- 25°C; ---. 25°C. Organisms: (1) coliforms; (2) Escherichia coli; (3) enterococci. Stresses: (a) heat; (b) freezing; (c) acid pH; (d) drying.

RESULTS

Figure 1 illustrates the effects of time and incubation temperature on resuscitation of injured coliforms, E. coli and enterococci. Optimum resuscitation parameters varied both with the nature of the stress and, to a lesser extent, with the organisms themselves. Heat-, freeze- and acid-stressed coliforms and E. coli tended to be resuscitated more effectively at 35°C than at 25°C. Conversely, cells exposed to drying were consistently recovered better at 25°C than at 35°C. We were unable to obtain an injured population of enterococci by exposure to an acid pH, and heating had only a minimal effect on these organisms; however, freeze- and dry-stressed enterococci were resuscitated at rates similar to coliforms and E. coli.

Table 2 shows the recovery of injured coliforms by both direct and resuscitative HGMF techniques and compares the performance of both these methods to the 5-tube MPN procedure. Resuscitation increased the HGMF-MPNs for all four stresses, though the increase over the direct HGMF results obtained for dry-stressed coliforms was not statistically significant. Recovery of freeze-stressed coliforms was significantly lower by the direct HGMF technique than by the 5-tube MPN method; however, the resuscitative HGMF technique provided recovery for this type of stress that was statistically equivalent to the conventional method. Recovery of heat-stressed coliforms was significantly higher by the resuscitative HGMF technique than by the conventional method.

Corresponding data for E. coli are presented in Table 3. The resuscitative HGMF technique recovered significantly greater numbers of injured E. coli than the direct HGMF method, regardless of the type of stress. The 5-tube MPN procedure was significantly better than the direct HGMF method in all cases; however, the resuscitative HGMF technique was statistically equivalent to the conventional method for all four types of stress.

The relationships between the direct and resuscitative HGMF-MPNs and the corresponding 5-tube MPNs for stressed E. coli are illustrated in Fig. 2 and 3. With the direct HGMF technique (Fig. 2), only 74 of 114 observations (65%) were within the 95% confidence limits of the 5-tube MPN, 37 (32%) were below and 3 (3%) were higher. Overall, the direct HGMF-MPN to 5-tube MPN ratio was 0.56. With resuscitation (Fig. 3), 90 of the 114 observations (79%) were within the 95% confidence limits of the 5-tube MPN, 16 (14%) were below and 8 (7%) were above. Overall, the resuscitative HGMF-MPN to 5-tube MPN ratio was 0.94.

Since we were unable to produce a significantly stressed population of enterococci by exposure to acid pH or to heat, the comparison between the HGMF method and the spread plate technique was limited to enterococci that had been exposed to freezing or drying. The results of this study are shown in Table 4. While resuscitation yielded a significant increase in the recovery of freeze-stressed enterococci over the direct HGMF technique, both the direct and the resuscitative HGMF procedures were statistically equal.

TABLE 2. Comparison of direct and resuscitative HGMF techniques and a 5-tube MPN method for enumeration of stressed coliforms in foods.

<table>
<thead>
<tr>
<th>Type of stress</th>
<th>Number of samples</th>
<th>MPN per g</th>
<th>HGMF to 5-tube MPN recovery ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct</td>
<td>Resuscitative</td>
<td>5-tube MPN</td>
</tr>
<tr>
<td>Heating</td>
<td>27</td>
<td>9.6 x 10⁵</td>
<td>1.2 x 10⁵</td>
</tr>
<tr>
<td>Freezing</td>
<td>29</td>
<td>4.5 x 10⁵</td>
<td>5.2 x 10⁵</td>
</tr>
<tr>
<td>Acid pH</td>
<td>30</td>
<td>6.6 x 10⁵</td>
<td>8.6 x 10⁵</td>
</tr>
<tr>
<td>Drying</td>
<td>27</td>
<td>5.1 x 10⁴</td>
<td>5.5 x 10⁴</td>
</tr>
</tbody>
</table>

²Resuscitation done as described in Table 1.
³Most probable number.
⁴Statistically significant increase over direct HGMF-MPN (ANOVA; α = 0.05).
⁵Significant difference between the methods (t-test for paired data; α = 0.05).

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TABLE 3. Comparison of direct and resuscitative HGMF techniques and a 5-tube MPN method for enumeration of stressed Escherichia coli in foods.

<table>
<thead>
<tr>
<th>Type of stress</th>
<th>Number of samples</th>
<th>MPN per g</th>
<th>HGMF to 5-tube MPN recovery ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Direct HGMF</td>
<td>Resuscitative HGMF</td>
</tr>
<tr>
<td>Heating</td>
<td>29</td>
<td>4.9 × 10⁵</td>
<td>9.1 × 10⁵</td>
</tr>
<tr>
<td>Freezing</td>
<td>30</td>
<td>2.1 × 10⁵</td>
<td>3.3 × 10⁵</td>
</tr>
<tr>
<td>Acid pH</td>
<td>30</td>
<td>3.0 × 10⁵</td>
<td>6.1 × 10⁵</td>
</tr>
<tr>
<td>Drying</td>
<td>25</td>
<td>3.0 × 10⁴</td>
<td>4.0 × 10⁵</td>
</tr>
</tbody>
</table>

a,b,c,d: See Table 2 for description of footnotes.

Figures 2 and 3 show scatter plots relating HGMF-MPNs with corresponding 5-tube MPNs for enumerating stressed E. coli in foods.

Discussion

Bacterial injury is usually expressed as the difference between counts obtained under selective and non-selective conditions (4,11). While this observation can be easily measured using pure cultures, this approach cannot be used to evaluate the effects of stress on specific groups of organisms within mixed populations. Most of our samples contained a mixed flora. Thus, we determined the effect of stress on indicator organisms by measuring the difference in counts obtained under direct selective conditions and those obtained by employing a resuscitation technique before selective incubation.

Our preliminary studies, which established optimum resuscitation parameters for each group of organisms and type of stress, produced some interesting observations. The results obtained at 25°C and 35°C for dry-stressed organisms (Fig. 1) suggested that injury resulting from this type of stress may be different from that produced by the other three stresses, thus requiring a different repair mechanism (17). Resuscitation beyond the optimum incubation period resulted in a reduced count of indicator organisms in many cases. This effect was most noticeable in the case of freeze- and dry-stressed E. coli, although it was observed with the other organisms (Fig. 1). This phenomenon was most likely due to the growth of other bacteria during the prolonged non-selective incubation period, resulting in a masking of the specific biochemical reaction on selective medium (11).

Our results confirmed that a period of resuscitation on a non-selective medium was required for maximum recovery of stressed coliforms from foods by the HGMF technique. The numbers of coliforms recovered by the resuscitative HGMF technique were not always statistically different from the direct HGMF method, although the resuscitative method produced numerically higher results than the direct method for all four types of stress. The reason for the superiority of the resuscitative HGMF technique over the 5-tube MPN for enumeration of heat-stressed coliforms (Table 2) is unclear. Possibly, the sodium lauryl sulfate in the presumptive broth was somewhat inhibitory to these organisms (17).

The effects of resuscitation on the recovery of E. coli were far more dramatic. Under all four stress conditions, the 5-tube MPNs were significantly higher than the direct HGMF-MPNs. The resuscitative HGMF technique not only yielded significantly greater recovery of stressed E. coli than the direct HGMF method, but resuscitation also produced E. coli HGMF-MPNs that were statistically equi-
TABLE 4. Comparison of direct and resuscitative HGMF technique and a spread plate method for enumeration of stressed enterococci in foods.

<table>
<thead>
<tr>
<th>Type of stress</th>
<th>Number of samples</th>
<th>Direct HGMF (MPN/g)</th>
<th>Resuscitative HGMF (MPN/g)</th>
<th>Spread plate (CFU/g)</th>
<th>HGMF to spread plate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>30</td>
<td>$4.8 \times 10^3$</td>
<td>$5.2 \times 10^3$</td>
<td>$4.9 \times 10^5$</td>
<td>0.98</td>
</tr>
<tr>
<td>Drying</td>
<td>27</td>
<td>$1.5 \times 10^6$</td>
<td>$1.4 \times 10^5$</td>
<td>$1.3 \times 10^5$</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*aResuscitation done as described in Table 1.

*bStatistically significant increase over direct HGMF count (ANOVA; α = 0.05).

*cSignificant difference between the methods (t-test for paired data; α = 0.05).

valent to the 5-tube MPN results.

Our observations on the requirements for resuscitation of injured E. coli correspond quite well to those reported by Holbrook et al. (12) and Mackey (13) with the membrane spread technique. Both studies recommended a repair period of 4 h at 37°C for recovering injured cells from frozen products. In addition, Holbrook et al. (12) recommended an identical resuscitation procedure for recovery of E. coli exposed to conditions of heat, acid pH or drying. Holbrook et al. (12) based their temperature recommendation on a comparison of the effectiveness of 22, 30 and 37°C for the repair of freeze-injured E. coli. Our own results show that, while their recommendations were appropriate for the recovery of heat- and acid-stressed E. coli, the use of 37°C is not optimal for recovery of dry-stressed organisms.

In our preliminary study, we were unable to induce significant sublethal injury in the enterococci by exposure to acid pH or heating. Therefore, our comparative studies were limited to freeze- and dry-stressed enterococci. While resuscitation resulted in a significant increase in the recovery of freeze-stressed enterococci by the HGMF technique, it had no beneficial effect on the recovery of enterococci that had been exposed to drying. This study shows that an HGMF technique can provide equivalent results to conventional 5-tube MPN and spread plate methods for enumerating injured indicator organisms in foods, provided that an appropriate resuscitation procedure is employed.

Our studies show that a resuscitation period of approximately 4 to 4.5 h on TSA/Mg at 35°C is optimal for the repair of acid-, heat- and freeze-stressed coliforms and E. coli; 2 h at 25°C was optimum for dry-stressed coliforms and E. coli. While use of a resuscitative HGMF procedure produced a higher recovery of freeze-stressed enterococci than the direct HGMF method, neither method differed significantly from the spread plate method. Since resuscitation did not improve the recovery of enterococci that had been exposed to drying, acid pH or heating by the methods used in this study, routine use of resuscitation may be unnecessary for the selective enumeration of enterococci from foods.

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REFERENCES


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mutants. Thus, phage may not be extending the lag phase of bacterial growth per se but rather lyse the parental strain allowing the expression of resistant mutants. These mutants would probably retain the spoilage potential of the parental strains.

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