Enumeration of Indicator Organisms in Foods Using the Automated Hydrophobic Grid-Membrane Filter Technique

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(Received for publication June 11, 1981)

ABSTRACT

The automated HGMF technique was compared against accepted traditional methodology for the recovery and enumeration of coliforms, Escherichia coli, enterococci and Staphylococcus aureus from a variety of naturally and artificially contaminated foods. The overall ratios of recovery of the HGMF relative to conventional methods were 0.88 for coliforms, 0.80 for E. coli, 0.81 for enterococci and 0.80 for S. aureus. Our results suggest that the automated HGMF system is a viable alternative to conventional most-probable-number and spread plate techniques for the isolation and enumeration of foodborne microorganisms on selective media; however, consideration must be given to modifying procedures for the optimal recovery of stressed cells by this automated membrane filtration technique.

For many years, membrane filtration (MF) techniques have been successfully applied to the selective enumeration of indicator organisms from water (2), but have found little application in food microbiology.

Sharpe and co-workers (19,26-29) and Entis et al. (6) recently demonstrated the practicality of applying membrane filtration techniques to the microbiological analysis of foods. Sharpe et al. (26,27,29) have shown that it is possible to obtain counts of Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Streptococcus faecalis, Streptococcus faecium, and Salmonella typhimurium with Hydrophobic Grid-Membrane Filters (HGMF) that are equivalent to the numbers obtained by using conventional plating or MF techniques when using freshly prepared inocula. Entis et al. (6) and Peterkin and Sharpe (19,28) established that almost any food can be rendered filterable without affecting the microbial integrity of the product by applying the appropriate treatment. Furthermore, the prefiltration unit described by Entis et al. (6) effectively clarified food suspensions, preventing the deposit of particulate matter on the surface of the membrane filter which could interfere with the enumeration of bacterial colonies, particularly with the ISO-GRID Sample Processor (3). In a recent publication (3), we demonstrated that an automated HGMF system produced aerobic plate counts and yeast and mold counts in food which were equivalent to or greater than counts obtained by using traditional analytical methods. This paper reports the results of a study undertaken to further evaluate this system for enumerating specific indicator organisms from a variety of foods.

MATERIALS AND METHODS

Sample preparation

Food suspensions were prepared by stomaching (Colworth Stomacher, Model 400, A. J. Seward, England) 10 g of food with 90 ml of0.1% peptone - 1.0% Tween 80 diluent. Appropriate 10-fold dilutions were made in the peptone-Tween diluent.

To obtain a reasonable number of valid comparisons, 10 g of each food, with the exception of the raw ground meat for coliform and E. coli analyses, were pre-weighed in Stomacher bags and artificially contaminated. The inocula used for spiking (Table 1) were prepared by washing overnight Tryptic Soy agar (TSA) slant cultures with peptone-Tween. Each spiked food was inoculated with 0.1 ml of an appropriately diluted mixed suspension. All spiked samples, except for the salads and sandwich spreads, were frozen for at least 3 days before analysis.

Filtration units, HGMFs and automated counting system

The filtration units, HGMFs and the automated counting system used in this study were developed by QA Laboratories Limited as previously described (6).

Media and bacterial enumeration

HGMF. Coliform counts in raw ground meats were obtained using m-FC agar (Difco) with rosolic acid added. All other coliform determinations were done using m-FC agar without rosolic acid. After incubation at 35 C for 24 ± 2 h, all blue colonies were counted as coliforms.
E. coli counts were made on Tryptone Bile agar (TBA) (16,24) after incubation at 44.5°C for 24 ± 2 h. Development of the indole reaction was achieved by overlaying the HGMF from TBA onto a piece of paper towelling saturated with 0.5% p-dimethylaminobenzaldehyde in 1 N sulfuric acid (A.N. Sharpe, personal communication). The developing pink to red indole reaction was "fixed" by exposing the filters to a low pressure, long wave UV lamp (Phillips TL 200 W O5) for 30 min (24).

Since all spiked samples were either frozen or acid-stressed, a resuscitation procedure was used for recovering coliforms and E. coli from these samples. After the foods had been filtered, the HGMFs were placed on TSA supplemented with 0.15% magnesium sulfate (TSA/Mg) and incubated for 2 h at 35°C. Following this resuscitation period, the filters were transferred to the appropriate selective medium and temperature for the remainder of the incubation time.

Enterococcus counts were obtained using m-Enterococcus agar (Difco) with an incubation period of 48 ± 4 h at 35°C. All pink to red colonies were counted and up to 10 colonies per filter were tested for catalase activity. All pink to red, catalase-negative colonies were considered presumptive enterococci (2).

S. aureus was enumerated on Baird-Parker medium supplemented with egg-yolk tellurite enrichment (Difco) (BPEY). After 48 ± 4 h incubation on m-Enterococcus agar at 35°C by using duplicate spread plates. All pink to red, catalase-negative colonies were counted as presumptive enterococci.

S. aureus counts were obtained by using duplicate BPEY spread plates and confirmed by the tube coagulase test according to Health Protection Branch Method MFA-21 (14).

Statistical evaluation All counts were converted to log10 values and a two-tailed t-test for paired data was performed (11).

RESULTS

The results are summarized in Table 2. As illustrated, 185 food samples representing five different food types were compared for total coliform recovery by the

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TABLE 1. Cultures used to prepare spiked samples.

<table>
<thead>
<tr>
<th>Spike</th>
<th>Organisms per spike</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coliforms</td>
<td>Escherichia coli</td>
<td>Health Protection Branch</td>
</tr>
<tr>
<td></td>
<td>Klebsiella pneumoniae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Citrobacter freundii</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterobacter aerogenes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterobacter agglomerans</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>E. coli - 5 strains</td>
<td>Raw sausages</td>
</tr>
<tr>
<td>Enterococci</td>
<td>Streptococcus faecalis</td>
<td>Health Protection Branch</td>
</tr>
<tr>
<td></td>
<td>Streptococcus faecium</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>S. aureus - 5 strains</td>
<td>Health Protection Branch</td>
</tr>
</tbody>
</table>

TABLE 2. Comparison of Iso-Grid™ HGMF and conventional methods for enumerating indicator organisms in food.

<table>
<thead>
<tr>
<th>Food type</th>
<th>ISO-GRID HGMF: Conventional method</th>
<th>Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coliforms b</td>
<td>E. coli b</td>
</tr>
<tr>
<td></td>
<td>n d</td>
<td>Ratio n</td>
</tr>
<tr>
<td>Ground meat</td>
<td>75</td>
<td>0.86</td>
</tr>
<tr>
<td>Raw sausages</td>
<td>ND d</td>
<td>56</td>
</tr>
<tr>
<td>Luncheon meat</td>
<td>28</td>
<td>0.74</td>
</tr>
<tr>
<td>Dried food</td>
<td>24</td>
<td>0.88</td>
</tr>
<tr>
<td>Cheese</td>
<td>30</td>
<td>1.10</td>
</tr>
<tr>
<td>Frozen foods</td>
<td>28</td>
<td>0.88</td>
</tr>
<tr>
<td>Salads</td>
<td>ND</td>
<td>25</td>
</tr>
<tr>
<td>Custards</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Overall</td>
<td>185</td>
<td>0.88</td>
</tr>
</tbody>
</table>

aIso-Grid™ Hydrophobic Grid-Membrane Filter, Trademark of QA Laboratories Limited.
bConventional analysis by 5-tube MPN.
cConventional analysis by duplicate spread plates.
dNumber of comparative samples.
eCombined data for ground meats, sausages and luncheon meats.
fSignificantly different, t-test for paired data (α = .05).
gNot done.
ISO-GRID™ HGMF method and a 5-tube MPN technique. The overall ratio of recovery by the HGMF method was 0.88. A t-test for paired data of the individual food types indicated that total coliform recovery by the automated HGMF method was not significantly different from the 5-tube MPN technique for any of the food types examined.

A total of 138 food samples representing four different food types were compared for E. coli recovery by the automated HGMF method and a 5-tube MPN technique. The overall ratio of recovery by the HGMF method was 0.80. A t-test for paired data of the individual food types showed that the HGMF method was not significantly different from the 5-tube MPN for E. coli recovery from ground meats, raw sausages, luncheon meats and cheeses. Recovery of E. coli from salads by the MPN technique was, however, significantly higher than by the HGMF method.

Forty-six frozen foods were examined for presumptive enterococci by the HGMF method and a spread plate technique. The HGMF ratio of recovery was 0.81. The t-test for paired data verified that recovery of presumptive enterococci by the two methods was not significantly different.

Four different food categories encompassing 114 individual samples were examined for recovery of S. aureus by the HGMF technique and a conventional spread plate method. The overall ratio of recovery by the HGMF was 0.80. A t-test for paired data of the individual food categories demonstrated that the HGMF method for recovery of S. aureus from meat (ground meat, raw sausages and luncheon meats) and salads was not significantly different from the conventional spread plate technique. Recovery of S. aureus from cheeses and custards was, however, statistically higher by the spread plate method.

**DISCUSSION**

Guthertz and Fruin (12) found that commercial automatic colony counters (ACC) could not accurately count bacterial colonies on selective media due to a lack of contrast between the colonies and the growth medium as well as the opacity of the media used. We have shown that the ISO-GRID™ automated scanning system effectively overcomes the difficulties posed to ACC's and is able to provide reliable counts of specific microorganisms on highly selective or differential media, even in the presence of high levels of background flora.

With the exception of our use of m-FC agar for total coliform enumeration, the media formulations used for the HGMF method were the same as those used for conventional plating techniques. M-Endo-LES agar has been the medium of choice for enumerating coliforms from water by the MF technique (2). In our automated HGMF system, however, we were unable to obtain accurate camera scans with m-Endo-LES agar because of the reflectivity of the gold-green metallic sheen from typical coliform colonies.

M-FC medium was originally designed for the enumeration of fecal coliforms from water (9). This medium contained no substances which were inhibitory to coliform bacteria but rather relied on an elevated incubation temperature for its selectivity (9,20). Rosolic acid was added to further inhibit the growth of non-fecal coliforms. Presswood and Strong (20) demonstrated that recovery of fecal coliforms was better on m-FC without rosoacid. Since m-FC medium was not inherently inhibitory to coliforms, and because lactose fermenting, gram-negative organisms produced distinct blue colonies on m-FC which were easily distinguished by the HGMF scanning system, we applied m-FC agar both with and without rosoacid to total coliform enumeration by the HGMF method. The results of our parallel study indicated that m-FC agar was a viable medium for obtaining coliform counts from foods with the automated HGMF system. Similarly, the Anderson-Baird-Parker technique for E. coli enumeration using TBA, described originally as a membrane-spread technique (16,24), was successfully adapted to the HGMF procedure. The combined use of m-FC agar for total coliform determinations and TBA for E. coli counts reduces the time for these evaluations to 1 day as compared with 2 to 6 days by the conventional MPN method.

The recovery and enumeration of injured or stressed microorganisms on selective media has been extensively reviewed (13,15,22,31). Liquid enrichment procedures, such as those in the coliform MPN method, allow for resuscitation of injured or stressed cells before their exposure to more selective conditions (22). Unfortunately these MPN methods are time-consuming, tedious and imprecise (13). Conventional spread or pour plate techniques using selective media, while providing a more precise count than the MPN, do not possess that inherent ability to resuscitate injured cells and therefore require the inclusion of an enrichment repair technique before selective enumeration. Liquid repair methods have been shown to result in increased bacterial counts when subsequently plated on solid selective media; however, such increases might also have been due to the multiplication of uninjured cells. Hence, the time/temperature parameters for liquid enrichments must be carefully controlled and specified for the method of detection subsequently used (13,22). Solid repair methods involving overlays of selective media with non-selective enrichments or vice-versa have become more popular because they produce less variable results. The ratios of selective to non-selective agar volumes must, however, be carefully controlled and the time and temperature for resuscitation must be specified for each group of organisms being enumerated (13).

Many microbiologists have observed and accept 80-90% recovery of microorganisms when using membrane filtration methods as compared with pour or spread plate techniques or MPN methods, especially if
the organisms had been exposed to stress. MF manufacturers responded by producing filters with increased surface pore size, theorizing that improved bacterial recovery would occur if the cells were more deeply imbedded in the filter and hence "bathed" in nutrient (30). Some improvement in bacterial recovery has been reported using these modified filters (10,17) although their use by single step or direct enumeration methods for stressed cells still have not produced results equivalent to MPN methods (5,25). We also tried to improve the recovery of stressed cells using HGMFs with a surface pore size of 0.65μm in a single step approach without success (unpublished data).

Two step procedures involving resuscitation on a non-selective medium followed by selective enumeration dramatically improve bacterial counts from stressed systems (22). The MF provides a most convenient approach for performing two-step enrichments. Once the sample suspension has been filtered, the membrane can be placed on a non-selective repair medium. Since the cells are localized on the filter, some cell multiplication can occur before the formation of visible colonies without the loss of quantitation (22). After the resuscitation period the filter can be transferred to any appropriate selective medium. Almost any non-selective medium can be used for resuscitation (22), however, the incubation conditions for optimal recovery of injured cells remains unsettled (5,13).

In our study, a 2-h resuscitation on TSA/Mg at 35°C worked reasonably well for most of the freeze-injured coliform and E. coli studies but was less effective for recovering acid-stressed organisms. The comparative HGMF-MPN data showed excessive scatter when graphically displayed (not shown) suggesting that our resuscitation procedure may have been sub-optimal when applied to acid-injured cells and/or combined with media and incubation conditions of high specificity, e.g., TBA at 44.5°C (4,21). The excessive scatter may also reflect differences in the bacterial populations being recovered by the HGMF and the MPN (8), particularly with the ground meat.

Recovery and enumeration of presumptive enterococci by the HGMF compared favorably with the spread plate method both in terms of overall recovery and in the linear relationship described by the two sets of data. A scatter plot of the HGMF-MPN data (Fig. 1) showed a typical distribution pattern about the theoretical line of equivalence but without the excessive scatter demonstrated by the coliform and E. coli results, suggesting a more uniform recovery of similar populations.

Media formulations which are effective for conventional spread or pour plate techniques may not always be as successfully applied to MF methods (32). Our experience with S. aureus recovery by HGMF illustrates this problem. The HGMF method recovered S. aureus from meat (ground meat, raw sausages, luncheon meats) and salads as effectively as the conventional spread plate, but was less efficient for S. aureus recovery from cheeses and custards when a freezing stress was applied.

The recovery of S. aureus by MF techniques has received very little attention (1,7). Standard Methods for the Examination of Water and Wastewater tentatively recommends the use of m-Staphylococcus agar for S. aureus enumeration by MF (2). This medium has been found to be neither sufficiently differential nor selective for the quantitative recovery of S. aureus (1,7). Food microbiologists have extensively evaluated selective media for recovery and enumeration of S. aureus. Invariably, Baird-Parker Egg Yolk (BPEY) medium has been found to provide the best recovery of S. aureus and is now widely accepted for use in a spread plate technique (23). Unfortunately, the recovery of S. aureus from cheeses and custards which have been exposed to a freezing stress, using the HGMF technique with BPEY was less than satisfactory, although the HGMF technique has been shown to recover microorganisms as effectively as conventional methods when using unstressed inocula (26,27,29).

In view of our observations, we have begun to focus our attention on modifications to media formulations and procedures to optimize recovery of stressed cells with the automated HGMF system. Our preliminary results support the contention that foodborne microorganisms are subject to a variety of unknown stresses (18,33) which may be exacerbated by the use of a single step MF procedure for their enumeration (5). Thus it would seem beneficial to employ a method for resuscitation whenever an MF technique for microbial enumeration (5).

Conventional membrane filtration methods are hindered in their application to food microbiology. At low dilutions of food the filters may become clogged with food particles and, hence, the limit of detection may not be low enough for practical use. The ISO-GRID pre-filtration system and food treatments used for the HGMF method have overcome this serious drawback to
application of the membrane filtration procedure for qualitative food microbiology (6,28). The automated HGMF procedure for quantitative microbial analysis in foods has previously been shown to provide reliable evaluations of microbial content in a wide variety of foods using relatively non-selective media (3). The study reported here demonstrates that the ISO-GRID™ HGMF system has the ability to detect and enumerate specific microorganisms on highly selective or differential media, even in the presence of high levels of background flora. This system can provide results in a much shorter period of time than can be achieved conventionally.

ACKNOWLEDGMENTS

The authors are grateful to Mr. P. Bolesczuk, Mrs. H. Shannon and Miss J. Muscat for their technical assistance. The authors also wish to thank Mr. A. Porrett and Mr. M. P. Entis for developing the computer software.

This study was supported in part by Health and Welfare Canada under contract ISZ79-00130.

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