Evaluation of a Selective Enumeration Method Most Probable Number Enumeration Method for Viable Listeria spp. in Dairy Products

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ABSTRACT

A most probable number (MPN) method for enumerating low numbers of Listeria spp. in dairy foods was developed by adapting the U.S. Food and Drug Administration (FDA) Listeria isolation methodology. Milk, cheese, and other milk products were diluted and homogenized in enrichment broth (1 g/10 ml). Homogenates were inoculated with L. monocytogenes Lm82, a streptomycin-resistant variant of strain Scott A, at <1 to 320 CFU/g and further diluted in FDA enrichment broth to give 0.1, 0.01, and 0.001 g of food sample per 10 ml. Dilution aliquots (10 ml) in triplicate or quintuplicate were incubated at 30°C for 48 h before being subcultured on Oxford agar at 35°C. Esculin-hydrolyzing colonies on Oxford agar were confirmed as the inoculum strain by their ability to grow on Trypticase soy agar containing streptomycin. Differences between inoculum and MPN values were evaluated by using tabulated 95% confidence limits. The calculated MPNs agreed with the inoculum levels in 91% (58 of 64) of noncheese dairy foods and in 49% (56 of 112) of 15 varieties of ripened soft cheeses. Competitive microflora affected by cheese age and the kind of milk used may account for the suboptimal performance of the MPN method with the cheeses.

Key words: Listeria, foodborne, MPN enumeration, selective enrichment

The U.S. Food and Drug Administration (FDA) regulates pathogens that cause foodborne infections at zero tolerance in each replicated 25-g analytical portion and thus does not need an official enumeration method for regulating Listeria monocytogenes. However, because enumeration data are essential for defining the as yet undefined infectious dose for foodborne listeriosis, FDA has optional direct plate count and most probable number (MPN) enumeration methods for Listeria spp. (6) in the event that foods incriminated in outbreaks or sporadic cases of listeriosis come within its purview, and for generating knowledge about the concentration of Listeria microorganisms in foods. In such instances, the FDA suggests quantitation by direct plate count on selective medium such as Oxford agar (3), LPM agar (9) or LPM modified by adding esculin and ferric ions (6). An MPN selective enrichment procedure is also suggested. In the MPN method 10, 1, 0.1, and 0.01 g of food are incubated in quintuplicate in FDA selective enrichment broth for 48 h at 30°C. The MPN enrichments are then streaked on Oxford agar and incubated 24 to 48 h. MPN methods are useful for quantitating less than 100 CFU of Listeria cells per g, and inherently allow for repair of injured bacteria (2).

Results of an International Dairy Federation-AOAC International (IDF-AOAC) collaborative study (13) showed that the FDA selective enrichment method generally enabled identification of low populations of Listeria cells in milk and milk products and indicated that it could be adapted into an MPN method. The present study is an evaluation of the performance of this MPN method with milk and milk products, including mold-ripened and non-mold-ripened soft cheeses. Soft ripened cheeses are known to be difficult matrices from which to isolate and enumerate Listeria cells (8).

MATERIALS AND METHODS

Culture

The L. monocytogenes strain Lm82 used in this study is a streptomycin-resistant variant of strain Scott A and was obtained from D. Datta (7). For a preliminary 3-tube MPN study with collaborators, cultures of Lm82 were grown in tryptose broth at 30°C for 18 to 20 h. The cell concentration was determined by direct microscopic count using a hemocytometer or by quantitatively diluting the culture with saline to just visible turbidity, i.e., about 10^8 CFU/ml.

For the 5-tube MPN study, colonies on Trypticase soy agar (TSA) (25°C, 72 h) were harvested and diluted in phosphate-buffered saline (PBS) to give a suspension with turbidity corresponding to a McFarland no. 2 standard (6 × 10^8 CFU/ml).

Preparation of selective enrichments

Four portions of each food (25 g or ml) were dispensed into sterile containers of a high-speed blender (Waring Products Division, Dynamics Corp. of America, New Hartford, CT) or a peristaltic-type blender (Stomacher Laboratory Blender, The Tekmar Co., Cincinnati, OH). A 225-ml volume of selective enrichment broth (6) containing 10 mg of acriflavin per liter was added at room temperature to each food portion. The culture was diluted in physiological saline (0.85%, wt/vol) in the preliminary study and in PBS in the later studies, to give nominal concentrations of 10^2 and

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10^3 CFU/ml. Aliquots (1 ml) of each diluted suspension were added to each pair in the four portions of each food, and the mixtures were blended until thoroughly dispersed (1 to 3 min). The actual concentration of the inoculum was confirmed by a colony count after incubation of appropriate dilutions for 48 h at 35°C on standard plate count agar (Difco Laboratories, Detroit, MI). Food-sample microflora counts were determined in a similar fashion, starting with a 1 in 10 homogenate of the sample in PBS as the first decimal dilution.

**MPN enumeration**

In the preliminary study the 3-tube MPN method was used. In later studies, a 5-tube MPN method was used to narrow the confidence limit range and to attempt to reduce incorrect predictions of inoculum numbers due to random fluctuation. Immediately after inoculation and blending of each food portion, a 3- or 5-tube MPN assay in the selective enrichment broth was set up for each portion at each inoculum level (10^3 and 10^2 CFU/25 g or ml of food) using inoculated homogenates containing 1, 0.1, 0.01, and 0.001 g of food. This concentration was achieved by using the blend directly or by diluting it in fresh enrichment broth. By either method, all MPN tubes contained a final volume of 10 ml. The MPN tubes were incubated at 30°C for 48 h. After incubation, 10-ml test portions to be examined for isolated colonies were streaked onto Oxford agar (3) and incubated at 30°C for 2 days. Any typical Listeria colonies were then streaked on TSA containing 200 μg of streptomycin sulfate per ml and incubated to confirm the Oxford isolates as Lm82. Conventional confirmatory tests (6) were not needed since isolate identities were unambiguous.

**Food matrices**

Ice cream, milk, cheese, and milk powder were purchased at local retail stores and maintained, as appropriate, at -10°C, 5°C, and room temperature.

**Data analysis**

Standard 3- and 5-tube MPN tables (11) were used to convert the sequence of numbers of Listeria-positive tubes at each level of a dilution series to MPN per gram or milliliter. For uncommon sequences, the MPN tables of Oblinger and Koburger (10) were consulted. The first 3 of each set of 5-tube MPN enumerations were also used to provide 3-tube MPN data. This procedure can be regarded as an extreme case of the procedure for dealing with missing tubes in a 5-tube MPN (11). To control bias, the set of 3 tubes to be used from each 5-tube set was chosen in advance. An MPN result was categorized as a significantly discrepant prediction when the corresponding inoculum concentration was not within the 95% confidence limit of the MPN. Factors such as mold versus nonmold cheese ripening and raw versus pasteurized cheese milks were analyzed. Biases resulting from analysis of different cheese types were weighted by converting the proportions of successful MPN predictions of inoculum concentrations to percentages.

Knowledge of the pasteurization status of the cheese milks used was obtained from the cheese labels. Absence of a raw-milk or pasteurized-milk ingredient statement on the cheese label or lack of complete label information because of a missing or damaged label was assumed to mean that the cheese milk had not been pasteurized. For balanced analyses, such putative nonpasteurized milk cheeses were arbitrarily reclassified as being pasteurized if other examined portions of that cheese type had labels listing pasteurized milk as an ingredient or if that type of cheese was generally known to be made from pasteurized milk. Thus, to compare the results of the MPN method with pasteurized and raw-milk cheeses, performances were stated as a range of percentages of successful predictions.

**RESULTS**

**Preliminary study**

In a four-laboratory preliminary trial, duplicate portions of locally obtained soft cheeses and other dairy foods were inoculated with *L. monocytogenes* at two levels (4 and 40 CFU/g). The *Listeria* in the quantitatively inoculated cheeses were enumerated by the 3-tube MPN method. For noncheese dairy foods, 87% (28 of 32) of the recoveries of *L. monocytogenes* were not significantly different from the values expected from the input levels. In contrast, for recoveries of *L. monocytogenes* from soft cheeses, only 31% (10 of 32) were not significantly less than that expected from the input levels, taking into account the 95% confidence limits of the observed MPN.

The fact that differences of observed from expected values were not always large suggested that use of 5-tube MPN enumerations would improve the method's efficacy. However, this was not the case, and results from the 3-tube multilaboratory study were reported along with those of the 5-tube single laboratory study.

**Milk, milk powder, and ice cream**

The pooled 3- and 5-tube MPN predictions of *L. monocytogenes* inoculum size were correct in 91% of instances (Table 1).

**Cheeses**

Pooled 3- and 5-tube MPN predictions of inoculum size were correct in about 50% of instances (Table 2). Comparison of 3-tube data (not shown) derived from 5-tube data from the same food portions, as described in Methods, did not affect this conclusion. Thus, reducing the greater random fluctuation due to the 3-tube method by using the 5-tube method did not improve prediction. The lower prediction success rate with cheeses suggests that something in cheeses interferes with the MPN determination.

Total microflora counts did not correlate with MPN enumerations. The enumeration discrepancy appeared to be affected during postpurchase aging of the cheeses. Table 3 shows a significant (*P* = 0.05) negative effect of short-term aging on MPN

**TABLE 1. Proportion of correctly predicted Listeria monocytogenes inoculum concentrations by the MPN method: milk and noncheese milk products**

<table>
<thead>
<tr>
<th>MPN predictions (correct/total)</th>
<th>Range of MPN/μg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Matrix</strong></td>
<td></td>
</tr>
<tr>
<td>≈1–&lt;12</td>
<td>12–320</td>
</tr>
<tr>
<td>Ice cream</td>
<td>13/16</td>
</tr>
<tr>
<td>Milk powder</td>
<td>8/10</td>
</tr>
<tr>
<td>Milk (pasteurized)</td>
<td>10/10</td>
</tr>
<tr>
<td>Total</td>
<td>31/36</td>
</tr>
</tbody>
</table>

* Number of batches per matrix: mean 4, range 3 to 5. Number of food portions per matrix: mean 10.6, range 8 to 14.
TABLE 2. Proportion of correctly predicted Listeria monocytogenes inoculum concentrations by the MPN method: cheeses

MPN predictions (correct/total) | Range of MPN<sup>a</sup> | 1-<12 | 12-320 |
--- | --- | --- | --- |
Matrix | | | |
Brie | 4/6 | 10/14 |
Camembert | 2/6 | 1/6 |
Cashel blue | ND | 2/4 |
Danblu | 2/2 | 8/10 |
Explorer | ND | 1/4 |
Gorgonzola | 3/6 | 3/6 |
Limburger | 4/6 | 1/2 |
Livarot | ND | 1/4 |
Osterzola | 2/2 | 2/2 |
Pavin | ND | 1/4 |
Pont L’Evêque | 2/2 | 2/2 |
Reblochon | ND | 0/4 |
Romadur | 0/2 | 0/2 |
Roquefort | ND | 1/4 |
Stilton | 2/6 | 2/6 |
Total | 21/38 | 35/74 |

<sup>a</sup> Number of samples per matrix: mean 4, range 2 to 10.

aging (7 days at 5°C) on the performance of the 5-tube MPN enumeration. This effect was not correlated with changes in microfloral concentration, which tended to rise during the 7-day refrigerated storage. However, prolonged storage, 54 days at 5°C, of Roquefort and Petit Pont L’Evêque cheeses did not affect the initially acceptable performance efficacy of the 5-tube MPN method with these two cheeses (data not presented).

Categorizing the results according to mold-ripened (n = 8) and non-mold-ripened (n = 7) cheese classes showed

TABLE 3. Effect of cheese storage time on the proportion of correctly predicted Listeria monocytogenes inoculum concentrations by the MPN method and on microflora levels

<table>
<thead>
<tr>
<th>Postpurchase storage time</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix</td>
<td>MPN prediction&lt;sup&gt;b&lt;/sup&gt; (correct/total)</td>
<td>Microflora (CFU/g)</td>
</tr>
<tr>
<td>Brie</td>
<td>2/2</td>
<td>7 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Camembert</td>
<td>1/2</td>
<td>3 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cashel blue</td>
<td>2/2</td>
<td>2 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Danblu</td>
<td>2/2</td>
<td>1 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Explorer</td>
<td>1/2</td>
<td>5 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gorgonzola</td>
<td>1/2</td>
<td>2 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Livarot</td>
<td>1/2</td>
<td>4 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pavin</td>
<td>0/2</td>
<td>3 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reblochon</td>
<td>0/2</td>
<td>4 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>Roquefort</td>
<td>2/2</td>
<td>4 x 10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stilton</td>
<td>2/2</td>
<td>4 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>13/22</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>b</sup> Inoculum counts 61 and 72 CFU/g at 7 and 14 days, respectively.

TABLE 4. Effect of milk pasteurization and mold ripening on the Listeria monocytogenes MPN enumeration prediction success rate

<table>
<thead>
<tr>
<th>Milk</th>
<th>Mold ripened (n = 8)</th>
<th>Not mold ripened (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurized milk</td>
<td>52–63</td>
<td>63–100</td>
</tr>
<tr>
<td>Raw milk</td>
<td>25–46</td>
<td>13–25</td>
</tr>
<tr>
<td>All milks</td>
<td>54</td>
<td>31</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Methods for determination of ranges.

that MPN predictions were correct in 54 and 31% of instances, respectively (Table 4). However, according to the manufacturers’ labels, a larger proportion of mold-ripened cheeses had been made with pasteurized milk than had non-mold-ripened cheeses. Allowing for this as described in Methods, the ranges in Table 4 suggest that MPN estimation discrepancies will be greatest with non-mold-ripened cheeses made from raw milk, and least with non-mold-ripened cheeses made from pasteurized milk. The effect of milk pasteurization was somewhat less with mold ripening, the separation of the ranges being less marked than with nonmold ripening.

DISCUSSION

Using artificial inoculation of food samples with pathogens as a way to study the efficacy of enumeration methods for naturally occurring foodborne pathogens involves making an important assumption, namely, that potential physical entrapment of naturally occurring pathogens can be overcome either by effective comminution of the food sample during homogenization and/or that the pathogen has the ability to grow out of sequestration. This study, like similar studies of others, has had to make this assumption since it is probably almost impossible to disprove.

The MPN selective enrichment method used in this study seems to be promising for enumerating low concentrations (<1 to 100 CFU/g) of Listeria cells in pasteurized milk and in noncheese dairy products made from pasteurized milk. Use of differential selective plating media such as those proposed by Heisick et al. (5) in place of Oxford agar could make the MPN method specific for L. monocytogenes and/or hemolytic Listeria spp. enumeration.

MPN selective enrichment methodology also seems promising for hard cheese varieties (4). More research is needed to determine the exact reason(s) for the mediocre performance of the MPN enrichment method with mold-ripened and smear-ripened soft cheeses. Recovery from soft cheeses is known to be difficult because of microfloral competition by selective agent-resistant strains, especially those of the enterococci. Possible contributing factors to microfloral interference are the mode of cheese ripening, postretail cheese aging, acidity effects of the cheese and the selective enrichment culture, and pasteurization or nonpasteurization of the cheese milk. Data on the apparent age effect in this study were equivocal. Acidity effects can be counteracted by using a more strongly buffered selective enrichment broth.
The goal of this study was to evaluate the performance of the MPN method by screening a variety of dairy products, except fermented milks and hard cheeses. Although retail factors, particularly those involving soft cheeses, adversely affected the experimental design with regard to data analysis, some indications were obtained about the effect of ripening modes and the use of raw milk in cheese production; many surface-ripened cheeses are traditionally made with raw milk in France (12). In theory, at least for microbiologically simple food systems, the effect of a competitor on the performance of the MPN method can be corrected mathematically by using a generalized linear model (1). However, a correction would depend on knowing the concentration of the competitor or competitors, since food microflora may be a mixture of competitors.

In the interim, for enumerating Listeria in cheeses, it may be necessary to include a control inoculated with L. innocua so that the observed Listeria population in a naturally contaminated soft cheese can be corrected. Alternatively, the upper confidence limit of the observed MPN value could possibly be used for correction until the MPN is made more reliable for soft cheeses, especially those that are mold ripened or made from raw milk.

ACKNOWLEDGMENTS

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REFERENCES