Comparison of Seven Plating Media for Enumeration of *Listeria* spp.

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The suitability of seven media for the enumeration of *Listeria* spp. was evaluated at 30°C for 48 h. The media tested were (i) the original McBride *Listeria* agar formulation with glycine; (ii) modified McBride agar containing glycine anhydride; (iii) LiCl-phenylethanol-moxalactam (LPM) agar; (iv) acriflavine-ceftazidime agar; (v) Rodriguez isolation agar (RISA); (vi) modified Vogel-Johnson (MVJ) agar; (vii) cyclohexanedione-nalidixic acid-phenylethanol agar; and tryptoce agar as control. A total of 66 organisms were used including 11 *Listeria monocytogenes* strains and 5 other *Listeria* spp. For *L. monocytogenes* strains only, all media performed highly similarly. Of the other *Listeria* spp., only two grew on MVJ agar and three each grew on LPM and RISA. Only LPM agar inhibited the 50 non-listeriae, including five yeasts, while MVJ agar inhibited all but one yeast. The McBride *Listeria* agar formulation that contained glycine anhydride was less selective than the original. When pure cultures of 10 bacteria (including one *L. monocytogenes* strain) were combined and plated on four media, *L. monocytogenes* colonies were easiest to enumerate on MVJ agar, followed by LPM and RISA. These media ranked in the same order when plated with homogenates of various foods to which was added *L. monocytogenes* Scott A, but LPM agar was the best overall since Scott A was inhibited by MVJ. Upon microscopic examination of listerial colonies from the plating media, atypical cell morphology was noted with cells being about twofold in size on LPM, MVJ, and acriflavine-ceftazidime agars. Overall, LPM agar was the most suitable of the media tested even though it was inhibitory to *Listeria grayi* and *Listeria murrayi*.

There is currently a great deal of interest in culture media and methods for the selective recovery of *Listeria* spp. from food specimens, which stems from relatively recent human outbreaks of listeriosis (9, 14, 26). These outbreaks emphasized the need for more effective detection and recovery methods for *Listeria monocytogenes*, especially from dairy products. While *L. monocytogenes* is the only species in the present time that has been associated with human listeriosis, *Listeria ivanovii*, *Listeria innocua*, and *Listeria seeligeri* have been recorded as suspect pathogens (5, 24). Because the listeriae are generally outnumbered in food products by other organisms, their selective recovery has been the subject of a large number of recent studies (1, 3, 7, 8, 16, 18, 19, 22, 25, 28). Only four media selective for listeriae had been described prior to 1983 (4, 20, 21, 23), but about a dozen formulations have been published in the past 4 to 5 years. Several groups of investigators have compared two or more of these media for specific types of food, using limited numbers of listerial strains (2, 10, 11, 13, 17, 27). No reports exist in which large numbers of organisms were employed to assess the performance of a large number of the proposed media.

The aim of the present study was to evaluate the performance of seven selective plating media for enumerating not only *L. monocytogenes* but other *Listeria* species in pure and mixed culture, and to assess the ease of recognizing *L. monocytogenes* against the normal microbial flora of selected foods.

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MATERIALS AND METHODS

**Culture media.** The seven selective plating media evaluated, and their abbreviations, are listed in Table 1. Both McBride formulations were used without blood. The MVJ agar consisted of Vogel-Johnson agar base (Difco Laboratories, Detroit, Mich.), with 20 ml of a 1% solution of potassium tellurite per liter, 40 mg of nalidixic acid per liter, and 20 mg of moxalactam per liter as selective agents. CNP agar contains the following ingredients per liter: tryptoce, 20 g; NaCl, 10 g; dextrose, 5 g; yeast extract, 2 g; Tris, 7 g; nalidixic acid, 45 mg; 1.2-cyclohexanedione, 3 g; phenylethanol, 1 g; and agar, 15 g. The control plating medium employed was tryptoce agar (TA; Difco). All media were prepared according to the author or manufacturer’s directions, dispensed into petri dishes (10 by 100 mm) at 15 ml per plate, and allowed to stand overnight before surface plating.

**Cultures and sample preparations.** A selected set of 30 bacteria was used in the quantitative study (Table 2). An additional 36 organisms were employed for the qualitative evaluations, and they were divided into the following groups: (i) six *Listeria monocytogenes* (ATCC 19111, 19112, 19114, 19115, 19116, and 19117); (ii) five lactic acid bacteria—*Lactobacillus delbrueckii* ATCC 9649, two *Enterococcus faecalis* isolates (ATCC 7080, WSU 40), *Streptococcus cremoris* ATCC 19257, and *Enterococcus faecium* ATCC 14432; (iii) eight gram-positive bacteria other than listeriae or lactics—*Bacillus megaterium* WSU 73, *Bacillus subtilis* ATCC 9799, *Micrococcus luteus* WSU 204, two *Staphylococcus aureus* isolates (196E and WSU 103), *Brevibacterium linens* ATCC 471, *Arthrobacter pyridinolus* WSU 503, and *Corynebacterium striatum* WSU 280; (iv) 12 gram-negative bacteria—*Alteromonas putrefaciens* ATCC 8071, *Aeromonas hydrophila* ATCC 7965, *Escherichia coli* ATCC 11775, *Citrobacter freundii* WSU 600, *Serratia liquefaciens* WSU 126, *Providencia stuartii* WSU 592, Pseudo-
monas fragi ATCC 4973, Pseudomonas geniculata ATCC 14150, Pseudomonas aeruginosa ATCC 17806, Pseudomonas putida ATCC 12633, and two Pseudomonas spp. (meat isolates); and (v) five yeasts—Candida guilliermondii ATCC 6260, Candida lipolytica WSU 8313, Kluyveromyces fragilis ATCC 34439, Rhodotorula rubra ATLC 9449, and Torulopsis candida ATCC 12790.

All cultures were maintained on TA slants and transferred to tryptose broth with incubation for ca. 18 h at 30°C before plating. The media were inoculated as described below.

**Spiral plating.** The 30 pure cultures for the quantitative evaluation were surface plated in duplicate on the media noted above, employing a Spiral Plater (model D; Spiral Systems, Inc., Bethesda, Md.). Cultures were diluted to a concentration of approximately 10^4 to 10^5 CFU/ml in Butterfield phosphate buffer. After incubation at 30°C for 48 h, colonies were enumerated with the aid of a specially designed counting grid, and bacterial cell populations per milliliter were determined with a specially programmed calculator (Spiral Systems, Inc.).

**Qualitative platings.** The 36 organisms employed only for the qualitative evaluation were diluted to ca. 10^4 CFU/ml in Butterfield phosphate buffer (pH 7.2) and streaked, eight per plate, with the aid of sterile cotton-tipped applicators. After incubation at 30°C for 48 h, the plates were observed for growth or inhibition of each organism.

**Mixed-culture studies.** (i) Experiment 1. The first mixed-culture experiment was designed to assess the growth response and ease of recognition and recovery of several listeriae from a defined mixed culture in skim milk. The cultures consisted of one Listeria strain (L. monocytogenes Scott A, L. monocytogenes CDC, or L. innocua) six gram-positive bacteria (B. subtilis, Brevibacterium taipei, Brochothrix thermosphacta, Lactobacillus fermentum, S. aureus, and E. faecium), two gram-negative bacteria (E. coli and P. putida), and one yeast (C. lipolytica). Cultures were grown as noted above and diluted in 2% sterile skim milk to yield a mixed culture of ca. 10^5 cells per ml each. The media tested (TA, MBG, LPM, RISA, and MVJ) were inoculated by the Spiral Plater and incubated at 30°C for 48 h. Confirmatory tests for suspect listerial colonies included wet-mount examinations, Gram stain, catalase reaction, and blue colony appearance under oblique lighting.

(ii) Experiment 2. The second mixed-culture experiment was designed to evaluate the qualitative recovery of *L. monocytogenes* from the background flora of several foods by four selective media—MBG, LPM, RISA, and MVJ. The foods employed were milk (3.2% fat content), fresh yogurt, tzatziki (made from sour cream, yogurt, cucumbers, and spices), mixed vegetables (broccoli, alfalfa, and carrots), fresh ground beef, and fresh scallops. Each food sample was prepared for inoculation with and without *L. monocytogenes* Scott A to an approximate level of 10^2 to 10^3 cells per g. The liquid foods were diluted directly in Butterfield buffer, and the solid foods were blended in a Stomacher 400 (Dynatech Laboratories, Alexandria, Va.) for 2 min before dilution. Along with a pure culture of strain Scott A (as a control), the specimens were surface plated by use of sterile bent glass rods. After incubation at 30°C for 48 h, plates were evaluated to determine whether *L. monocytogenes* could be recognized and enumerated against the background flora.

## RESULTS

**Medium performance with pure cultures.** Recovery rates of 30 bacterial species or strains that were surface plated on the eight media are presented in Table 2. By analyzing these data and comparing geometric means and standard deviations of recovery rates for all *Listeria* spp. tested, we found that MBG, MBGA, ACA, and CNPA ranked closest to TA (the control medium), whereas LPM agar, RISA, and MVJ agar did not support quantitative recovery of all tested species. However, when considering only the *L. monocytogenes* strains tested, nearly all media performed equally well with respect to total numbers recovered. The exceptions were RISA, which displayed some inhibition of four of the five strains tested, and MVJ agar, which completely inhibited one strain (microcolonies were observed on the latter after 72 h). With regard to the other listeriae tested, the media varied widely in their capacities to support growth. *Listeria grayi* and *Listeria murayi* were best recovered on ACA but were completely inhibited on LPM, RISA, and MVJ. MVJ agar also inhibited *L. seeligeri*. With regard to MBG and MBGA, the latter supported better recovery of the *Listeria* spp. other than *L. monocytogenes*, while both media performed equally well in their qualitative recovery of this organism.

There were significant differences in the size of colonies formed on the various media. CNPA produced the largest colony diameters. MBG supported large colonies, whereas the very small colonies on MBGA were barely enumerable. When plates of the media (except RISA and MVJ agar) were viewed by oblique lighting, the characteristic blue appearance of listeriae was easiest to see for colonies formed on CNPA, followed by LPM agar and TA. Listeria colonies on ACA were yellowish-green in color. On MVJ agar, they appeared as shiny, small, black-to-the-edge colonies on a light red background (tellurite positive, mannitol negative). RISA produced small dark-green colonies (esculin hydrolysis) surrounded by a blackened zone.

Microscopic observations revealed that atypical cell morphology was enhanced on LPM, MVJ, and ACA, where the individual cells were about twofold in size. The media exhibited different capacities to inhibit bacteria other than *Listeria* spp. Recovery of the 12 non-listeria, gram-positive bacteria was best on MBGA, MBG, CNPA,

### TABLE 1. Summary of the seven selective plating media employed

<table>
<thead>
<tr>
<th>Medium</th>
<th>Abbreviation</th>
<th>Modification or selective ingredients</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acriflavine-ceftazidime agar</td>
<td>ACA</td>
<td>Acriflavine, ceftazidime</td>
<td>1</td>
</tr>
<tr>
<td>Cyclohexanedione-nalidixic acid-phenylethanol agar</td>
<td>CNPA</td>
<td>1.2-cyclohexanedione, nalidixic acid, phenylethanol</td>
<td>Jay*</td>
</tr>
<tr>
<td>Lithium-phenylethanol-moxalactam</td>
<td>LPM</td>
<td>Moxalactam, phenylethanol</td>
<td>16</td>
</tr>
<tr>
<td>McBride <em>Listeria</em> agar</td>
<td>MBG</td>
<td>Original formula</td>
<td>21</td>
</tr>
<tr>
<td>Modified McBride agar</td>
<td>MBGA</td>
<td>Glycine anhydride in lieu of glycine</td>
<td>21</td>
</tr>
<tr>
<td>Modified Vogel-Johnson</td>
<td>MVJ</td>
<td>Potassium tellurite, nalidixic acid, moxalactam</td>
<td>4</td>
</tr>
<tr>
<td>Rodriguez isolation agar</td>
<td>RISA</td>
<td>Nalidixic acid, acriflavine</td>
<td>25</td>
</tr>
</tbody>
</table>

TABLE 2. Counts of 30 bacteria on eight media after incubation at 30°C for 48 h

<table>
<thead>
<tr>
<th>Organism and source</th>
<th>Bacterial counts (log10 CFU/ml) on medium:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA</td>
</tr>
<tr>
<td>Listeria monocytogenes ATCC 35152</td>
<td>5.28</td>
</tr>
<tr>
<td>L. monocytogenes ATCC 15313</td>
<td>5.10</td>
</tr>
<tr>
<td>L. monocytogenes Scott A</td>
<td>5.16</td>
</tr>
<tr>
<td>L. monocytogenes V-7</td>
<td>5.15</td>
</tr>
<tr>
<td>L. monocytogenes CDC</td>
<td>5.18</td>
</tr>
<tr>
<td>L. grayi ATCC 19120</td>
<td>4.39</td>
</tr>
<tr>
<td>L. innocua ATCC 33090</td>
<td>5.09</td>
</tr>
<tr>
<td>L. ivanovii ATCC 19119</td>
<td>5.46</td>
</tr>
<tr>
<td>L. murrayi ATCC 25401</td>
<td>4.33</td>
</tr>
<tr>
<td>L. seeligeri ATCC 35967</td>
<td>4.60</td>
</tr>
<tr>
<td>Geometric mean</td>
<td>4.97</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.38</td>
</tr>
</tbody>
</table>

- Acinetobacter calcoaceticus ATCC 17910
- Enterobacter cloacae ATCC 23355
- Escherichia coli ATCC 25922
- Hafnia alvei WSU 83
- Pseudomonas aeruginosa ATCC 27853
- P. fluorescens ATCC 13325
- Salmonella typhimurium ATCC 14028
- Serratia liquefaciens WSU 123
- Geometric mean: 4.47 <1.30b
- Standard deviation: 0.93 <1.56

- Growth but colonies too small to enumerate (enumeration possible only after 96 h).
- No colonies on plates (<20/ml).

and ACA, while RISA recovered only one of these organisms and LPM and MVJ agar recovered none. Regarding inhibition of the eight gram-negative bacteria, the performance of MBGA was poorest, while LPM and MVJ agars inhibited all strains.

To further evaluate the seven media, an additional set of 36 organisms was tested, and the growth or inhibition data for these strains were combined with those in Table 2 to yield the results presented in Table 3. Overall, LPM agar performed the best by inhibiting all organisms (including five yeasts) other than the listeriae. MVJ agar allowed only one organism to grow: *C. lipofera*. No inhibition of the yeasts was effected on ACA; RISA inhibited *R. rubra*. Interestingly, one of the two *E. coli* strains grew on MBG, MBGA, and ACA, while the other one did not. None of the six strains of *Enterococcus* spp. tested grew on LPM and MVJ agars, whereas two strains grew on RISA and all strains could be recovered quantitatively from MBG, MBGA, ACA, and CNPA. Furthermore, *S. aureus* strains were controlled by LPM, MVJ, and RISA, while strain 196E grew on ACA and all three tested strains grew on MBGA, MBG, and CNPA.

**Mixed-culture studies.** In the mixed-culture studies described in experiment 1, MBG yielded heavy growth and four different organisms could be distinguished easily: *B. subtilis*, *E. coli*, *P. putida*, and *S. aureus*. It was somewhat difficult to distinguish between listerial and enterococcal colonies. Both were small in diameter (0.5 mm) and appeared white to bluish-white by oblique lighting. In addition, *B. subtilis* caused substantial problems by its spreading growth which masked other colonies on the plate. Two distinct and well-isolated colony types were found on LPM agar, easily distinguished as a *Listeria* and a pseudomonad. Both colony types were uniformly about 1 mm in diameter. The individual cells of the pseudomonad were atypical in shape by microscopic examination. RISA supported the growth of four different organisms, among which *Listeria* colonies could be enumerated due to their typical appear-
ance. The enterococci appeared as small colonies, whereas the pseudomonad formed a brownish, medium-sized colony type. Easy to differentiate was *C. lipolytica*, having white colonies with no background coloration of the medium. No appreciable colony formation was observed on MVJ agar after 48 h. However, after 72 h two colony types could be enumerated: *Listeria*, which formed small black colonies, and *C. lipolytica*, which formed small white colonies. One *L. monocytogenes* strain (Scott A) appeared to be inhibited on this medium, forming only a few isolated colonies.

Overall, from the mixed-culture evaluation of the media, the listeriae were easiest to recognize and enumerate on MVJ agar, followed by LPM and RISA. MBG did not prove satisfactory for this purpose.

**Recovery from inoculated foods.** In experiment 2, the four most promising plating media (MBG, LPM, RISA, and MVJ) were tested for their recovery of *L. monocytogenes* Scott A from the contaminating background flora of selected foods. Plating of the pure culture of Scott A indicated inhibitory properties of MVJ agar for this strain, since no colonies formed in 48 h at 30°C. Also, no growth was obtained on any medium including TA from the plated yogurt samples, with or without *Listeria* spp.

Upon the plating of milk samples containing ca. 10³ listeriae per ml on LPM and MBG agars, listeriae were present on MBG, but they were masked by the larger background flora. On the other hand, LPM agar facilitated recognition and recovery of listerial colonies while small colonies of lactic acid bacteria did not interfere with their recognition. With respect to the other media tested, no background growth from milk was observed on RISA and MVJ agar, while RISA allowed easy recognition of listeriae. The tzatziki flora formed pinpoint colonies on MBG, but not on other media. However, this did not interfere with the recovery of the much larger *Listeria* colonies on this medium. The natural flora of vegetables contained high numbers of different organisms since two or more different types were observed on each medium, except MVJ. Enumeration of listeriae was easiest on LPM agar, somewhat more difficult on RISA, and not possible on MBG, which did not suppress the contaminants sufficiently while enhancing growth of the listeriae.

Both fresh ground beef and fresh scallops yielded high colony counts on MBG, fewer on RISA, and only a few colonies on LPM agar. Recognition of suspect listerial colonies was possible only on LPM, while RISA and MBG were not sufficiently selective.

Overall, the best medium to suppress the natural flora of the tested food products was MVJ agar followed by LPM agar and RISA. However, LPM agar was most satisfactory for recovery and enumeration of listeriae, since MVJ agar proved to be inhibitory to the tested strain of *L. monocytogenes*.

**DISCUSSION**

In the quantitative assessment of the seven selective media employing 30 bacterial species or strains, the Spiral Plater proved to be a time-saving and reliable operating tool for the determination of cell numbers. Results were accurate and reproducible, and the variation between replicates was 5%. We found it particularly useful for repeated platings of the same inoculum on many plates, which assures reliable quantitative results. The Spiral Plater has been found to be comparable to, if not better than, the standard plate count by several investigators, among whom are Jarvis et al. (15), and it has received Association of Official Analytical Chemists approval as an official method.

ACA affected the highest recovery of listeriae, with numbers being close to those on TA. However, almost all gram-positive bacteria, some gram-negative strains, and the five yeasts all formed colonies on this medium. In addition to its lack of selectivity, this medium did not work with oblique lighting since the colonies were yellow to greenish in color. Oblique lighting was unreliable for other media, a fact that has been noted by McClain and Lee (22).

LPM and MVJ agars were the only two media that inhibited the 10 lactic acid bacteria in pure culture. The latter proved very useful because of its differential capacity. However, it did not facilitate quantitative recovery of all listeriae although the formulation we used differed from that of Buchanan et al. (2, 3). Their original formulation contains 20 mg of bacitracin per liter but it was found to be too inhibitory to listeriae. To take advantage of tellurite as a differential agent, we tried a formulation of LPM agar with added tellurite and also a formulation of Baird-Parker agar employing the same agents used in MVJ agar. However, all of the tested listerial strains were inhibited by both medium formulations. The considerable difference between LPM and LPM plus tellurite suggests that tellurite is the critical ingredient in the formulation. It is unclear as to why listeriae did not grow on modified Baird-Parker agar since they grew on MVJ agar. It might be due to the high concentration of glycine and LiCl in conjunction with tellurite and other ingredients.

Moxolactam, the broad-range cephalosporin antibiotic used in LPM and MVJ agars, proved to be highly useful, controlling not only the gram-negative bacteria but also most of the other gram positives. It appears to be the agent of choice for further development of media selective for listeriae, and it was used in two recently published medium formulations (8, 28).

With respect to the many selective agents used for listerial media, we tried a medium originally designed as a selective enrichment broth (6) by adding 1.5% agar, but it was inhibitory to all listeriae tested and was excluded from further studies. This medium employs acriflavine and poly-
myxin B as selective agents, both of which have been shown to be inhibitory at certain concentrations (25).

Media, such as MVJ agar, that are highly selective and partially suppressive to uninjured listeriae are not useful for the recovery and enumeration of injured cells, which might be present in certain foods such as pasteurized milk (6). Furthermore, listeriae are usually far outnumbered by other microorganisms that occur naturally in specific types of food or biological specimens. This suggests the need for a recovery and selective enrichment procedure prior to enumeration.

In regard to colony and cell morphology of listeriae on selective media, it was reported by Gray and Killinger (12) that some listerial strains tend to dissociate in culture. We found this particularly true for strain Scott A, and it was sometimes troublesome to enumerate colonies of this strain when using oblique lighting. Furthermore, strain Scott A showed a varying growth response on the same media, especially when plated in mixed cultures, making it difficult to enumerate. As noted above, listerial colonies displayed atypical cellular morphology when grown on LPM, ACA, and MVJ agars. This might have been due to the cell wall-active antibiotics, and care should be exercised when using microscopic observations to confirm suspect listerial colonies from selective plating media. The behavior of strain Scott A in mixed culture, along with its aberrant morphology on selective media, may be related to the production of extracellular substances by certain other organisms. These phenomena may be of significance in explaining the difficulty of recovering L. monocytogenes from dairy products, as well as the varying results obtained in thermal destruction studies. Further research is being conducted in this area.

In summary, LPM agar was found to be the most effective of the media evaluated; the differential capacity of MVJ agar was particularly useful. MBGA was much less satisfactory than MBG.

LITERATURE CITED


