ARTICLES

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Detection of Nonpathogenic and Pathogenic *Listeria* Species by Use of a Chromogenic Agar

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SUMMARY

A selective and differential chromogenic plating medium (R & F *Listeria* spp./*Listeria monocytogenes* Plating Medium [LSPM]) has been developed that simultaneously differentiates presumptive colonies of both the nonpathogenic *Listeria* species (*L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi*) and the pathogenic species (*L. monocytogenes* and *L. ivanovii*) on a single plate in 42–48 h at 35°C. Unlike chromogenic media that produce only a single color in detecting the presence of all *Listeria* species on the basis of ß-glucosidase activity, or those that specifically detect the two pathogenic species by phosphatidylinositol-specific-phospholipase C activities (PI-PLC), LSPM contains a combination of indoxyl-derivative chromogenic substrates with which colonies of nonpathogenic *Listeria* species are pink because of their β-glucosidase activity, and pathogenic species are blue-green to blue-violet, depending on the strain-specific balance of β-glucosidase and PI-PLC activities on an agar with an opaque white background. On LSPM, 39 pure culture strains of *L. monocytogenes* yielded blue-green to blue-violet colonies 1–2 mm in diameter with or without surrounding precipitates in 42–48 h, and 4 strains of *L. ivanovii* yielded dark blue-green colonies with dark precipitates, whereas all of the nonpathogenic *Listeria* strains yielded pink colonies 1–2 mm diameter without precipitates. The ability to differentiate *Listeria* spp. from *L. monocytogenes* over a broad range on the same plate of LSPM was demonstrated at ratios of 1:1 to 100:1 of *L. innocua* to *L. monocytogenes*. Additionally, a high level of selectivity by this plating medium was evidenced by the lack of growth by common species of five gram-positive genera (*Bacillus*, *Staphylococcus*, *Lactobacillus, Pediococcus,* and *Enterococcus*), and eight gram-negative genera (*Escherichia, Enterobacter, Citrobacter, Shigella, Morganella, Providencia, Pantoea,* and *Klebsiella*). Two yeast genera, *Zygosaccharomyces* and *Candida*, also failed to grow at 35°C.

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TABLE 1. The colony morphologies of *Listeria* and other bacteria growing on LSPM for 42 to 48 h at 35°C

Bacterial Species	Number of Strains	Colonial Morphology
Listeria monocytogenes	39	Convex, 1–2 mm, blue-green to blue-violet, ± precipitate
Listeria ivanovii	4	Convex, 1–1.5 mm, dark blue-green, large ppt.
Listeria innocua	6	Convex, 1–2 mm, pink, no ppt.
Listeria welshimeri	2	Convex, 1–2 mm, pink, no ppt.
Listeria seeligeri	1	Convex, 1–2 mm, pink, no ppt.
Listeria grayi	1	Convex, 1–2 mm, pink, no ppt.
Bacillus cereus/thuringiensis	3	No growth
Enterococcus spp.[a]		No growth
Gram positive spp.[b]		No growth
Gram negative spp.[c]		No growth
Yeasts spp.[d]	3	No growth

[a]*Enterococcus faecalis, E. faecium,* and *E. avium*

[b]*Includes: Bacillus circulans* and *B. subtilis; Staphylococcus aureus, S. epidermidis,* and *S. saprolyticus; Lactobacillus acidophilus* and *L. plantarum;* and *Pediococcus cerevisiae*

[c]*Includes: E. coli* (2 strains) and *E. coli* O157:H7 (1 strain); *Enterobacter aerogenes; Citrobacter freundii; Shigella sonnei; Morganella morganii; Providencia alcalifaciens; Pantoea agglomerans: Enterobacter sakazakii; Klebsiella pneumoniae* and *K. ozaenae*

[d]*Zygosaccharomyces bailii* and *Z. rouxii; Candida albicans*

INTRODUCTION

The presence of any *Listeria* spp. in a food sample is interpreted as an indicator of the general hygiene used in its production and also suggests that such organisms might be masking the presence of the pathogen *L. monocytogenes*. In 2003, the US Food and Drug Administration (FDA) modified *Listeria* methodology by encouraging the use of one of the newer chromogenic differential selective agars (i.e., BCM [presently R & F], ALOA, CHROMagar Listeria, or Rapid'L. mono) that differentiate the two pathogenic spp., *L. monocytogenes* and *L. ivanovii*, from the nonpathogenic spp. (*L. innocua, L. seeligeri, L. welshimeri, and L. grayi),* as long as it is run in parallel with one of the prescribed selective agars (i.e., Oxford, PALCAM, LPM plus esculin and ferric iron, and MOX) *(3).* The FDA-prescribed selective media identify all the *Listeria* spp. without differentiation on the basis of β-D-glucosidase (esculinase) activity. Of the suggested newer chromogenic differential selective agars listed above, R & F and Rapid'L. mono both contain the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate (X-IP), which differentiates *L. monocytogenes* and *L. ivanovii* from the nonpathogenic spp. by detecting phosphatidylinositol-specific phospholipase-C (PI-PLC) activity, but do not differentiate the other *Listeria* spp. ALOA and CHROMagar Listeria, however, detect both β-glucosidase activity by the hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside [X-β-D-glucoside]), and PI-PLC activity by incorporation of L-α-phosphatidylinositol, a non-chromogenic substrate that produces a white precipitate with a halo around blue colonies of the two pathogens, and thus differentiate *L. monocytogenes* and *Listeria* spp. on the same plate *(6).*

The differentiation of *L. monocytogenes* and *Listeria* spp. by two positive chromogenic reactions on a single plate in 42–48 h at 35°C *(5),* using a selective and differential chromogenic plating medium (R & F *Listeria* spp./*Listeria monocytogenes* Plating Medium [LSPM]), is described here. The three chromogenic substrates responsible for these reactions have indoxyl moieties halogenated in different positions: 5-bromo-6-chloro-3-indoxyl-β-glucopyranoside (Magenta-β-glucoside; 0.033 g/l) and 6-chloro-3-indoxyl-β-glucopyranoside (Salmon-β-glucoside; 0.030 g/l) for β-glucosidase, and X-IP (0.29 g/l) for PI-PLC, producing two different colors upon specific hydrolysis. By a critical balancing of the levels of these three substrates, this mechanism permits presumptive colonies of nonpathogenic *Listeria* spp. to appear as pink colonies, and the pathogenic *Listeria* species as blue-green to blue-violet colonies on a single plate, with the latter dependent upon the strain-specific balance of β-glucosidase and PI-PLC activities. Additionally, to better discern the light pastel pink colonies of the nonpathogenic *Listeria* spp. and the blue-green to blue-violet colonies for the pathogenic *Listeria* spp. on the plates, they are viewed against an opaque white background in the agar produced by addition of titanium dioxide (TiO_2) to the medium. Colonies of *L. monocytogenes* are detectable on a plate in a 100-fold excess of *L. innocua*.

MATERIALS AND METHODS

Growth of organisms and media

All bacterial strains (see Table 1) were maintained on brain heart infusion agar (BHIA; Acumedia, Lansing, MI) slants

FIGURE 1. Pure culture of *Listeria monocytogenes growing* on LSPM for 42–48 h at 35°C

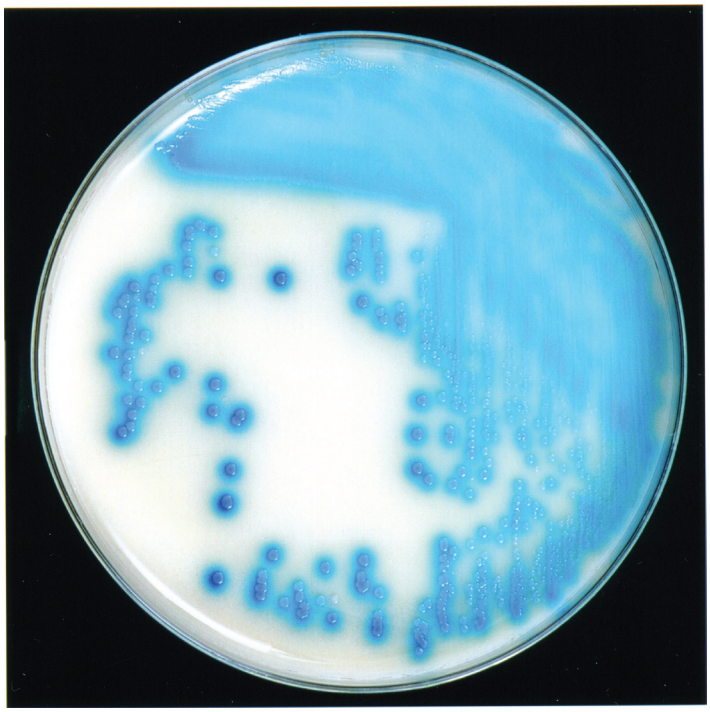
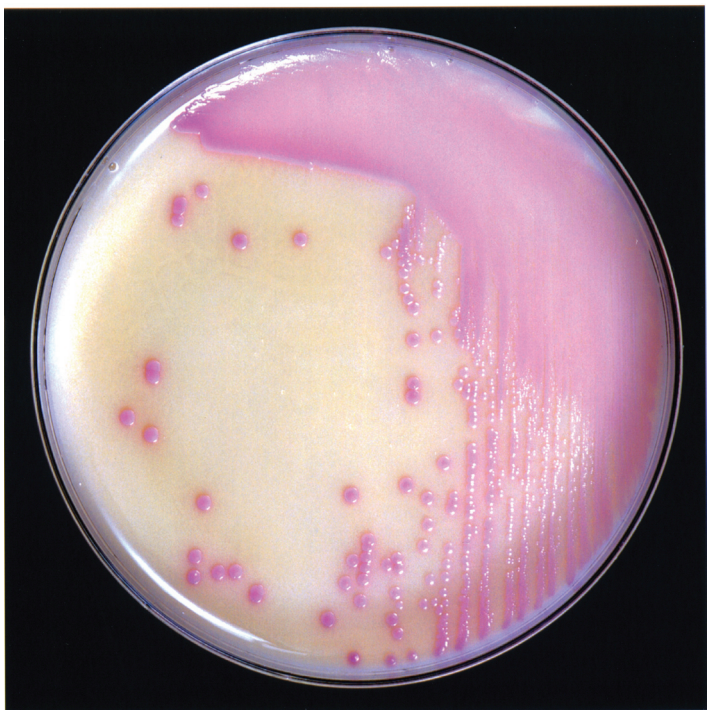


FIGURE 2. Pure culture of *Listeria innocua* growing on LSPM for 42–48 h at 35°C



at 4 to 6°C and transferred monthly. For the 39 *L. monocytogenes* strains tested, the origin of isolation included pork, environmental (meat and dairy processing plants), clinical, rabbit, poultry, sheep, bovine brain tissue, beef, milk, and cheese; the serotypes represented included 1/2a, 1/2b, 1/2c, 3a, 3b, 4a, 4b, 4c, 4d, and 4e. For inoculation studies, cells were grown in either brain heart infusion broth (BHIB) or APT (All Purpose Tween; Difco Laboratories, Detroit, MI) broths at 35°C for 24 h to insure that they were in the stationary phase. Loopfuls of cells were streaked on the plates and incubated at 35°C for 48 h.

Media preparation

The LSPM was prepared according to manufacturer's directions. Briefly, 3.0 g of TiO_2 is added to 1 liter of deionized water and swirled. To this suspension 62.0 g of LSPM powder (R & F Products, Downers Grove, IL) is added with swirling, and the mixture is autoclaved for 10 min. Upon removal from the autoclave, the suspension is swirled to re-suspend the TiO_2 and cooled to 50°C before adding the supplements. After another swirl, plates are poured that are stable in the dark for up to 30 days at 4°C.

Colony morphology and species characteristics

After 42–48 h of incubation at 35°C, colonies growing on the plates were examined for morphology, growth, and color against the opaque white background of the agar.

RESULTS AND DISCUSSION

On LSPM, 39 pure culture strains of *L. monocytogenes* yielded blue-green to blue-violet colonies 1–2 mm diameter with or without surrounding precipitates in 42–48 h, and 4 strains of *L. ivanovii* yielded dark blue-green colonies with dark precipitates, whereas all of the nonpathogenic *Listeria* strains yielded pink colonies 1–2 mm diameter without precipitates (Table 1). The percent recovery for three *L. monocytogenes* strains (ATCC 19114, 19116 and 19117) on LSPM compared with recovery on Tryptic soy agar (Acumedia) ranged from 63.3 to 70.0% (data not presented). The suppression of growth

FIGURE 3. Mixed culture of *Listeria innocua* (pink) and *Listeria monocytogenes* (blue-green) at a 1:1 ratio growing on LSPM for 42–48 h at 35°C

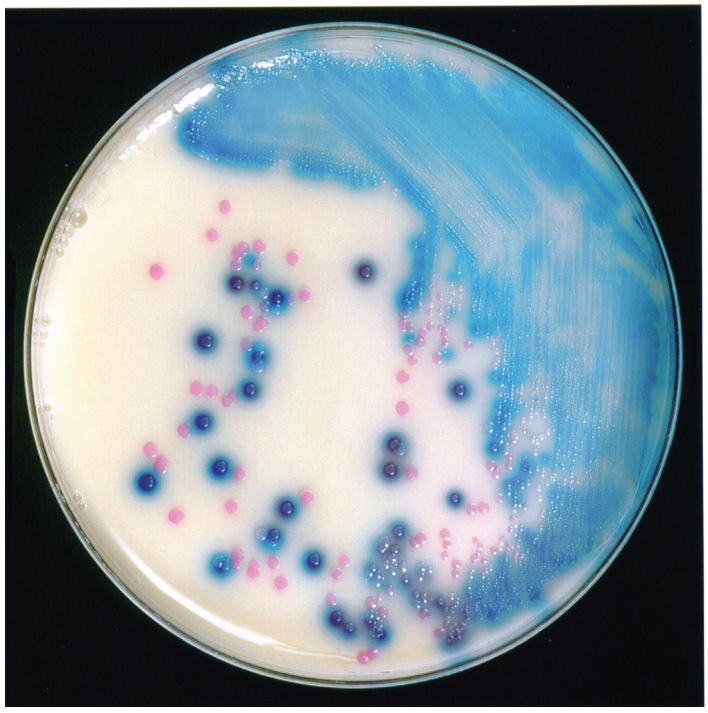
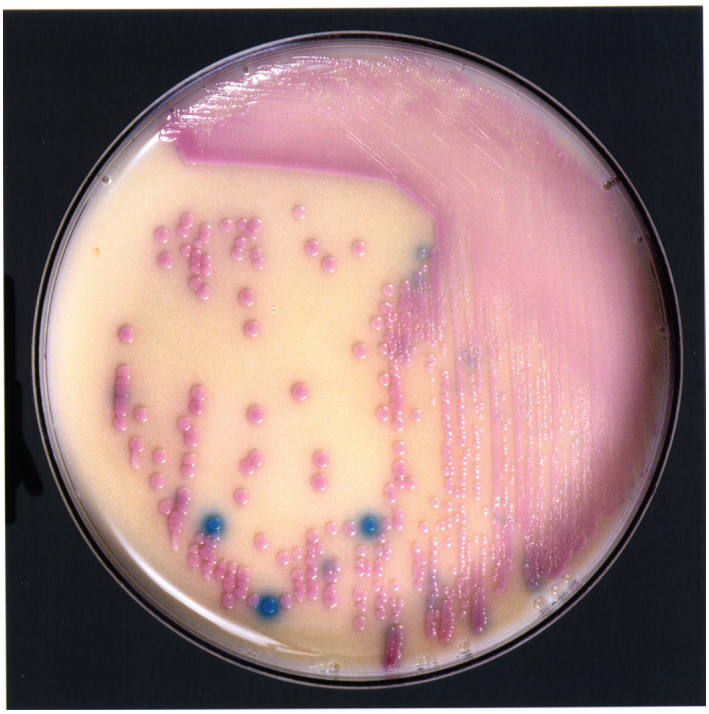


FIGURE 4. Mixed culture of *Listeria innocua* (pink) and *Listeria monocytogenes* (blue-green) at a 100:1 ratio growing on LSPM for 42–48 h at 35°C



observed for five common gram-positive genera (*Bacillus*, *Staphylococcus*, *Lactobacillus, Pediococcus,* and *Enterococcus*), as well as eight gram-negative genera (*Escherichia, Enterobacter, Citrobacter, Shigella, Morganella, Providencia, Pantoea,* and *Klebsiella*), demonstrates the high selectivity of LSPM. Two yeast genera, *Zygosaccharomyces* and *Candida*, also failed to grow in LSPM at 35°C. The highly selective properties of LSPM prevent the growth of potentially false positive organisms such as *Enterococcus* and *Bacillus* species that are positive for β-glucosidase or PI-PLC activity.

Plating on LSPM of pure cultures of *L. innocua* and *L. monocytogenes* showed that the three chromogenic substrates in the medium are capable of generating water-insoluble positive color reactions for both β-glucosidase (pink) and PI-PLC activities (blue-green to blue-violet) that permit *Listeria* spp. and *L. monocytogenes* to be readily differentiated (Figs. 1 and 2). Using mixed cultures, *L. innocua* and *L. monocytogenes* are differentiated by positive color reactions on a single plate over a wide range on LSPM incubated for 42–48 h at 35°C, with respective ratios of 1:1 and 100:1 (Figs. 3 and 4). Previously, media capable of detecting both β-glucosidase and PI-PLC activities have been reported to be superior for *L. monocytogenes* detection in a variety of foods when compared with standard methods and protocols currently in use *(1, 2, 4, 6, 7)*. In these media, however, PI-PLC activity is detected by a non-chromogenic substrate (L-α-phosphatidylinositol) and not X-IP in order to avoid a color conflict with the X-glucoside used to detect β-glucosidase. This non-chromogenic substrate produces a white precipitate and halo around blue colonies of *L. monocytogenes* and *L. ivanovii*.

Although use of the LSPM medium has not been fully evaluated against a standard protocol for the differentiation of nonpathogenic and pathogenic *Listeria* species, the formation of two discrete positive color changes, plus the use of X-IP instead of a non-chromogenic substrate (L-α-phosphatidylinositol) for PI-PLC detection, should result in a clearer and more accurate differentiation of *Listeria* species than is possible in media without these properties. Studies are now being conducted to determine the effectiveness

of LSPM in comparison with USDA and FDA recommended plating media on various food products.

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