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Selective-Enrichment Procedure for Isolation of *Listeria monocytogenes* from Fecal and Biologic Specimens

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A selective-enrichment procedure (SEP) was developed to isolate *Listeria monocytogenes* from fecal and biologic specimens. This procedure was compared with direct plating with McBride listeria agar and 2-, 4-, and 8-week cold-enrichment procedures in recovering *L. monocytogenes* from mouse fecal, liver, and brain specimens. Although the SEP occasionally did not isolate the organism from specimens proved positive by the other procedures, the SEP isolated *L. monocytogenes* from about two and five times as many specimens as the cold-enrichment and direct-plating procedures, respectively.

The occurrence of three recent food-associated outbreaks of listeriosis has prompted increased concern for detecting *Listeria monocytogenes* in food, environmental, and biologic specimens (5, 7, 9). Listeriosis is a severe and often fatal illness, with clinical manifestations that typically include neonatal sepsis or meningitis, sepsis or meningitis in the immunocompromised patient, and puerperal sepsis or "flu-like" illness during pregnancy (1, 2, 4, 6). The mortality rate for listeria infections is generally about 30% (5, 7, 9).

Attempts to isolate *L. monocytogenes* from positive specimens by directly plating suspect material onto conventional media occasionally fail, but isolation is generally successful when the specimens undergo a period of cold enrichment (1, 3, 10). Hence, it is recommended that a portion of the specimen be stored at 4°C and recultured periodically for 2 to 3 months if initial attempts at isolation are unsuccessful (1, 3, 10). Because this lengthy procedure is not amenable for routine testing of food or biologic specimens by microbiological laboratories, there is indeed a need for a shortened enrichment procedure for isolating *L. monocytogenes*. The objective of this study was to develop and verify the efficacy of such an enrichment procedure.

Twenty-two isolates of *L. monocytogenes* were used individually to infect mice for fecal, liver, and brain test specimens. Most were human isolates, and these included: Scott A, Murray B, F5416, F5380, F5260, and F5234 (obtained from J. Lovett, Food and Drug Administration, Cincinnati, Ohio); Butler, 1186, 1179, 1625, Gerber, 1969, 202B, I_1 1490, and 137B (obtained from R. Kaplan, Rush-Presbyterian-St. Lukes Medical Center, Chicago, Ill.); and 43325, 6616, and 25784 (obtained from J. Archer, Wisconsin State Laboratory of Hygiene, Madison). Isolates from raw milk, V7 and V37CE, were obtained from J. Lovett. Isolates of veterinary origin, VET-1 and SAM-2, were obtained from C. Czuprynski (University of Wisconsin, Madison). After receipt, cultures were subcultured once, then frozen in skim milk, and held at −70°C until required.

Each culture of *L. monocytogenes* was grown in 100 ml of tryptose broth (Difco Laboratories, Detroit, Mich.) in a 500-ml sidearm Erlenmeyer flask, which was three times evacuated to 20 in. of Hg and replaced with an atmosphere of 5% O_2–10% CO_2–85% N_2. Cultures were grown with agitation (100 gyrations per min) at 37°C for 48 h. Cells were then washed three times with 0.01 M phosphate-buffered saline (PBS), pH 7.2, concentrated by centrifugation (8,000 × *g*, 15 min), and adjusted spectrophotometrically to the appropriate concentration (1×10^6 to 2×10^7 CFU/ml) with PBS. Cell numbers were confirmed by plate counts on tryptose agar (Difco) after incubating at 37°C for 48 h.

Mice (Sprague Dawley-ICR), weighing 20 to 25 g, in a group of 10 per strain of *L. monocytogenes*, were each inoculated perorally via a 20-gauge cannula with 0.5 ml of test culture. Fecal specimens were collected from each mouse at 7, 11, and 14 days postinoculation by holding each mouse individually in a cage over filter paper for 3 h. The fecal pellets (total weight, 0.2 to 0.4 g per animal) of each animal were diluted (1:5) in PBS, homogenized by macerating pellets with the wooden end of a sterile cotton swab, and mixed for about 1 min with a Vortex mixer. All specimens were then assayed for *L. monocytogenes*. Additionally, at 14 days postinoculation, mice were surgically exposed, and liver and brain tissues were aseptically removed. Each tissue specimen was homogenized in 0.1% peptone (1 part tissue to 9 parts peptone) for 1 min by a Polytron tissue homogenizer (Brinkman Instruments, Inc., Westbury, N.Y.) and then assayed for *L. monocytogenes*.

All fecal, liver, and brain specimens were assayed for *L. monocytogenes* by three procedures: (i) direct surface plating of 0.1-ml portions of each specimen preparation in duplicate onto McBride listeria agar (MLA) (8), (ii) cold enrichment by adding one-half of the remaining specimen sample to tryptose broth (100 ml), and (iii) enrichment by adding the other half of the sample to a selective-enrichment medium (100 ml) for 1 day.

Specimen preparations directly plated onto MLA were incubated in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C for 48 h. At least three colonies typical of those formed by *L. monocytogenes* (bluish gray, translucent, slightly raised, 0.5 to 1.5 mm in diameter, and weakly beta-hemolytic) per plate were selected for confirmation. Confirmatory tests included: characteristic tumbling motility (when grown in tryptose broth at 21°C) determined microscopically, Gram stain reaction (positive), catalase reaction (positive), beta-hemolysis, and biochemical tests (8).

Specimen preparations added to tryptose broth for cold enrichment were incubated at 4°C and assayed for *L. monocytogenes* by surface plating 0.1-ml portions of enrichment culture onto duplicate MLA plates at 2, 4, and 8 weeks of

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TABLE 1. Isolation of *L. monocytogenes* by different procedures from specimens of perorally inoculated mice

Strain	Dose[a]	No. of mice[b]	No. of specimens positive for *L. monocytogenes*:														
			7-day feces			11-day feces			14-day feces			Brain			Liver		
			DPP[c]	CEP[d]	SEP	DPP	CEP	SEP	DPP	CEP	SEP	DPP	CEP	SEP	DPP	CEP	SEP
Scott A	1.3×10^6	9	8	3	7	0	1	3	0	0	1	0	0	0	0	1	0
V7	1.4×10^6	9	4	3	4	1	3	4	2	1	3	0	2	2	0	1	3
Murray B	1.2×10^7	10	0	3	3	0	1	2	0	1	1	0	2	1	0	1	2
V37 CE	2.0×10^7	9	0	2	2	0	1	1	0	0	1	0	0	0	0	2	3
F5416	3.1×10^6	8	2	2	3	0	1	1	0	0	2	0	0	0	0	0	0
F5380	2.6×10^6	6	0	0	3	0	1	1	0	0	1	0	0	0	0	0	0
F5260	2.4×10^6	10	0	2	3	0	2	2	0	0	0	1	1	2	0	1	1
F5234	2.0×10^6	10	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0
6616	2.5×10^6	9	0	0	3	2	0	3	0	0	0	0	1	1	0	2	1
25784	2.5×10^6	8	0	0	2	1	0	0	0	0	0	0	1	1	0	0	0
43325	4.2×10^6	10	0	0	3	0	0	1	0	0	0	0	1	1	0	3	3
137B	2.5×10^6	8	1	1	1	0	0	0	0	0	0	0	1	1	0	1	1
202B	3.5×10^6	10	0	0	0	0	2	2	0	1	2	0	0	0	0	0	0
1179	5.2×10^6	10	0	1	2	0	1	2	0	0	0	0	0	1	0	0	1
1186	2.1×10^6	10	0	1	1	0	2	1	0	0	1	0	0	0	0	0	0
1625	5.0×10^6	10	0	1	1	0	0	1	0	0	0	1	1	2	1	1	1
1969	5.0×10^6	9	0	0	2	0	0	1	0	0	0	0	0	0	0	0	1
Butler	4.6×10^6	10	0	0	3	0	0	1	0	0	1	0	0	0	0	2	1
Gerber	3.5×10^6	10	0	1	1	0	0	0	0	0	0	0	1	1	0	0	1
I_1 1490	3.7×10^6	10	0	0	2	0	0	1	0	0	1	0	0	0	0	0	0
SAM-2	1.9×10^6	9	0	0	2	0	0	2	0	0	1	0	0	1	0	0	0
VET-1	1.8×10^6	8	1	0	3	1	0	2	0	0	0	0	0	1	0	0	1

[a] Number of *L. monocytogenes* in 0.5 ml of PBS perorally administered to each mouse.
[b] Ten mice were challenged with each strain; however, specimens were obtained only from survivors.
[c] DPP, Direct-plating procedure.
[d] CEP, Cold-enrichment procedure.

incubation. Incubation conditions for MLA plates and confirmatory tests for *L. monocytogenes* were the same as those described above.

Specimen preparations receiving the selective-enrichment procedure (SEP) were incubated in a selective enrichment medium composed of 0.9% tryptose broth, 5% defibrinated sheep blood, 0.5% glucose, 0.15% dipotassium phosphate, polymyxin B (16 IU/ml), acriflavine hydrochloride (10 μg/ml), and nalidixic acid (40 μg/ml). Each specimen preparation was added to 100 ml of enrichment medium, which was held in a 250-ml sidearm Erlenmeyer filter flask. After adding the test specimen, each flask was evacuated three times to 20 in. Hg and replaced with an atmosphere of 5% O_2–10% CO_2–85% N_2. Enrichment cultures were incubated with agitation (100 gyrations per min) at 37°C for 24 h and then assayed for *L. monocytogenes* by surface plating duplicate MLA plates with the same incubation conditions and confirmatory tests described above.

Initial approaches taken to evaluate the efficacy of the SEP for isolating *L. monocytogenes* used raw milk as the test medium. By using 100 ml of selective-enrichment medium, the SEP consistently isolated the organism from 25-ml raw milk samples inoculated with <10 *L. monocytogenes* per milliliter of sample when several different strains of *L. monocytogenes* were individually tested (data not presented). However, isolating pathogens from naturally contaminated sources is often more difficult than isolating them from artificially inoculated foods. Hence, studies were done to test naturally contaminated specimens.

Fifty raw milk samples obtained from bulk tanks at farms that supply milk to the University of Wisconsin-Madison milk processing facility were assayed for *L. monocytogenes* by the three procedures described above. All of the samples were negative for *L. monocytogenes* by all three procedures. Because the prevalence of the organism in milk from these farms appeared to be quite low, this approach was deemed impractical for evaluating the efficacy of the three isolation procedures.

A third approach was taken to compare the three isolation procedures, using as test samples fecal and tissue specimens from mice perorally inoculated with different strains of *L. monocytogenes*. Although the nature of *Listeria* contamination of these specimens may be different from that which occurs in animals with a naturally acquired, active listeria infection, specimens from inoculated mice are likely to contain equivalent numbers and types of background microbial flora that are present in specimens from naturally infected mice.

Ten mice were inoculated with each strain of *L. monocytogenes*. Some animals died before test specimens and data could be obtained (Table 1). Results from surviving mice suggest that only a small portion of those animals were infected by the organism, since isolations were generally made from only one to three animals in each group.

Results indicate that of the three isolation procedures evaluated, the SEP was superior for isolating *L. monocytogenes* from all three types of specimens (Table 1). Occasionally the organism was not isolated by the SEP from a specimen found to be positive for *L. monocytogenes* by one of the other procedures; however, this was infrequent (occurring in <5% of all of the *L. monocytogenes*-bearing specimens). Overall, the SEP isolated *L. monocytogenes* from about two and five times as many specimens as the cold-enrichment and direct-plating procedures, respectively.

In this study, tissue, fecal, and environmental specimens were diluted, to allow for appropriate distribution of specimens for comparison of different isolation procedures. In practice, this dilution is unnecessary. Rather, each specimen

(preferably no more than 25 g/100 ml of enrichment medium) can be macerated directly with selective-enrichment medium before incubation.

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