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Highly Selective Medium for Isolation of *Listeria monocytogenes* from Food†

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A new selective medium (Al-Zoreky–Sandine listeria medium [ASLM]) was formulated to recover *Listeria monocytogenes* from food specimens; the medium completely inhibited common food microflora. Recognition of *Listeria* colonies is evident by black discoloration of the medium due to esculin hydrolysis without need for special illuminating equipment. The medium contains acriflavin, ceftazidime, and moxalactam as selective agents. Compared with Listeria Selective Agar, ASLM was equally effective in recovering *L. monocytogenes*. However, ASLM inhibited micrococci, enterococci, and gram-negative bacteria, especially a strain that mimicked *L. monocytogenes* on Listeria Selective Agar. The new medium was able to recover heat injured cells with only 15% less count than the nonselective medium.

Recent food outbreaks of listeriosis (6, 9, 12, 15, 20) have emphasized the need for a highly selective medium to detect *Listeria monocytogenes*, a human and animal pathogen. Listeriosis has a mortality rate of up to 33% (5, 17) and is highest for pregnant women, infants, and immunocompromised persons. Fifty percent lethal doses of *L. monocytogenes* are as low as 5 cells for immunocompromised mice, whereas the 50% lethal dose for normal mice is 10^5 to 10^7 cells (7).

The natural flora of food may complicate the detection of this pathogen with the media now available. Antibiotics and chemicals are being used in most media in an attempt to recover listeriae while inhibiting other flora (1, 5, 6, 8). However, interference from background microorganisms, difficulty in recognition of listeriae, and the need for special lighting for identification have emphasized the need for research on *Listeria* isolation media (3, 10, 11, 13, 24). A monoclonal antibody-based enzyme-linked immunosorbent assay has been described, but the test requires the presence of high levels of listeriae (>10^4 CFU/ml) in foods for positive detection (2, 19, 21).

It was the objective of this study to evaluate a newly developed medium, Al-Zoreky–Sandine listeria medium (ASLM), for isolation of *L. monocytogenes* from foods, especially to determine the amount of interference from naturally present bacteria.

MATERIALS AND METHODS

Cultures and strain maintenance. *L. monocytogenes* Scott A, Jalisco, and ATCC 7644 and *Listeria innocua* were used after being activated in brain heart infusion (BHI) broth (Difco Laboratories) at 37°C for 18 to 24 h. All strains were maintained between transfers on tryptic soy agar (TSA) slants at 2 to 5°C.

Test media. The experimental medium (ASLM) consisted of Listeria Selective Agar (LSA) Base (CM856; Oxoid Ltd.), which contains the following (per liter): Columbia blood agar base, 39 g; esculin, 1 g; ferric ammonium citrate, 0.5 g; and lithium chloride, 15 g. After the base medium was autoclaved at 121°C for 15 min and tempered to 45°C in a water bath, the following inhibitory agents (filter sterilized, 0.2-μm-pore-size filter) were aseptically added to 1 liter: 5 ml of aqueous absolute ethanol (1:1) containing 200 mg of cycloheximide (Sigma Chemical Co.), 2.5 ml of an aqueous solution containing 50 mg of ceftazidime pentahydrate (Glaxo), 2.5 ml of an aqueous solution containing 10 mg of acriflavin hydrochloride, and 2 ml of a 1% solution of moxalactam (Sigma) in phosphate buffer (pH 6.1). The final pH was 7 ± 0.1.

The other medium used for comparative purposes was LSA, the so-called Oxoid agar, with an antibiotic supplement (Oxoid); this medium gave the highest recovery of *L. monocytogenes* in comparison with other well known listeria media (20). TSA was used as the reference agar (nonselective).

Spiral plating. Quantitative evaluation of recovered *L. monocytogenes* was carried out with a Spiral Plater (model D; Spiral Systems) unless otherwise mentioned. Active cultures were diluted to 10^4 to 10^5 CFU/ml. Incubation was at 37°C for 48 h, and the number of cells was determined with a counting grid.

Pure culture comparison. Each *L. monocytogenes* (Scott A, Jalisco, or ATCC 7644) or *L. innocua* strain was activated and surface plated (Spiral Plater). Both ASLM and Listeria Selective Agar (LSA) were used to enumerate the pathogen. The nonselective medium TSA was the control. Statistical analysis was conducted to evaluate any significant differences between the media by using analysis of variance (Statgraphic version 4.0, Oregon State University).

Selectivity of ASLM. Several microorganisms (Table 1), including *L. monocytogenes* strains as positive controls, were activated in BHI broth for approximately 24 h at 37°C. Samples (0.1 ml) of the cultures were spread onto the surface of TSA, ASLM, and LSA with a glass rod. Plates were incubated at 37°C for 48 h. Plates were examined for microbial growth. For lactic acid bacteria, MRS agar was used as a reference medium, and KF streptococcal medium was used as a control medium for *Streptococcus faecalis*.

Recovery of *L. monocytogenes* from food. Raw milk obtained from the Oregon State University dairy farm was artificially contaminated with *L. monocytogenes* (ATCC

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TABLE 1. Selectivity of ASLM for listeriae

Microorganism	Growth in[a]:		
	TSA	ASLM	LSA
Gram-negative bacteria			
Pseudomonas aeruginosa ATCC 419	+++	−	−
Escherichia coli V517	+++	−	−
Salmonella typhimurium	+++	−	−
Yersinia enterocolitica ATCC 23715	+++	−	−
Aeromonas hydrophila ATCC 7965	+++	−	−
Campylobacter jejuni ATCC 29428[b]	+++	−	−
Gram-positive bacteria			
L. monocytogenes ATCC 7644	+++	+++	+++
L. monocytogenes Scott A	+++	+++	+++
Lactobacillus bulgaricus CH_2	NT	−	−
Lactobacillus lactis LAR 28	NT	−	−
Staphylococcus aureus[c]	+++	−	+++
Staphylococcus isolate[d] (raw milk)	+++	−	+++
Lactococcus lactis ATCC 7962	NT	−	−
Lactococcus diacetylactis 18-16	NT	−	−
L. diacetylactis 26-2	NT	−	−
Streptococcus faecalis GF590	NT	+	+++
S. faecalis CG110	NT	−	+++
Streptococcus thermophilus CR5	NT	−	−

[a] Samples of 0.1 ml of 24-h cultures were spread onto the surface of the agar: +++, heavy growth; −, no growth; +, very slight growth after 48 h of incubation; NT, not tested (see Materials and Methods).
[b] Grown at 37°C in Campy Pak (BBL Microbiology Systems).
[c] Esculin negative.
[d] Esculin positive.

7644 or Scott A) at 10^4 to 10^5 CFU/ml. Cells were plated on ASLM and LSA (Spiral Plater). In addition, the level of natural microflora was determined by using TSA. Plates were incubated at 37°C for 48 h. Similarly, pasteurized milk (2% fat) was contaminated at 10^4 to 10^5 CFU/ml. Also, samples of raw and pasteurized milk not contaminated with *L. monocytogenes* were plated in the above-mentioned media as a control.

In other experiments, samples of raw and pasteurized milk were incubated at 20°C for 18 h or at 2 to 5°C for 10 days to enhance growth of the natural flora before being artificially contaminated with *L. monocytogenes*. Cottage cheese was also inoculated with *L. monocytogenes* at approximately 10^4 CFU/ml (ATCC 7644) or 10^5 CFU/ml (Scott A), and samples were surface plated on TSA, ASLM, and LSA with a glass rod. Uninoculated control cottage cheese was plated as well.

Heat-injured cells. The procedure of Golden et al. (13) was employed with *L. monocytogenes* ATCC 7644 as the test organism. Cells (~10^8 CFU/ml) were heated (52°C for 15 min) in a water bath in tryptose-phosphate broth (TPB). After heating and cooling the organism was diluted in TPB to give approximately 10^2 to 10^6 CFU/ml. The Spiral Plater was used, and plates were incubated at 37°C for 48 h. Both heat-injured and noninjured cells (controls) were plated in ASLM. The control medium used was TSA.

RESULTS AND DISCUSSION

Colonial appearance of *L. monocytogenes* and productivity on ASLM. *L. monocytogenes* (Scott A, Jalisco, and ATCC 7644) or *L. innocua* grew on both LSA and ASLM as black colonies surrounded by black, which was due to esculine hydrolysis (8, 12). Inspection of listeriae on ASLM further revealed that they had small dark centers; the rest of the colony appeared dark green. Also, these colonies exhibited sunken centers. No significant difference ($P > 0.01$) was found between the number of listeria colonies appearing on either medium (Table 2). Another independent study (data not shown) with both media also indicated that ASLM and LSA gave the same recovery of *L. monocytogenes* (M. A. Daeschel [Department of Food Science and Technology, Oregon State University, Corvallis], personal communication). Also, ASLM was not inhibitory to the three *L. monocytogenes* strains used when compared with the nonselective medium. Other media have been found inhibitory to *Listeria* spp. (13, 18, 24) and to provide poor colony recognition (3).

TABLE 2. Comparison of recovery of *L. monocytogenes* and *L. innocua* on TSA, ASLM and LSA media

Strain	Recovery[a] (log_{10} CFU/ml) in:		
	TSA	ASLM	LSA
L. monocytogenes			
Scott A	5.01	5.04	4.99
ATCC 7644	5.19	4.98	5.0
Jalisco	5.15	5.15	5.10
L. innocua	4.63	4.5	4.63

[a] Spiral plate counting with incubation at 37°C for 48 h. Each value represents the average of two replicates in duplicate.

Selectivity of the test medium. ASLM was strongly inhibitory (100% inhibition) for all nonlisterial microorganisms tested (Table 1). Both strains of *L. monocytogenes* grew in LSA and ASLM used as positive controls. In fact, both staphylococci and *S. faecalis* GF590 and CG110 grew well on LSA and gave positive esculin reactions, indicated by the black discoloration of the medium; also, one esculin-negative staphylococcus grew luxuriously on LSA but was inhibited on ASLM. The black color on LSA medium can be misleading for identification of listeriae, since staphylococci and enterococci may be present in food or clinical specimens and give false-positive readings on LSA. Most important is that complete inhibition of all organisms tested was achieved in ASLM, even though high number of cells were spread onto the surface. With prolonged incubation (>48 h), *S. faecalis* GF590 grew very slightly in ASLM to produce pinpoint colonies, whereas a complete black discoloration of LSA plates occurred in less than 24 h of incubation. Furthermore, strains of aerobic spore formers and diphtheroids will cause black discoloration in LSA (8). Other media suggested for listeriae also have been found unable to suppress *Enterococcus* spp. and micrococci (1, 16, 18, 24). In addition, the use of Henry illumination to view *Listeria* colonies on LPM and MMA media (11) can be misleading, since some members of a natural flora can give the same blue color as that produced by *Listeria* spp. (5, 16). The combination of inhibitory agents used in ASLM (moxalactam, ceftazidime, and acriflavin) proved superior in inhibiting a variety of microorganisms while allowing listeriae to grow. For more details concerning the inhibitory substances used in different listerial media, refer to Cassiday and Brackett (5).

Recovery of *L. monocytogenes* from food. Result for recovery of *L. monocytogenes* from food are summarized in Table 3. More cells of ATCC 7644 were recovered on ASLM than on LSA. However, both media gave comparable results with the Scott A strain. Other selective media have been reported to have different recovery rates for isolation of *L. monocytogenes* from various foods and clinical specimens (8, 10, 13,

TABLE 3. Efficacy of direct plating on ASLM for recovering *L. monocytogenes* Scott A and ATCC 7644 from food[a]

Strain	Recovery medium	$\log_{10}$ CFU at indicated inoculum per ml or g of:		
		Raw milk	Pasteurized milk[b]	Cottage cheese
ATCC 7644	ASLM	4.30	5.07	4.12
	LSA	4.00	5.01	4.03
Scott A	ASLM	4.46	5.05	4.3
	LSA	4.49	5.17	4.38

[a] Food was contaminated artificially with 10^4 to 10^5 CFU of *L. monocytogenes* per ml. Control samples (without added listeriae) contained between 10^4 and more than 10^6 CFU of natural flora per ml.

[b] Pasteurized milk was incubated at 20°C for 20 h before being inoculated with *L. monocytogenes*.

20). It is noteworthy that recovery of *L. monocytogenes* from food with ASLM was efficient, since a high number (~10^6 CFU/ml) of background microflora also was present.

Incubation of raw milk at 20°C for 20 h or 2 to 5°C for 10 days before inoculation with *L. monocytogenes* made detection of the pathogen impossible on LSA, since many staphylococci emerged (>10^6 CFU/ml) that were esculin positive (black discoloration of the medium) (Fig. 1). Furthermore, a gram-negative bacterium isolated from cabbage that grew slowly at 25°C and not at all at 30°C and that gave an esculin-positive reaction on LSA was completely inhibited on ASLM. In this regard, other recommended media for *L. monocytogenes*, AC and MVJA (1, 4), allowed the growth of other natural contaminants present in raw milk and gave colony characteristics similar to those of *L. monocytogenes* (data not shown). Also, microscopic examination of staph-

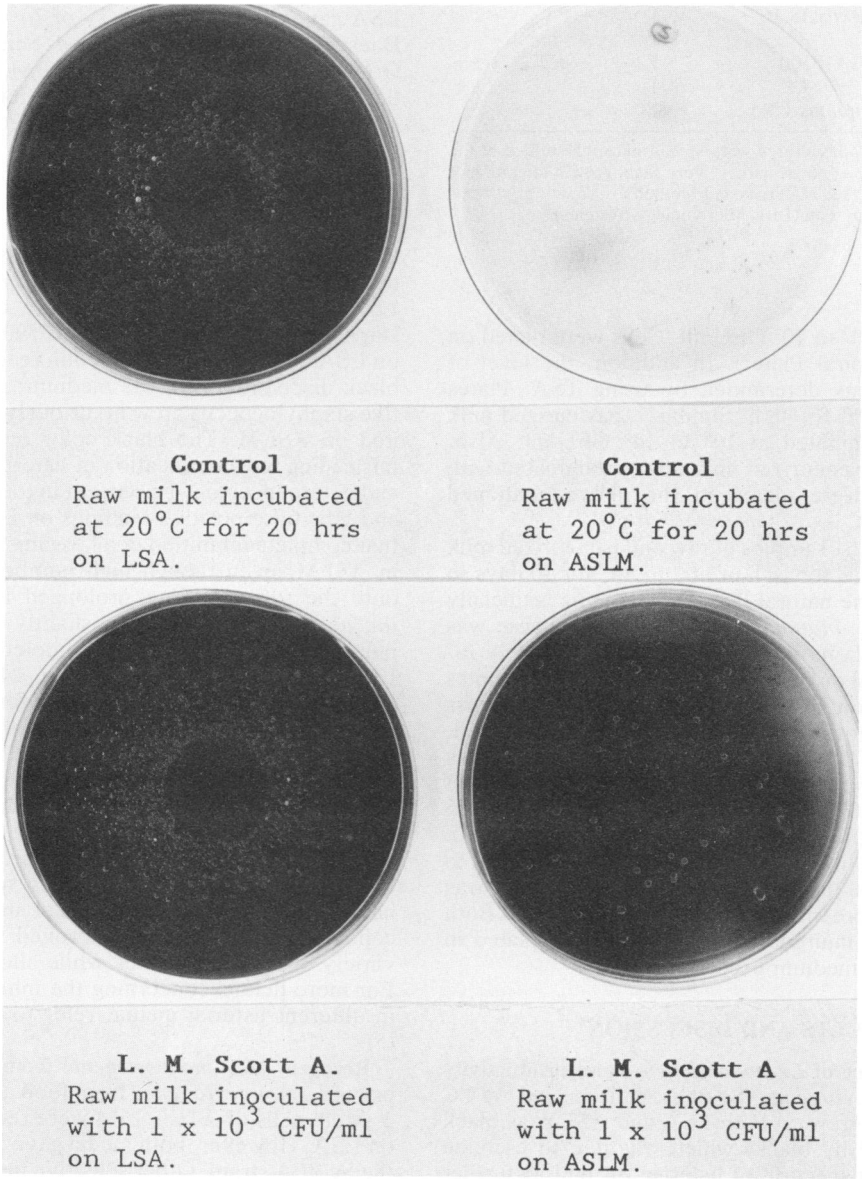

FIG. 1. Appearance of *L. monocytogenes* Scott A colonies when spread on LSA and ASLM in comparison with control plates of these two media. Raw milk samples were preincubated at 20°C for 20 h (controls) before inoculation with *L. monocytogenes*.

TABLE 4. Efficiency of direct plating on ASLM in the recovery of heat-injured *L. monocytogenes* ATCC 7644

Treatment	Medium	$\log_{10}$ CFU/ml recovered in the following dilution[a]:		
		10^{-2}	10^{-4}	10^{-6}
No injury	TSA	>6[b]	4.4	2.7
	ASLM	>6	4.4	2.5
Heat injury	TSA	>6	4.2	<2.6[b]
	ASLM	>6	3.55	<2.6

[a] The organism was diluted as indicated before and after heat injury at 52°C for 15 min. Values are the means of two replicates in duplicate.
[b] Spiral Plater is recommended for counts between 400 and 10^6 CFU/ml.

ylococci grown in LSA was misleading, since under these conditions this organism had unusually long cells that tended to stain gram negative. By culturing these isolates in BHI broth, cells were recovered as typical gram-positive cocci.

Raw milk tested in the present study was free of *L. monocytogenes* even after prolonged cold storage. This pathogen, however, has been recovered from raw milk at levels of 1.5 and about 7% in Canada and the United States, respectively, and as high as 45% in Spain (9, 22). The high number of natural contaminants encouraged by preincubation in our study did not complicate the detection and identification of *L. monocytogenes* in ASLM, although the interference of contaminating organisms has been noted with other media (1, 3, 6, 8, 10, 18).

Recovery of heat-injured cells. Heat injury of LM ATCC 7644 caused approximately 15% lower colony count in ASLM when compared with that of the nonselective medium TSA (Table 4). An attempt to resuscitate cells after heat injury and before plating may increase the number of cells recovered. It has been stated that 1,000 times lower count was achieved when *L. monocytogenes* was heat injured at 52°C for 60 min and then plated on a medium containing sodium chloride (14, 23). It also has been noted that heat injury of this pathogen caused a 20 to 95% decrease in the count, depending on the strain and medium, in comparison with the count on a nonselective medium (16). Similarly, other media have been found to give poor recovery of injured listerial cells (13, 24).

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