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A New Selective Medium for Isolating *Listeria* spp. from Heavily Contaminated Material

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Food-associated outbreaks of human listeriosis have emphasized the importance and necessity of screening food for the presence of *Listeria* isolatesselective agar medium combining acriflavine (10 mg/liter) with ceftazidime (50 mg/liter) was developed. A total of 1,099 cheese production specimens were cultured, from which 157 *Listeria* isolates. (14.3%) grew. When compared with modified McBride agar, the acriflavine-ceftazidime agar recovered more *Listeria* isolates (98 versus 65%, $P < 0.001$) more rapidly (57% after 48 h of incubation of the enrichment broth versus 35%, $P < 0.01$) and in greater amounts. Acriflavine-ceftazidime selective agar medium proved to be a highly sensitive medium to recover *Listeria* spp. from heavily contaminated food products.

Recent food-associated outbreaks of listeriosis (3, 4, 11) and the subsequent revived interest in the epidemiology of the disease have reemphasized the need for an effective selective medium for the isolation of *Listeria* spp. from food, environmental, and biological materials.

Antibiotics, as well as dyes, have been successfully used in selective media (5–7). *Listeria* spp. are generally highly resistant to broad-spectrum cephalosporins, antibiotics which are effective inhibitors of most bacteria other than *Listeria* spp. This fact led us to develop a new medium in which ceftazidime, a broad-spectrum cephalosporin, was combined with the dye acriflavine.

In preliminary experiments (E. S. Bannerman, W. Kamm, M. Galazzo, L. Tissières, and J. Bille, Abstr. 46th Annu. Meet. Swiss Soc. Microbiol., abstr. no. P3, 1987), we established the optimal concentrations for ceftazidime and acriflavine as 50 and 10 mg/liter, respectively, by testing their effects, both alone and in combination, on the growth of *Listeria* spp. and representative flora normally encountered in contaminated material. In broth, the combination of acriflavine and ceftazidime totally inhibited *Staphylococcus epidermidis*, *Escherichia coli*, and *Enterococcus faecalis*. *Listeria monocytogenes* and *Listeria innocua* were not affected, but *Listeria seeligeri* was slightly inhibited. We then added this combination to an agar base to prepare a solid medium. The selective properties of this new medium were assessed and compared with those of modified McBride (McB) agar by examining over 1,000 specimens from cheese or material used during cheese production.

MATERIALS AND METHODS

Specimens. The following types of specimens were studied: 429 swabs from rinds of soft cheeses, 597 swabs from working surfaces and other material used in the production of cheese, and 73 nasal swabs from dairy personnel.

All swabs were immediately put into tubes containing 6 ml of enrichment broth and transported to the laboratory.

Media. The enrichment broth used was that recommended by the U.S. Food and Drug Administration and consisted of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 0.6% yeast extract (BBL) supplemented with a filter-sterilized aqueous solution of acriflavine hydrochloride (final concentration, 15 mg/liter; Sigma Chemical Co., St. Louis, Mo.), an aqueous solution of nalidixic acid (final concentration, 40 mg/liter; Sigma), and a 40% ethanol-water solution of cycloheximide (final concentration, 50 mg/liter; Sigma).

Acriflavine-ceftazidime (AC) agar was prepared as follows. After being autoclaved, the following inhibitory substances were added aseptically to a concentration of 44 g/liter of cooled (45 to 48°C) Columbia agar base (Gibco Ltd., Paisley, Scotland): (i) 2.5 ml of a membrane filter-sterilized aqueous solution containing 10 mg of acriflavine hydrochloride and (ii) 2.5 ml of a membrane filter-sterilized aqueous solution containing 50 mg of ceftazidime pentahydrate (Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom).

Modified McB agar was prepared by the method described elsewhere (8) and consisted of 35.5 g of phenylethanol agar (Difco Laboratories, Detroit, Mich.), 10 g of glycine anhydride (Sigma), and 0.5 g of lithium chloride in 1,000 ml of distilled water, to which 200 mg of cycloheximide per liter (12) was added after autoclaving and cooling. Blood agar plates containing defibrinated human blood (5%) were used as a third and nonselective medium.

Culture procedure. The tubes containing the swabs in the U.S. Food and Drug Administration enrichment broth were incubated at 30°C for 48 h. A loopful of broth was then streaked onto each blood agar plate, a modified McB agar plate, and an AC agar plate. The plates were incubated at 35°C for 48 h and were then examined for the presence of *Listeria* colonies. These appeared on McB agar as blue to greyish blue colonies when examined by Henry transillumination (12) and on AC agar as small, yellow, glistening, and translucent colonies. The density of growth was estimated as weak (when the colonies covered up to only one-third of the streaked surface), heavy (when they covered the whole streaked surface), or moderate (when the colonies covered more than one-third but less than the total streaked surface).

After the first subculture, the tubes containing the swabs were stored at 4°C for cold enrichment, and additional subcultures were done at 7-day intervals for 4 weeks.

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TABLE 1. Comparative recovery of *Listeria* spp. on two solid selective media

Species (no. of isolates)	No. of isolates growing on:		
	AC agar	McB agar	McB and AC agars
L. monocytogenes (88)	38	2	48
L. innocua (49)	7	1	41
L. seeligeri (20)	10	0	10

Suspicious colonies were identified to the genus and species level by conventional procedures (1, 9). For each isolate, the time at which it was detected, the medium on which it grew, and the density of growth were recorded.

RESULTS

A total of 1,099 samples comprising 429 swabs from the rinds of soft cheeses, 597 swabs from material used during cheese production, and 73 nasal swabs from dairy personnel were cultured. *Listeria* isolates were recovered from 157 specimens (14.3%), of which 88 were identified as *L. monocytogenes*, 49 were identified as *L. innocua*, and 20 were identified as *L. seeligeri*. These isolates were recovered from the rinds of soft cheeses (110 specimens), the environment (44 specimens), and three nasal swabs.

Table 1 compares the recovery of the 157 isolates from the two selective agar media. A total of 154 isolates were recovered from AC medium (98%), while only 102 were recovered from McB medium (65%), a statistically significant difference ($P < 0.001$). Table 2 shows the differences in recovery times between the two *Listeria* selective media. Of the 99 isolates recovered from both AC and McB agars, 52 were detected at the same subculture on both media, 37 were detected earlier on AC agar, and 10 were detected earlier on McB agar. The differences in the densities of growth on the two media are shown in Table 3. It should be noted that no *Listeria* spp. were detected on the nonselective blood agar plates. The plates were usually overgrown with up to six species of bacteria and fungi.

DISCUSSION

The isolation of *Listeria* spp. from cheese has recently been documented during and after food-associated outbreaks of human listeriosis (2, 3, 13). Generally, enrichment broth has to be combined with selective media (7, 8, 10) because of the low number of *Listeria* spp. and the great extent of contamination in the specimens (12).

TABLE 3. Differences in densities of growth for 99 *Listeria* isolates recovered on both selective media

Species	No. of isolates showing various degrees of growth[a] on:					
	AC medium			McB medium		
	+	++	+++	+	++	+++
L. monocytogenes	4	27	17	34	10	4
L. innocua	4	15	22	22	10	9
L. seeligeri	2	2	6	6	0	4

[a] +, Weak; ++, moderate; +++, heavy.

The present study evaluated a new selective medium for the isolation of *Listeria* spp. from cheese and compared it with the medium generally used by other researchers in this field (2, 13).

AC agar was found to be superior to McB agar in the following respects. (i) AC agar medium was more sensitive, allowing the detection of about 50% more isolates of *Listeria* spp. than McB agar did. This sensitivity was probably due to the fact that AC agar inhibited more bacterial species than McB agar did. (ii) AC agar was more selective. With the exception of very few enterococci, members of the family *Enterobacteriaceae*, yeasts, and molds, AC agar inhibited all organisms other than *Listeria* spp. In most instances, the typical colonial morphology allowed easy detection of *Listeria* spp. On McB agar, however, up to four bacterial species often grew in various densities. The growth of such a mixed bacterial population was probably the reason that on McB agar the isolation rate was lower and the density of growth was weaker than on AC agar. (iii) AC agar recovered *Listeria* spp. more rapidly than McB agar did. Of the isolates recovered from the two selective media, 57% were detected on AC agar after 48 h, while only 35% were detected on McB agar, a difference which could be an important factor during an outbreak of listeriosis.

After a recent report of a new selective agar medium for the recovery of *Listeria* spp. from beef (6), we compared that medium, which contained moxalactam as an antibiotic, with AC agar and found it to be significantly ($P < 0.001$) inferior to AC agar in the recovery of *Listeria* spp. from cheese (data not shown).

Our results suggest that AC agar is a very effective selective medium for detecting *Listeria* spp. in heavily contaminated specimens, and an assessment with contaminated human samples, such as stool, vaginal, and rectal swabs, is now under way. The availability and the use of an effective selective medium could contribute to a better understanding of the epidemiology of listeriosis.

TABLE 2. Time of recovery of 99 *Listeria* isolates on two solid selective media

Medium	Species	No. of isolates (%) recovered after 48 h at 30°C	No. of isolates recovered after additional cold enrichment for[a]:			
			7 days	14 days	21 days	28 days
AC	*L. monocytogenes*	23	12	4	5	4
	L. innocua	25	2	2	3	9
	L. seeligeri	8	0	1	1	0
	Total	56 (57)	14 (71)	7 (78)	9 (87)	13 (100)
McB	*L. monocytogenes*	11	15	10	2	10
	L. innocua	19	3	5	3	11
	L. seeligeri	5	0	4	0	1
	Total	35 (35)	18 (54)	19 (73)	5 (78)	22 (100)

[a] Numbers in parentheses indicate cumulative percentages.

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LITERATURE CITED

1. **Bortolussi, R., W. F. Schlech III, and W. L. Albritton.** 1985. *Listeria*, p. 205–208. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
2. **Breer, C.** 1986. Das Vorkommen von Listeria in Kaese, p. 230–233. Proceedings of the Second World Congress on Foodborne Infections and Intoxications, Berlin. Institute of Veterinary Medicine, Robert von Ostertagsinstitut Bundesgesundheitsamt, Berlin.
3. **Centers for Disease Control.** 1985. Listeriosis outbreak associated with Mexican-style cheese—California. Morbid. Mortal. Weekly Rep. **34**:357–359.
4. **Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reingold.** 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. **312**: 404–407.
5. **Hayes, P. S., J. C. Feeley, L. M. Graves, G. W. Ajello, and D. W. Fleming.** 1986. Isolation of *Listeria monocytogenes* from raw milk. Appl. Environ. Microbiol. **51**:438–440.
6. **Lee, W. H., and D. McClain.** 1986. Improved *Listeria monocytogenes* selective agar. Appl. Environ. Microbiol. **52**:1215–1217.
7. **Mavrothalassitis, P.** 1977. A method for the rapid isolation of *Listeria monocytogenes* from infected material. J. Appl. Bacteriol. **43**:47–52.
8. **McBride, M. E., and K. F. Girard.** 1960. A selective method for the isolation of *Listeria monocytogenes* from mixed bacterial populations. J. Lab. Clin. Med. **55**:153–157.
9. **Rocourt, J., A. Schrettenbrunner, and H. P. R. Seeliger.** 1983. Différenciation biochimique des groupes génomiques de *Listeria monocytogenes* (sensu lato). Ann. Microbiol. (Paris) **134A**:65–71.
10. **Rodriguez, L. D., G. S. Fernández, J. Fernández, F. Garayzabal, and E. R. Ferri.** 1984. New methodology for the isolation of *Listeria* microorganisms from heavily contaminated environments. Appl. Environ. Microbiol. **47**:1188–1190.
11. **Schlech, W. F., III, P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome.** 1983. Epidemic listeriosis—evidence for transmission by food. N. Engl. J. Med. **308**:203–206.
12. **Seeliger, H. P. R.** 1961. Listeriosis. S. Karger, Basel.
13. **Terplan, G., R. Schoen, W. Springmeyer, I. Degle, and H. Becker.** 1986. *Listeria monocytogenes* in Milch und Milchprodukten. Dt. Molkerei-Ztg. **41**:1358–1368.