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Comparison of Four Procedures to Detect *Listeria* spp. in Foods

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ABSTRACT

A comparison was made of four procedures to detect *Listeria* spp. in two food categories. The study comprised 309 assays, 71 on milk from both infected and uninfected cows, and 238 on ten types of fresh vegetables. A sample was considered positive if it could be detected by at least a single method and if isolates could be confirmed as *Listeria* spp. The procedures detected 98-100% of the positive milk samples. Recovery from vegetable samples ranged from 45 to 86%, probably because of low levels of *Listeria* spp. in the presence of mixed flora. The ELISA procedure of the Organon Teknika® corporation detected 68% of the 44 positive vegetable samples; the GENE-TRAK®DNA probe, 45%; the U.S. Food and Drug Administration (FDA) culture procedure, 75%; and the FDA probe procedure, 86%. Recovery was higher with LiCl-phenylethanol-moxalactam agar (FDA probe procedure) than with modified McBride Agar (FDA culture procedure).

Outbreaks of human listeriosis (*1,2,9*) have caused much concern to the food industry and health professionals alike. As a result, analytical procedures for detecting low levels of *Listeria* spp. in foods are currently being developed. The testing systems now available commercially are intended to be rapid, sensitive and specific.

Our study compared the enzyme-linked immunosorbent assay (ELISA) procedure of the Organon Teknika®Corporation, Durham, NC (*8*); the DNA probe procedure of GENE-TRAK®Systems, Framingham, MA (*4*); the Food and Drug Administration (FDA) culture procedure using modified McBride agar (FDA-MMA) (*6,7*); and the FDA *Listeria* DNA probe procedure using LiCl-phenylethanol-moxalactam (FDA-LPM) (Atin Datta, FDA, Washington, DC, personal communication). The foods used in the study were 71 cow's milk samples and 238 fresh vegetables, for a total of 309 samples.

MATERIALS AND METHODS

Vegetables

Ten types of the market vegetables were tested, including potatoes (70 tests), radishes (68 tests), mushrooms (16 tests), carrots, cabbage, broccoli, cauliflower, lettuce, tomatoes, and cucumbers (12 tests each). These vegetables were purchased at two local Minneapolis supermarkets.

Milk

Seventy-one milk samples were analyzed, including 59 samples from cows experimentally infected with *L. monocytogenes* and 12 samples from uninfected cows.

Inoculation of Cattle

Culture preparation. The Scott A strain of *L. monocytogenes* (serotype 4b), which was initially isolated from a patient during the 1979 Massachusetts outbreak (*2*) was obtained from Dr. Joseph Lovett (FDA, Cincinnati, OH) and was used throughout this study for inoculation of cattle. The strain was maintained on trypticase soy agar with 0.6% yeast extract (TSA-YE) and incubated microaerophilically (10% CO_2, 80% N_2, 10% O_2) at room temperature.

Dairy cattle and inoculation schedule. Holstein dairy cattle weighing 500-600 kg and approximately 3-7 years of age were used. The animals were part of a project to produce high titers of *Listeria*-infected milk and thus had received multiple intramammary route injections deep into the cisterna of the teat canal. The inoculation schedule, dose (CFU/dose) and numbers of quarters of the mammary gland inoculated are summarized in Table 1. The cows were monitored regularly for *L. monocytogenes*. Animals which served as uninfected controls were housed in barns removed from the *Listeria*-infected herd.

Enumeration of bacteria. Milk samples (100 µl) were plated in duplicate on brain heart infusion agar and McBride's agar, each containing 5% defibrinated bovine blood. Plates were incubated microaerophilically at 30°C for 2 d before bacteria were enumerated.

Testing procedures

For each of the four testing procedures, 25 g of sample was mixed in 225 ml of Listeria enrichment broth (LEB) (*6,7*) and incubated at 30°C. For the FDA-LPM procedure, the first 17 milk samples tested were not enriched but were plated directly.

FDA-MMA procedure

The FDA culture procedure of incubating enrichments for 7 d at 30°C and streaking on MMA at 24 h and 7 d has been previously been reported (*6,7*).

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TABLE 1. *Inoculation schedule and dose (log_{10} CFU/dose) of* L. monocytogenes *administered to lactating Holstein dairy cows via the intramammary route.*

Date	No. of cows inoculated	No. of quarters inoculated/cow	Total dose (CFU)/cow
Sept 29	7	1	10^7
Oct 6	3	3	10^7
14	1	3	10^7
	1	1	10^7
20	2	4	10^8
26	4	3	10^7
Nov 2	6	4	10^8
9	5	4	10^8
	1	4	10^7
16	4	4	10^8
23	4	4	10^8
30	3	4	10^8
Dec 7	3	4	10^8
15	3	4	10^2
	3	1	10^3
24	3	4	10^2
	3	1	10^3
Jan 5	1	4	10^2
	3	4	10^3
	3	1	10^4
12	4	4	10^3
	3	1	10^4

Organon Teknika procedure

The Listeria-Tek® system, an ELISA procedure that uses monoclonal antibody-coated microtiter wells, was supplied by Organon Teknika Corp. and has been previously described *(8)*.

Vegetables. A 1-ml portion of each of the 48-h LEB cultures was placed in flowing steam for 20 min (verbal instructions, Organon Teknika). Upon reaching room temperature, 100 µl of the steamed samples and controls (both positive and negative) was placed in designated wells and mixed with 100 µl of conjugate. The microelisa strips were then incubated at 37°C for 1 h. After incubation, the wells were emptied and washed 7 times with PBS. The substrate was placed in each well (100 µl/well) and held at room temperature for 30 min. Stop solution was added (100 µl/well) and the absorbance was recorded by the microelisa reader. Positive test samples were streaked on MMA and LPM agar *(5)*. Cultures were identified according the FDA procedure *(6,7)*.

Milk. The procedure for milk was the same as for vegetables, except that after the LEB was incubated for 24 h, 10-ml portions were transferred to 90 ml of tryptose phosphate-1% sodium pyruvate broth for further enrichment at 30°C for 24 h (as per manufacturer's instructions). Subsequently, 1-ml portions of these enrichment cultures were steamed and processed as in vegetable format.

GENE-TRAK procedure

In the GENE-TRAK procedure, all samples were processed as previously described *(4)*.

FDA hemolysin gene probe procedure for L. monocytogenes *(FDA-LPM)*

Enriched 24-h samples were streaked to LPM *(5)* agar plates. The first 17 milk samples tested were not enriched but were plated directly. The plates were incubated at 30°C for 48 h. After incubation, typical *Listeria* spp. colonies were picked and streaked to TSA-YE plates. The plates were incubated at 30°C for 24 h.

The TSA-YE plates were examined for typical *Listeria* spp. colonies. A colony was picked and tested for catalase activity, ß-hemolysin, gram stain, motility, and fermentation of 0.5% xylose and rhamnose *(6)*.

Confirmation of Listeria monocytogenes *by hemolysin gene probe*

Sterile individual Whatman No. 541 filters (82 cm) were placed on top of the colonies on the TSA-YE plates and pressed with a sterile glass rod (hockey stick). After 2 h, the filters were lifted from the agar plates, placed (colony side up) on top of wetted (with 2 ml 0.5N NaOH, 1.5N NaCl) Whatman No. 1 filter papers inside a 100 x 15 mm plastic petri dish *(3)*. The petri dish was placed inside a 600 watt microwave oven (Dual Wave II, General Electric, Louisville, KY) and radiated for 30 s to lyse the *Listeria* spp. cells (Atin Datta, personal communication). The filters were then transferred to fresh plastic petri dishes with Whatman No. 1 filters (wetted with 2 ml 1 M Tris (pH 7), 2 M NaCl) and allowed to set for 5 min. All filters were air dried [*(3)*; Atin Datta, personal communication].

Colony hybridization. Filters were hybridized by using a 50 ml hybridization mixture (28.9 ml distilled water; 15 ml 20x standard saline citrate (SSC), 5 ml 50x Denhardt's solution; 0.1 ml 0.5 M EDTA, pH 8.0; and 1 ml sonicated boiled calf thymus DNA) *(3)*. Individual filters were placed in 100 x 15 mm plastic petri dishes and 5 ml of the hybridization mix was added to each petri dish. One million counts of ^{32}P-labeled *L. monocytogenes* hemolysin gene probe was added (*3*; Atin Datta, personal communication). The plates were incubated at 37°C for 24 h. After incubation the hybridized filters were washed in 6x SSC for 5-10 s. They were placed in fresh plastic petri dishes and covered with 6 ml of fresh 6x SSC and incubated at 50°C for 1 h. This procedure was repeated once more for a total wash time of 2 h. The filters were then rinsed in 2x SSC for 5-10 s and air dried *(3)*.

Autoradiography. The filters were taped onto an 8 x 10-inch sheet of white paper and placed in a Kodak X-ray film cassette holder with intensifying screens. An 8 x 10-inch piece of stiff plastic was placed over the filters in the cassette *(3)*. In the darkroom the X-ray film was placed on top of the plastic. The entire cassette was put into a plastic bag, the end was taped and the bag was placed in a -70°C freezer for 24 h. The film was developed and observed for black spots in the area where *L. monocytogenes* colonies were present *(3)* (Fig. 1).

RESULTS AND DISCUSSION

A total of 309 samples (238 vegetable and 71 milk) were tested. Table 2 lists the number of samples that were positive by each procedure. Organon Teknika produced 22 false positives. We learned during the course of this evaluation that careful washing of the wells is essential to avoid cross contamination that could result in false-positive reactions. Although the instructions call for six washings, we had better results with seven washings. After incubation of the enrichment cultures, the manipulation time to obtain readings for Organon Teknika was approximately 2.5 h as compared with approximately 6 h for GENE-TRAK.

None of the four procedures detected all of the positive samples. A sample was scored as positive if it could be

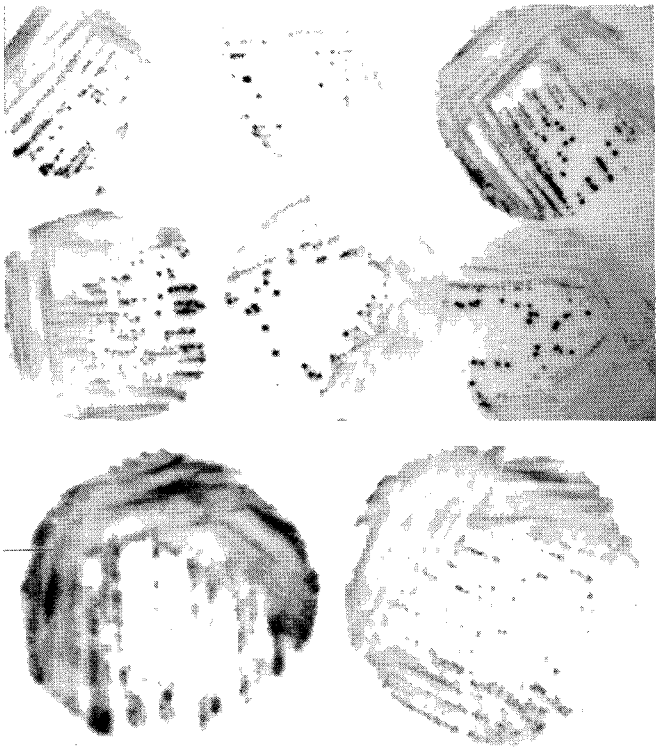

Figure 1. *Autoradiogram A with results of milk from cows purposely infected with* L. monocytogenes. *Autoradiogram B with results of* L. monocytogenes *on fresh market radishes.*

TABLE 2. *Number of samples found positive for* Listeria *spp. from 309 samples tested.*

Procedure	No. of positive samples	No. of false positive[a] samples	Actual No. of positive samples (%)
FDA-MMA	92	0	92 (30)
FDA-LPM	96	0	96 (31)
GENE-TRAK	78	0	78 (25)
Organon-Teknika	111	22[b]	89 (29)

[a]False positive = positive absorbance reading, but negative by all other methods. No *Listeria* spp. isolated.

[b]Five false-positive milk samples; 17 false-positive vegetable samples.

detected by at least one of the four procedures and an isolate could be confirmed as *Listeria* spp. A correlation matrix (Fig. 2) shows the number of times a specific procedure was positive when another procedure was negative and vice versa. A comparison of the FDA-MMA, FDA-LPM and Organon Teknika procedures with that of GENE-TRAK showed only one false-negative result (FDA-LPM). That sample was not enriched for the FDA-LPM procedure but instead was plated directly on LPM agar according to the FDA DNA probe direct plating method. GENE-TRAK had 14 more false negatives than did FDA-MMA and at least twice as many as either Organon Teknika or FDA-LPM (7 and 5, respectively). (Note: GENE-TRAK specifies that their assay is intended for dairy and environmental samples.) Twelve of the 14 samples were not detected as positive by the FDA-MMA procedure until after the LEB had incubated for

CORRELATION MATRIX

Positive

Negative	FDA MMA	Gene-Trak	FDA LPM	Org.-Tek.
FDA MMA		0	9	4
Gene-Trak	14		19	11
FDA LPM	5	1		4
Org.-Tek.	7	0	11	

Figure 2. *Correlation matrix. False negatives of each procedure are compared with FDA-MMA, the FDA culture procedure; the GENE-TRAK DNA probe; FDA-LPM, the FDA DNA probe; and Organon Teknika, the ELISA procedure.*

7 d. The *Listeria* spp. contamination apparently had not reached the concentration level necessary for detection by GENE-TRAK after its prescribed incubation procedure. FDA-MMA, GENE-TRAK, and Organon Teknika gave more false-negative results than did the FDA-LPM procedure, even though FDA-LPM had only a 24-h incubation time for the LEB. This result demonstrates the superior capability of the LPM agar to isolate *Listeria* spp., as previously reported *(5)*.

Of the 309 samples, 206 were negative by all four procedures and were considered to be free of *Listeria* spp. contamination. Fifty-nine of the positive samples (i.e., positive by at least one of the four procedures) were milk and 44 were vegetables (19 potatoes and 25 radishes). Table 3 shows the number of positive milk and vegetable samples detected by each procedure.

The ability of the procedures to detect low levels of *Listeria* spp. contamination in the presence of mixed flora may best be illustrated by the positive vegetable samples (Table 3). The low levels of *Listeria* spp. contamination in these samples was demonstrated by the presence of fewer colonies on the isolation agars, and by the fact that 12 of the

TABLE 3. *Number of milk and vegetable samples positive[a] for* Listeria *spp.*

Procedure	No. of positive milk samples (total 59 positive[a])	No. of positive vegetable samples (total 44 positive[a])
FDA-MMA	59 (100%)	33 (75%)
FDA-LPM	58 (98%)[b]	38 (86%)
GENE-TRAK	58 (98%)	20 (45%)
Organon Teknika	59 (100%)	30 (68%)

[a]Positive by at least one of the four procedures.

[b]One positive milk sample was not detected by the FDA-LPM procedure; however, this sample was tested without the 24-h enrichment step.

samples were not found positive by the FDA-MMA procedure until after the enrichment had incubated for 7 d. The FDA-MMA procedure was the only one of these procedures with a 7-d incubation period.

Even though the FDA-LPM procedure had only a 24-h incubation period, it isolated *Listeria* spp. more frequently than the FDA-MMA (Table 3). The only difference between the two procedures (to the point of isolation) was the type of isolation agar used (MMA vs. LPM) and the additional 7-d streak in the FDA-MMA procedure. This again indicates the superior selective quality of the LPM agar, as previously reported *(5)*. At the time of this study, GENE-TRAK used MMA agar only; however, both GENE-TRAK and Organon Teknika currently require streaking on LPM agar, but only if the sample is first detected positive by DNA probe (GENE-TRAK) or ELISA (Organon Teknika).

Adjustments of the incubation period should improve all of the procedures. At times, 24-48 h incubation had been too short and 7 d too long to obtain the detectable level of *Listeria* spp. The detectability probably depends on the initial *Listeria* spp. count and condition as well as the levels and types of background microorganisms present.

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