*Journal of Food Protection, Vol. 50, No. 3, Pages 188-192 (March 1987)*


# *Listeria monocytogenes* in Raw Milk: Detection, Incidence, and Pathogenicity

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(Received for publication August 22, 1986)

ABSTRACT

To determine the incidence of *Listeria monocytogenes* in raw milk, an isolation method was evaluated and used to analyze milk from three areas of the United States. The incidence varied by area from 0% in California to 7% in Massachusetts, with an overall incidence of 4.2%. The highest incidence found in any area during a single sampling period was 12% in Massachusetts in March 1985. During that same sampling, the incidence for all *Listeria* species was 26%. Of the 27 *L. monocytogenes* strains isolated during the survey, 25 were pathogenic in adult mice. One of three *Listeria ivanovii* isolated was pathogenic. No other isolates demonstrated pathogenicity.

Two recent outbreaks of listeriosis have focused attention on the potential health problem of milkborne *Listeria monocytogenes*. In a 1983 Massachusetts outbreak pasteurized milk was incriminated as the vehicle, although *L. monocytogenes* was isolated only from raw milk *(8)*. All patient isolates were serotype 4b, whereas a variety of serotypes were isolated from milk. In a California outbreak in 1985, a Mexican-style cheese was the vehicle *(12)*. *L. monocytogenes*, serotype 4, was isolated from patients, the manufacturing plant environment and the cheese. Mortality rates for both outbreaks were approximately 30%.

These incidents are not the first time milk has been reported as the bacterial source in listeriosis, although previous reports were sporadic and involved small numbers of people *(3, 5, 9, 13, 20)*. *L. monocytogenes* can cause mastitis in cows *(9)*, and a naturally infected cow was reported as shedding *L. monocytogenes* into milk at concentrations of 2,000 to 20,000 cells/ml *(13)*.

Some reports have indicated that *L. monocytogenes* can survive pasteurization *(1, 3, 5, 17)*. Recently reported thermal destruction kinetics for one of the more heat-resistant isolates from the Massachusetts outbreak supports the adequacy of the current pasteurization process guidelines of the Food and Drug Administration for destroying *L. monocytogenes* in whole milk *(2)*. This finding is at odds with others who report finding *L. monocytogenes* in pasteurized milk offered for public sale *(7)*. The organism has also been shown to survive to final product when added to milk used to make nonfat dry milk *(6)* and cottage cheese *(19)*.

The Massachusetts outbreak of listeriosis highlighted two urgent needs: *(a)* a need for an isolation method that was shorter than the cold enrichment procedure used during the investigation of that incident *(10)*, and *(b)* a need to know the incidence of *Listeria* in the raw milk supply. This report describes the laboratory trial of a shortened enrichment procedure for isolating *L. monocytogenes* from milk and its use in analyzing farm-bulk-tank milk from three widely separated areas of the United States for the incidence of *Listeria* species.

Since this work began, two limited surveys for *Listeria* have been reported. During the Massachusetts outbreak of 1983, Hayes et al. *(10)* examined milk from farms supplying the suspect dairy plant. Of the 121 raw milk samples analyzed using the cold enrichment method, 12% were found to contain *L. monocytogenes*. Dominguez-Rodriguez et al. *(4)* examined 95 samples of raw milk from west and central Spain and found that 45% contained *L. monocytogenes*. Other *Listeria* species found were *L. innocua* (15.8%), *L. welshimeri* (3.1%), and *L. seeligeri* (1%).

## MATERIALS AND METHODS

### *Bacterial cultures*

The *L. monocytogenes* cultures used in the laboratory trials of the isolation media were supplied by the Centers for Disease Control, Atlanta, GA. Two strains were patient isolates obtained during the 1983 Massachusetts outbreak. Two strains were isolated from raw milk during the investigation of the same outbreak. All other cultures were isolated from humans unrelated to any outbreak. The serotypes represented are 1 (not sub-typed), 1A and 4B.

### *Raw milk samples*

The raw milk used in the laboratory trials of the isolation media was obtained from the bulk tank of a local dairy. The raw milk analyzed for *Listeria* during the survey was obtained from farm bulk tanks in three areas of the United States. The California samples were supplied by the Bureau of Milk and Dairy Foods Control, Department of Food and Agriculture. Milk from the Massachusetts area (including Vermont) was sup-

plied by Agri-Mark, a marketing organization serving several hundred farms in the Northeast. Samples from the Tri-State area around Cincinnnati, OH, were supplied by Milk Marketing, Inc., serving several hundred farms in northern and central Kentucky, southwestern Ohio and southeastern Indiana. Cooperating organizations were requested to sample a farm only once. Shipments from California and Massachusetts were by air express in refrigerated cartons. All samples were maintained at 4°C during shipment and storage. Analysis was always begun within 1 week of sampling.

*Enrichment broth*

The enrichment broth (EB) used was Trypticase soy supplemented with 0.6% yeast extract, 15 mg of acriflavin HCl/L, 40 mg of naladixic acid/L, and 50 mg of cycloheximide/L. This formula is a modification of the enrichment broth described by Ralovich et al. *(18)*. The EB culture was incubated at 30°C, which was reported by Mavrothalassitis *(14)* to be optimum for *Listeria* enrichment from heavily contaminated samples.

*Isolation agar*

McBride agar was modified by removing blood and adding cycloheximide *(15)*. This modified McBride (MM) agar consisted of 35.5 g of phenylethanol agar/L supplemented with 10 g of glycine anhydride/L, 0.5 g of lithium chloride/L, and 200 mg of cycloheximide/L.

*Isolation procedure*

Twenty-five milliliters of milk, either inoculated or natural, was mixed with 225 ml of EB and incubated at 30°C for 48 h. At 24 and 48 h, EB culture was streaked directly onto MM agar. In addition, 1 ml of EB culture was added to 9 ml of 0.5% KOH, mixed briefly, and streaked onto MM agar. Plates were incubated for 48 h at 35°C before they were examined for typical colonies using 45° incident-transmitted light *(11)*. Uninoculated milk and EB were negative controls, and inoculated EB was a positive control.

Five colonies per plate were picked and confirmed by the following procedures. Isolates recovered during the laboratory trials were checked for morphology, motility, gram stain, catalase production, hemolytic activity on horse blood agar, and API 20S profile. Isolates from the survey were also tested for nitrate reduction, urease production, $H_2S$ production (TSI), MR-VP reaction, and ability to ferment with acid production dextrose, esculin, maltose, mannitol, rhamnose, and xylose. Isolates giving responses typical for *L. monocytogenes* were serotyped, and all *Listeria* sp. isolates were tested for mouse pathogenicity.

*Laboratory trials of isolation method*

*L. monocytogenes* strains were grown overnight at 30°C in Trypticase soy-yeast extract broth, and dilutions were made in 0.1% peptone water. Concentrations of $10^0$, $10^1$, and $10^2$ cells/ml were made for each strain used in each of three raw milk samples. The isolation procedure was previously described.

*Field survey for* Listeria *species incidence in raw milk*

Samples were taken from individual farms by the cooperating government or marketing organization. The samples were analyzed by the procedure previously outlined. In addition, the first 50 samples from the Tri-State area were analyzed in parallel by both the cold enrichment method *(10)* and our 30°C enrichment method.

*MPN procedure for enumerating* Listeria *in selected samples*

Ten milliliters of milk was added to 90 ml of EB, and 1, 0.1, and 0.01 ml of milk was added to 10 ml of EB. All cultures were prepared in triplicate and incubated at 30°C for 24 h. Cultures were then streaked onto MM agar before and after KOH treatment. Plates were incubated at 35°C for 48 h and examined for typical colonies. Isolates were confirmed as previously described. Most-probable-number values were derived as described in the second edition of the *Compendium of Methods for the Microbiological Examination of Foods (16)*.

*Serotyping*

All isolates identified as *L. monocytogenes* were transferred twice on Tryptose agar slants and incubated for 24 h at 35°C. The last transfer included two tubes; the bacterial growth in both was harvested after the last 24-h incubation period in a total of 3 ml of Difco FA buffer to a 16 × 125-mm screw-cap tube. This tube was heated at 80°C for 1 h, and the bacteria were pelleted by centrifugation. Approximately 2.2 ml of the buffer was removed, and the bacteria were resuspended in the remaining buffer and used in the slide agglutination reaction. The sera (Difco) consisted of types 1 and 4 only.

*Mouse pathogenicity*

All *Listeria* isolated were tested for pathogenicity in 16- to 18-g Swiss White mice. Isolates were grown for 24 h at 35°C in Trypticase soy-0.6% yeast extract. A culture was concentrated 10-fold by centrifugation and resuspended in 0.1% peptone diluent. Five mice per culture were inoculated i.p. with 0.1 ml containing $10^9$ cells. Mice were observed for 1 week, and deaths were recorded.

## RESULTS AND DISCUSSION

*Laboratory trials of the isolation method*

Three *L. monocytogenes* concentrations in raw milk were used to evaluate the recoverability of the method. Eight bacterial strains (Table 1) were added individually to three raw milk samples, providing 24 opportunities for recovery at each inoculum level. The percentages of recovery at each concentration level, culture treatment, and incubation time are shown in Table 1. Percent recovery was significantly greater ($P \leq 0.01$) at 24 h than at 48 h, with or without KOH treatment, at all inoculum levels except $10^0$ cells/ml. Dilution (1/10) in 0.5% KOH significantly increased the percent recovery ($P \leq 0.01$) of *Listeria* at 24 h but not at 48 h.

Since these data were generated, new evidence (G. Agello, P. Hayes, and J. Feeley. 1986. A comparison to two methods for isolating *Listeria monocytogenes* from cheese. Abstracts of the Annual Meeting, American Society for Microbiology, Washington, DC, p. 5) indicates that extending the incubation period to 7 d may allow better recovery of environmentally stressed *Listeria* from milk and milk products. We now recommend streaking EB culture onto MM agar after 1 and 7 d of incubation at 30°C.

At the level of $10^2$ cells/ml, *L. monocytogenes* was recovered more than 90% of the times attempted when EB culture was diluted in 0.5% KOH before streaking onto MM agar (after 24 h). At the lowest concentration, $10^0$

TABLE 1. *Percent recovery[a] for* L. monocytogenes *inoculted into milk at three concentration levels with varied culture time and treatment.*

| Inoculum level | 24-h incubation | | | 48-h incubation | | |
|---|---|---|---|---|---|---|
| (cells/ml)[b] | Without KOH | With KOH | Total | Without KOH | With KOH | Total |
| 212 | 83 | 96 | 90 | 75 | 79 | 77 |
| 21 | 67 | 79 | 73 | 58 | 63 | 60 |
| 2 | 33 | 58 | 46 | 33 | 42 | 38 |

[a]Number of isolations/number of isolation opportunities × 100.
[b]Average of 24 trials at each inoculum level.

TABLE 2. *Isolation of* Listeria *from raw milk in California.*

| *Listeria* species | No. positive samples/100 samples examined | |
|---|---|---|
| | April 1985 | Total |
| *L. monocytogenes* | 0 | 0 (0.0)[a] |
| *L. innocua* | 4 | 4 (4.0) |
| *L. ivanovii* | 1 | 1 (1.0) |
| *L. welshimeri* | 0 | 0 (0.0) |
| *L. seeligeri* | 0 | 0 (0.0) |
| Total | 5 | 5 (5.0) |

[a]Incidence expressed as percent of total samples examined.

TABLE 3. *Isolation of* Listeria *from raw milk in Massachusetts.*

| *Listeria* species | Number of positive samples[a] | | |
|---|---|---|---|
| | March 1985 | July 1985 | Total |
| *L. monocytogenes* | 12 | 2 | 14 (7.0)[b] |
| *L. innocua* | 17 | 2 | 19 (9.5) |
| *L. ivanovii* | 0 | 0 | 0 (0.0) |
| *L. welchimeri* | 0 | 0 | 0 (0.0) |
| *L. seeligeri* | 0 | 0 | 0 (0.0) |
| Total | 26[c] | 4 | 30[c] (15.0) |

[a]From 100 samples/period.
[b]Incidence expressed as percent of total samples taken.
[c]Figure adjusted to reflect multiple isolations.

cells/ml, the recovery rate was 58% when KOH treatment was used after a 24-h incubation of EB culture. One-third of the inoculated *Listeria* could be recovered under the worst performance conditions at the lowest concentration examined ($10^0$ cells/ml).

*The survey for* Listeria *incidence in raw milk*

To compare the chosen isolation method with the cold enrichment method already in use *(10)*, the first 50 farm samples taken in the Tri-State area (October, 1985) were analyzed in parallel by both methods. The only departure from the procedure of Hayes et al. *(10)* was that the primary and secondary enrichments were streaked onto modified McBride agar only. Our method detected *L. monocytogenes* in two samples and *L. innocua* in 4 of the 50 samples. No *Listeria* were detected by the cold enrichment method.

One-hundred samples from California were analyzed in April 1985. No *L. monocytogenes* was detected. *L. innocua* was found in four samples, and *L. ivanovii* in one (Table 2). We had planned to sample California milk more than once, but after the finding of Mexican-style cheese contaminated with *Listeria*, many laboratories began to sample that state's raw milk supply. Duplicating the work of others was not considered useful. While no organized report has been published by the many agencies involved, the informal reports support our findings.

Table 3 lists data from sampling in Massachusetts during March and July 1985. A total of 200 samples was analyzed: 100 during each period. Thirty of the total samples contained *Listeria* species, nearly half of which were *L. monocytogenes*. The highest incidence (26%) was noted during the March sampling. Twelve of the 26 isolates were *L. monocytogenes*. In July, only 4% were positive for *Listeria*, half of which were *L. monocytogenes*.

Three-hundred and fifty samples were analyzed from the Tri-State area: 50 were analyzed in October, 1984, and 100 each in February, May and August 1985 (Table 4). The greatest variety of species was found in Tri-State milk; five *Listeria* species were detected in February; the most common isolate was *L. innocua*. Unlike the Massachusetts data, the *L. monocytogenes* incidence varied little, remaining around 5%, although the total species incidence reached 25% in February.

A seasonal variation in incidence was present in both the Massachusetts and Tri-State data. Incidence was lowest in hot weather months, but peaked during cold weather months. The explanation for the seasonal variation may be related to feeding practices, herd management or some unknown factors affecting either the animal-bacteria relationship, the bacteria-environment relationship or both.

The incidence data for all *Listeria* species are summarized by sampling area (Table 5). Table 6 summarizes the *L. monocytogenes* isolations. Of the 27 isolates, 25 were pathogenic for mice. Most Tri-State isolates were serotype 4, whereas 12 of 14 Massachusetts isolates were serotype 1. The two *L. monocytogenes* isolates that failed to demonstrate pathogenicity in mice were both serotype 4.

The incidence rate found for *L. monocytogenes* in Massachusetts is similar to that found by Hayes et al. *(10)*

TABLE 4. *Isolation of* Listeria *from raw milk in the Tri-State area.*

| *Listeria* species | Number of positive samples[a] | | | | |
|---|---|---|---|---|---|
| | Oct. 1984 | Feb. 1985 | May 1985 | Aug. 1985 | Total |
| *L. monocytogenes* | 2 | 4 | 5 | 2 | 13 (3.7)[b] |
| *L. innocua* | 3 | 15 | 8 | 1 | 27 (7.7) |
| *L. ivanovii* | 0 | 2 | 0 | 0 | 2 (0.6) |
| *L. welshimeri* | 1 | 5 | 0 | 0 | 6 (1.5) |
| *L. seeligeri* | 0 | 1 | 0 | 0 | 1 (0.3) |
| Total | 6 | 25[c] | 13 | 3 | 47[c](13.1) |

[a]From 100 samples/period (except 50 in Oct. 1984).
[b]Incidence expressed as percent of total samples taken.
[c]Figure adjusted to reflect multiple isolations.

TABLE 5. *Isolation of* Listeria *from raw milk: incidence summary.*

| *Listeria* species | Incidence[a] | | | |
|---|---|---|---|---|
| | Tri-State | Massa-chusetts | Califor-nia | Total |
| *L. monocytogenes* | 3.7 | 7.0 | 0.0 | 4.2 |
| *L. innocua* | 7.7 | 9.5 | 4.0 | 7.7 |
| *L. ivanovii* | 0.6 | 0.0 | 1.0 | 0.5 |
| *L. welshimeri* | 1.5 | 0.0 | 0.0 | 0.9 |
| *L. seeligeri* | 0.3 | 0.0 | 0.0 | 0.1 |
| Total | 13.1[b] | 15.0[b] | 5.0 | 12.6[b] |

[a]Incidence expressed as percent of total samples (650) examined.
[b]Figure adjusted to reflect multiple isolations.

TABLE 6. *Isolation of* Listeria monocytogenes *from raw milk.*

| Sample source | No. of samples | No. of isolates | No. pathogenic | Serotype | | |
|---|---|---|---|---|---|---|
| | | | | 1 | 4 | NT[a] |
| California | 100 | 0 | 0 | 0 | 0 | 0 |
| Tri-State | 350 | 13 | 12 | 4 | 9 | 0 |
| Massachusetts | 200 | 14 | 13 | 12 | 1 | 1 |
| Total | 650 | 27 | 25 | 16 | 10 | 1 |

[a]NT-not typable.

in 1983. Of the 121 samples they examined, 12% were positive for *L. monocytogenes*. They did not look for other *Listeria* species. Domínguez-Rodriguez et al. *(4)* found a much higher incidence of *L. monocytogenes* (45%) in the milk from Spain. They recovered *L. monocytogenes* with much greater frequency than any of the other *Listeria* species except *L. grayi*. Domínguez-Rodriguez et al. *(4)* did not find *L. murrayi, L. dentrificans,* or *L. ivanovii*. Since our isolation procedure rejected those isolates using mannitol or reducing nitrate, we would not have detected *L. murrayi, L. grayi,* or *L. denitrificans*.

This study used the MPN procedure to quantify the *Listeria* concentration in four of the farm bulk tank samples previously determined to be positive. The procedure detected *L. monocytogenes* at concentrations of <1/ml (data not shown). Although we presently have no data that evaluate the detectability of the MPN procedure, our results indicate that when *Listeria* is present in bulk tank milk, concentrations are in the magnitude of 1 organism/ml or less. This finding further supports the adequacy of the present pasteurizatioin process that is based on the destruction of 12 log units of bacteria.

In summary, the enrichment procedure used here is sensitive to the level of $10^0$/ml for isolating *Listeria* from raw milk. By this method, *Listeria* species have been shown to be common in raw milk obtained from farm bulk tanks in the United States. All five of the established *Listeria* species were detected. For all areas, the overall incidence for the five species was 12.6%, and the *L. monocytogenes* incidence was 4.2%. The incidence of *Listeria* sp. varies seasonally, and the concentration in raw milk is low--probably <1 cell/ml.

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