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Isolation of *Listeria monocytogenes* from Raw Milk

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During a recent outbreak of listeriosis, we examined 121 raw milk samples and 14 milk socks (filters). *Listeria monocytogenes* was recovered from 15 (12%) of 121 milk specimens and 2 (14%) of 14 milk socks. The optimal processing method consisted of cold enriching diluted milk for 1 month with culture to selective broth, followed by plating.

In an outbreak of human listeriosis that occurred in Massachusetts and Connecticut during July and August 1983, pasteurized whole or 2% milk was implicated epidemiologically as the vehicle of transmission of *Listeria monocytogenes* (4). Although raw milk has previously been suggested as a vehicle of transmission in listeriosis (3, 5, 8, 11, 15), pasteurized milk has not. An inspection of the plant in which the implicated milk was processed uncovered no evidence of improper pasteurization. While investigating this outbreak, we obtained raw-milk samples from individual farms, the milk cooperative, and the pasteurizing plant to establish the source of the contamination and determine the prevalence of *L. monocytogenes* in the raw milk.

Because there were no established standard methods for the isolation of *L. monocytogenes* from raw milk, we processed the samples by several methods to increase our chances of recovering listeriae.

Milk samples were collected from three different sources. Forty samples (1 liter each) of raw milk were taken from the trucks that collect milk daily from the dairy farms and transport it to the milk cooperative. The capacity of each truck was 2,500 gal (ca. 9,462 liters). Seventy-two samples of raw milk were also collected from 6,000-gal (ca. 22,710-liter)-capacity trucks that transported the milk from the cooperative to the pasteurizing plant. In addition, nine samples of raw milk were collected from bulk tanks (2,000 to 4,000 gal [ca. 7,570 to 15,140 liters]) on four farms where bovine listeriosis had been recently diagnosed. Portions of 14 milk socks that were used at the pasteurizing plant to filter out large debris were also collected. Approximately 6,000 gal of milk had been filtered through each sock. All samples were shipped by air on ice and arrived cold in Atlanta within 24 h.

The milk was shaken thoroughly, and 0.1 ml of each sample was plated directly onto McBride *Listeria* agar (1) and gum base nalidixic acid (GBNA) medium (10). Then each milk sample was diluted 1:5. For the shipments from the cooperative, 50 ml of milk was added to 200 ml of nutrient broth no. 2 (Oxoid Ltd., Columbia, Md.). For the shipment from the pasteurizing plant, 50 ml of milk was added to 200 ml of the same broth made with 0.1 M MOPS (3-[*N*-morpholino]propanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.) buffer. We intended to hold the pH at 7.2 by using this buffer. Both the diluted and undiluted milk samples were held at 4°C (cold enrichment) and plated to the previously mentioned media after 1 week or 1 month. After cold enrichment (6) for 1 month, a sterile polyester fiber-tipped swab was dipped into each milk sample and inoculated into a tube of Stuart transport medium (Difco Laboratories, Detroit, Mich.), which was then held at 25°C for 1 week before being plated to the two media (9). In addition, after cold enrichment for 1 month, a 1:10 dilution of each milk sample was made in a selective broth consisting of nutrient broth no. 2 with nalidixic acid (100 μg/ml) and potassium thiocyanate (3.75%), which was incubated at 35°C overnight before being plated (14). The milk socks were placed in 200 ml of nutrient broth (without MOPS) and held at 4°C for a month. A swab of the broth was then inoculated into Stuart transport medium, and a 1:10 dilution of the broth was made into the selective broth. The pHs of all milk samples, diluted and undiluted, and of the milk socks were monitored weekly and adjusted to a range of 6.6 to 7.0 with 10 M potassium hydroxide.

All plates were incubated for 48 h at 35°C and then examined by the Henry method of oblique lighting (7). Suspect colonies were streaked to Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% sheep blood and incubated for 18 h at 35°C. Isolates were biochemically confirmed as *L. monocytogenes* and serotyped by the Reference Bacteriology Section, Centers for Disease Control.

To determine the sensitivity of this method, we artifically seeded raw milk with serially diluted concentrations (0.75 to 10^5 organisms per ml) of a recently isolated strain of *L. monocytogenes* type 4b. Each sample of artificially seeded milk was processed as described above.

We were able to detect *L. monocytogenes* in undiluted milk seeded with 0.75 organisms per ml on immediate direct plating. Likewise, for up to 5 weeks, when we halted the experiment, we recovered *L. monocytogenes* from diluted and undiluted milk seeded with 0.75 organisms per ml after primary cold enrichment by direct plating and after both secondary enrichment methods.

Of the 121 raw milk samples, 15 (12%) were positive for *L. monocytogenes*. All 15 isolates were from diluted milk; only 2 isolates were from undiluted milk ($P = 0.0001$; Table 1).

No isolates were made by direct plating of the undiluted milk before cold enrichment. Direct plating after 1 or 4 weeks of cold enrichment at 4°C yielded only one isolate (made from undiluted milk at 1 week; Table 1).

The isolation procedure that yielded the greatest number of positive milk samples (12) was dilution of the milk followed by primary enrichment at 4°C for 1 month, second-

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TABLE 1. Influence of dilution on the isolation of *L. monocytogenes* from 121 milk samples

Wk at 4°C	No. of specimens positive from milk	
	Undiluted	Diluted
0	0	ND[a]
1	1	0
4[b]	0	0
4[c]	2	15[d]

[a] ND, Not done.
[b] Primary enrichment only.
[c] Secondary enrichment in Stuart transport medium and in selective broth before plating.
[d] $P = 0.0001$, Sign test.

TABLE 2. Recovery of *L. monocytogenes* from 15 positive diluted-milk samples, with different enrichment procedures and isolation media

Procedure	No. of specimens positive with:		
	McBride agar	GBNA agar	Both media
Primary enrichment at 4°C	0	0	0
Secondary enrichment[a]			
Stuart transport medium only	3	6	7
Selective broth only	9	12	12
Both secondary enrichments	11	15	15

[a] Preceded by 1 month of primary enrichment at 4°C.

ary enrichment in the selective broth, and plating on GBNA agar (Table 2). There was no statistically significant difference in the number of positive milk samples found by using selective broth only (12 isolates) versus Stuart transport medium only (7 isolates) or two secondary enrichments (15 isolates) (Table 2). The use of Stuart transport medium and the selective broth versus cold enrichment gave significantly more isolates ($P = < 0.025$ and $P < 0.005$, respectively; McNemar test).

There was no statistically significant difference detected in the number of positive milk samples when two plating media were used. All 15 milk samples were positive on GBNA agar, while only 11 were positive on McBride *Listeria* agar (Table 2). Milk samples found positive on both plating media yielded essentially the same number of *L. monocytogenes* colonies.

We isolated a variety of serotypes, including 1a, 3b, 4b, and 4a,b. Although we attempted to determine statistically if any particular isolation technique selected a specific serotype of *L. monocytogenes*, the number of positive milk samples was too small to do so. The MOPS did not adequately buffer the milk, and we had to closely monitor and adjust the pHs of all the milk samples.

Of the 14 milk socks, 2 (14%) were found positive for *L. monocytogenes*, 1 by using Stuart transport medium and the other by using the selective broth.

There is very little information in the literature on the prevalence of *L. monocytogenes* in raw milk. Schultz (12) examined 1,004 raw-milk samples from individual cows and detected *L. monocytogenes* in only 10 samples. He estimated that the sensitivity of his method required the presence of 10^5 to 10^7 organisms per ml of milk before *L. monocytogenes* could be detected. It is highly unlikely that any milk sample in our study contained 10^5 to 10^7 *L. monocytogenes* cells per ml because of the large dilution resulting from pooling the raw milk. Although the sensitivity of our method for detecting artificially seeded listeriae in raw milk was extremely good, it has questionable predictability for naturally infected milk, since *L. monocytogenes* may well exist intracellularly in leukocytes and not be evenly distributed throughout the milk.

The purpose of diluting the milk was to impede spoilage and provide a bacteriologic medium supportive of the growth of *L. monocytogenes*. Another reason for diluting the milk was to reduce the lactose concentration and prevent a drop in pH, because it had been reported that growth of *L. monocytogenes* stops at pH 5.6 and that it is difficult to isolate *L. monocytogenes* from specimens with high lactic acid concentrations (2, 13, 15). Dilution appears to enhance the isolation of listeria. Fifteen isolates were made from diluted milk and only two from undiluted milk ($P = 0.0001$). It is not clear what the predominant factors are which cause the enhancement.

Our experience with secondary enrichment extends and supports the findings of Watkins and Sleath (14), who found that a two-stage enrichment procedure enhances the isolation of *L. monocytogenes* from sewage and sewage sludge. Secondary enrichments yielded 15 positive milk specimens versus only 1 when primary enrichment was used. The secondary enrichment methods differed in the time and temperature of incubation. What effect each of these variables had on the recovery of *L. monocytogenes* is unknown. We do not know what the recovery of *L. monocytogenes* would have been if we had used Stuart medium and the selective broth before cold enrichment or after 1, 2, or 3 weeks of cold enrichment. This procedure might have shortened the time required for isolation.

There was no statistical difference in the number of samples positive on McBride *Listeria* agar versus Martin GBNA medium. McBride *Listeria* agar inhibited molds better, and Martin GBNA medium enabled better detection of the *Listeria* colonies, which had a distinct blue color under oblique lighting. Unfortunately, neither medium is currently available commercially.

The recovery rate of listeriae from 14% of the milk socks is surprisingly similar to the 12% positivity rate of the raw-milk samples. However, processing milk socks does not appear to be an efficient way to screen for the presence of listeriae in large volumes of raw milk. Only one of the positive milk socks had been used to filter milk that was also found positive, while five positive milk samples were filtered through five milk socks that were found negative.

In conclusion, dilution of the milk 1:5 in nutrient broth and primary cold enrichment, followed by secondary enrichment in the selective broth, and plating on GBNA agar resulted in the best rate of recovery of listeria.

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