APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1987, p. 1433–1438
0099-2240/87/071433-06$02.00/0

Vol. 53, No. 7

Survival of *Listeria monocytogenes* in Milk during High-Temperature, Short-Time Pasteurization

MICHAEL P. DOYLE,[1]* KATHLEEN A. GLASS,[1] JOHN T. BEERY,[1] GARY A. GARCIA,[2] D. JEFFREY POLLARD,[2] AND RONALD D. SCHULTZ[2]

Food Research Institute,[1] and Department of Pathobiological Sciences, School of Veterinary Medicine,[2] University of Wisconsin–Madison, Madison, Wisconsin 53706

Received 8 December 1986/Accepted 31 March 1987

Milk from cows inoculated with *Listeria monocytogenes* was pooled for 2 to 4 days and then heated at 71.7 to 73.9°C for 16.4 s or at 76.4 to 77.8°C for 15.4 s in a high-temperature, short-time plate heat exchanger pasteurization unit. *L. monocytogenes* was isolated from milk after heat treatment in six of nine pasteurization trials done at 71.7 to 73.9°C and in none of three trials done at 76.4 to 77.8°C. An average of 1.5 to 9.2 *L. monocytogenes* cells was seen in each milk polymorphonuclear leukocyte before heat treatment in 11 of 12 pasteurization trials. Noticeable degradation of leukocytes with intracellular listeriae was detected in unpasteurized milk after 3 days of storage at 4°C, and by 4 days of storage leukocytes had deteriorated to cellular debris, suggesting that holding unpasteurized milk refrigerated for 4 or more days would eliminate a protective effect leukocytes may provide for increasing heat resistance of *L. monocytogenes*. Results indicate that under the conditions of this study, *L. monocytogenes* can survive the minimum high-temperature, short-time treatment (71.7°C, 15 s) required by the U.S. Food and Drug Administration for pasteurizing milk.

A recent outbreak of listeriosis in Massachusetts was associated by epidemiologic studies with drinking a specific brand of pasteurized whole or 2% milk (9). Listeriosis was detected in dairy cows of some of the herds that produced the implicated milk. Inspection of the dairy plant that processed the milk revealed no evidence of faulty pasteurization. Hence, questions were raised about the possibility of *Listeria monocytogenes* surviving pasteurization.

Studies done to evaluate the thermal resistance of *L. monocytogenes* have produced conflicting results (1, 3, 6). The most comprehensive study on heat resistance of *L. monocytogenes* was recently reported by Bradshaw et al. (3), who used raw whole milk inoculated with laboratory-cultured *L. monocytogenes* and heated in sealed glass tubes to determine *D*-values for thermal inactivation of the organism. Their studies revealed that the *D*-value at 71.7°C for the most heat-resistant strain evaluated was 0.9 s, indicating that 15 $\log_{10}$ of *L. monocytogenes* per milliliter of raw milk would be killed if heated at 71.7°C for 15 s, which is the minimum heat treatment milk must be given for high-temperature, short-time pasteurization according to the process guidelines of the U.S. Food and Drug Administration (FDA) (10).

L. monocytogenes is an intracellular parasite and can be present within leukocytes in *L. monocytogenes*-contaminated milk. Some investigators suggest that an intracellular state may confer heat resistance to the organism and allow some listeriae within leukocytes to survive pasteurization (9). Earlier studies on the thermal resistance of *L. monocytogenes* in milk failed to test this hypothesis. Thus, it was the objective of this study to determine whether intracellular *L. monocytogenes* in milk from *L. monocytogenes*-inoculated cows could survive high-temperature, short-time pasteurization treatment in a small-scale plate heat exchange pasteurization unit.

* Corresponding author.

MATERIALS AND METHODS

Inoculation of cows and production of *L. monocytogenes*-contaminated milk. Four Holstein cows, ranging in age from 3 to 6 years old and each producing 22.7 to 27.2 kg of milk per day, were selected for this study on the basis of their low serum agglutination titers (<1:20) to *L. monocytogenes* Scott A (serotype 4b). This organism was originally isolated from a blood culture of a patient involved in an outbreak of listeriosis linked to drinking pasteurized milk (9). It also was the strain used to inoculate the animals to provide *L. monocytogenes*-contaminated milk. The cows were housed for the duration of the study in an isolation facility maintained by the School of Veterinary Medicine, University of Wisconsin–Madison.

Each time the animals were inoculated, the bacterial inoculum was freshly prepared by growing *L. monocytogenes* Scott A in tryptose broth (Difco Laboratories, Detroit, Mich.) at 37°C for 24 h and washing the culture three times in phosphate-buffered saline (PBS; 0.01 M sodium phosphate [pH 7.2], 0.15 M NaCl), using centrifugation (8,000 × *g*, 12 min, 4°C) to harvest the cells. Cells were spectrophotometrically (A_{500}) adjusted to the appropriate concentration with PBS and were enumerated to confirm cell numbers by plating serial (1:10) dilutions onto tryptose agar plates that were then incubated at 37°C for 48 h.

Because of the absence of reports on the way in which to establish a listeria infection in experimentally infected animals that would result in continued excretion of listeriae in milk, each of the four cows was inoculated at different sites with different numbers of bacteria. Surgery was performed on one animal in which 2×10^{11} listeriae were inoculated into the mesenteric lymph nodes and Peyer's patches of the small intestine, and 2×10^{11} were inoculated into the tonsils. This animal developed a fever of 42.8°C and bloody diarrhea and died 3 days postinoculation. A second animal was inoculated with 10^{11} listeriae in the left front quarter of the udder via the teat canal and also with 10^7 listeriae in the supramammary

lymph node. A third animal was inoculated with 2×10^9 listeriae in the tonsil, and a fourth animal was inoculated with a similar dose in the tonsil and also fed 5×10^{11} listeriae into the rumen via a stomach tube. The latter three animals survived but developed fevers between 40 and 41.6°C and had reduced feed intake and milk production for weeks to days postinoculation. Three weeks after initial inoculations, the animals were each inoculated with 1×10^5 to 2×10^5 listeriae into the left front quarter of the udder via the teat canal. Five days later, milk was collected for the first pasteurization studies. Two weeks later and thereafter at approximately weekly intervals for 4 weeks, each animal was inoculated with 10^5 to 10^7 listeriae via the teat canal into a different quarter of the udder from the previous inoculation.

Milk samples from each quarter of each animal were enumerated twice weekly for *L. monocytogenes* by plating serial (1:10) dilutions onto McBride *Listeria* agar (MLA) and incubating the agar plates by procedures described below.

Cows were milked twice daily, and the milk was collected in 10-gallon (37.9-liter) cans that were brought (usually within 1 to 2 h) to the Food Research Institute for storage at 4°C. Depending on the amount of milk produced by the cows, milk was stored over a 2- to 4-day period (usually 2 days; rarely 3 or 4 days) for pasteurization studies.

Analysis of milk. Before each pasteurization study, approximately 30 gallons (113.6 liters) of milk from the most recent milkings was pooled in a stainless-steel holding tank and agitated for 5 min. A 1-liter sample of milk was then taken for the following analyses.

(i) Somatic cell count. Direct microscopic somatic cell count was determined by the standard method (11, 13). For each sample, circular 1-cm^2 films were prepared and stained, and the cells in a horizontal strip of each film were counted.

(ii) Fat. Milk fat was determined by the Babcock fat test described in *Standard Methods for the Examination of Dairy Products* (5).

(iii) Standard plate count. The standard plate count was determined by the standard method (15).

(iv) Total solids. Total milk solids were determined by the atmospheric oven procedure described in *Standard Methods for the Examination of Dairy Products* (5). Samples were first partially dried on a steam bath and then heated in an oven at 100°C for 3 h.

(v) pH. The pH was determined with a pH meter (model 140; Corning Scientific Instruments, Medfield, Mass.) with a Corning combination electrode.

***L. monocytogenes*. (i) Plate counts.** *L. monocytogenes* was enumerated by plating milk serially (1:10) diluted in PBS onto MLA with 5% defibrinated sheep blood. MLA plates were incubated in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C for 48 h. At least five colonies typical of those formed by *L. monocytogenes* (bluish gray, translucent, slightly raised, 0.5 to 1.5 mm in diameter, and weakly β-hemolytic) per plate with the highest dilution of countable colonies were selected for confirmation. Confirmatory tests were the same as those described previously (8), except serology was done with *Listeria* O type 4 antiserum (Difco) only.

(ii) Counts after disrupting leukocytes. Two 30-ml portions of milk in 50-ml centrifuge tubes in ice-water were treated to free intracellular *L. monocytogenes* from leukocytes. One portion was sonicated at 200 to 250 W with a Braunsonic 1510 sonicator (B. Braun Instruments, South San Francisco, Calif.) by using four 30-s bursts with a 30-s delay between each burst. The sonicator probe was 1 cm in diameter. The other portion was homogenized for 2 min with a Polytron tissue homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.) at a speed setting of 5 with a 1-cm probe. Each portion of milk was serially diluted and plated onto MLA by procedures described above to enumerate *L. monocytogenes*.

(iii) Microscopic counts within PMNL. Milk (500 ml) was added to 1 liter of PBS and centrifuged ($3,300 \times g$, 30 min). The supernatant fluid was removed, and the cell pellet was gently resuspended in 250 ml of PBS. The cells were again sedimented by centrifugation, the supernatant fluid was removed, and 75 ml of fixative (paraformaldehyde-periodate-lysine [12]) was added and allowed to react with the cell pellet for 18 h at 4°C. The center of the pellet was removed and embedded by the cold glycol methacrylate (JB-4; Polysciences, Warrington, Pa.) method (4). The block of cell pellet was cut to a depth of 60% to obtain at least 12 2-μm sections.

Two adjacent sections from each depth of about 10, 20, 30, 40, 50, and 60% of the block were treated as a matched set. One section was mounted on a slide and stained with azure A (14). This section was used to determine the ratio of polymorphonuclear leukocyes (PMNL) to somatic cells. The complementary section was mounted on a slide and reacted with immunoperoxidase-conjugated antibody by the procedure described by Beery et al. (2) to determine the number of *L. monocytogenes* cells within PMNL. *Listeria* O antiserum type 4 (Difco) was used as the antibody in this procedure for detecting intracellular *L. monocytogenes*. Slides were examined microscopically and cells (PMNL, somatic cells, and *L. monocytogenes* cells within PMNL) were counted in a vertical strip at the center of each specifically stained section. The average of six counts per cell pellet was used to calculate the number of *L. monocytogenes* within PMNL per milliliter of milk, using the following equation: number of *L. monocytogenes* within PMNL/milliliter of milk = (average number of *L. monocytogenes* in PMNL/microscopic field) × [(average number of PMNL per microscopic field)/(average number of somatic cells per microscopic field)] × total number of somatic cells/milliliter of milk).

After heat treatment, milk (100-ml portions) was sampled from the pasteurizer at approximately 2-min intervals. A total of three milk samples for each trial was assayed for surviving *L. monocytogenes* by five procedures. These included two direct plating procedures in which 0.1-ml portions of each sample were surface plated onto 10 plates each of tryptose agar and MLA. The plates were incubated in a microaerobic environment (5% O_2, 10% CO_2, 85% N_2) at 37°C for 48 h. Each sample was also assayed by three enrichment procedures including: (i) cold enrichment, (ii) the FDA enrichment procedure (J. Lovett, D. W. Francis, and J. M. Hunt, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, P17, p. 253; J. Lovett, personal communication), and (iii) the selective enrichment procedure of Doyle and Schoeni (7).

For cold enrichment, 25 ml of milk was mixed with 100 ml of tryptose broth, which was then held at 4°C and tested at 1, 2, 3, 4, 6, and 8 weeks of incubation by plating at each sampling time 0.1 ml of enrichment culture on each of duplicate plates of MLA. For the FDA procedure, 25 ml of milk was added to 225 ml of enrichment broth (composed of [per liter]: 30 g of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), 6 g of yeast extract, 15 mg of acriflavin hydrochloride, 40 g of nalidixic acid, and 50 mg of cycloheximide) and held at 30°C. At 24 and 48 h of incubation, enrichment cultures were both surface plated (0.1 ml)

directly onto duplicate plates of MLA and alkali treated (1 ml into 9 ml of 0.5% KOH) and then surface plated onto duplicate plates of MLA. For the selective enrichment procedure, 25 ml of milk was added to 100 ml of selective enrichment broth (7) and incubated with agitation (100 gyrations per min) at 37°C under microaerobic conditions. At 1, 2, 3, 5, and 7 days of incubation, 0.1-ml portions of enrichment culture were surface plated in duplicate onto MLA plates. MLA plates were incubated as described above, and colonies typical of those formed by *L. monocytogenes* were selected for confirmation. Isolates were confirmed as *L. monocytogenes* by procedures described above.

Pasteurization trials. A total of nine pasteurization trials were done in the temperature range of 71.7 to 73.9°C, and three trials were done at 76.4 to 77.8°C. None of the milk was processed through a clarifier or homogenizer.

Raw milk (ca. 113.6 liter) for each trial was added to a 150-liter stainless-steel holding tank in a refrigerated room separate from the room with the pasteurization unit. After mixing with an agitator (50 rpm) for 5 min, milk was pumped through 3.8-cm (inner diameter) braid-reinforced vinyl tubing (FDA grade) into the balance tank of the pasteurization unit. All components of the pasteurization unit were new and used for the first time in this study. Milk was pumped through the unit with a positive displacement timing pump (Pasilac A/S, Silkeborg, Denmark) which was located between the raw regenerator section and the heater section of the pasteurizer. The timing pump was calibrated and sealed by Wisconsin Department of Agriculture Division of Food and Standards inspectors such that milk heated at 71.7 to 73.9°C and 76.4 to 77.8°C was held in the holding tube for 16.4 and 15.4 s, respectively. The pasteurizer (model 217SB-1; Cherry-Burrell, Cedar Rapids, Iowa) was a plate heat exchanger consisting of 8 Thermaflex plates (each 0.17 m^2) in the regeneration section, 11 plates in the heating section, and 8 plates in the cooling section. Cold, running tap water was used in the cooling section.

The holding tube was 5.0 m long by 3.6 cm (inner diameter) stainless steel and was designed according to specifications of the Grade A Pasteurized Milk Ordinance (10). An indicating thermometer and recorder controller bulb, both calibrated for accuracy, were located at the end of the holding tube. A recorder controller (Taylor Instrument Co., Rochester, N.Y.) recorded the temperature of milk leaving the holding tube. Two Pasilac flow diversion valves were interconnected between the end of the hold tube and the raw milk section of the pasteurizer. The response time of the flow diversion valves was 1.2 s, but there was no delay between movement of the two valves. The flow diversion valves were never activated during any of the pasteurization trials.

After leaving the cooling section of the pasteurizer, milk flowed through Tygon tubing (3.8-cm inner diameter) to a Plexiglas hood with an activated UV light. The hood was located over a stainless-steel sink with a drain. Milk samples were taken directly from the tubing about 30 to 60 cm from the end. Samples were obtained by inserting a 13-gauge needle connected to a sterile 60-ml syringe into the tubing and withdrawing the sample as the milk flowed through. Blood agar exposure plates were placed in the hood near the sampling area to detect airborne contaminants; no microbial contaminants were detected on any exposure plates after incubation at 37°C for 48 h. The temperature of the milk when sampled ranged from 24 to 34°C.

After each pasteurization trial, equipment was cleaned and sanitized by a rigorous scheme which included: (i) flushing the unit with water at 82.2°C for 10 min; (ii) circulating 1.1 kg of Monarch BW-90 caustic detergent (H. B. Fuller Co., Minneapolis, Minn.) per 100 liters of water at 82.2°C for 25 to 30 min; (iii) rinsing with water for 10 min; (iv) circulating 1.8 liters of Monarch MP-2 acid (phosphoric acid; H. B. Fuller Co.) per 100 liters of water at 82.2°C for 25 to 30 min; and (v) flushing the unit for 10 min with water. At the start of each trial, the unit was sanitized by: (i) flushing the unit with water at 82.2°C for 10 min; (ii) circulating 45 g of Antibac B (sodium dichloro-*O*-triazinetrione; Diversy-Wyandotte, Wyandotte, Mich.) per 75 liters of water at 82.2°C for 10 min; and (iii) flushing the unit for 10 min with water. Immediately after the pasteurization unit was sanitized, the Tygon tubing at the end of the pasteurizer was soaked in 10% bleach for a minimum of 10 min.

Storage effects on PMNL with intracellular *L. monocytogenes*. Milk (ca. 25 liters) from the morning milking of three cows was stored in a 38-liter stainless-steel milk can at 4°C and sampled at 0, 1, 2, 3, and 4 days to determine the effect of refrigerated storage on the integrity of *L. monocytogenes*-infected PMNL. Before each sampling the milk was stirred for about 5 min with a sterile stainless-steel mixing bar. Each sample of milk (500 ml) was processed and examined microscopically by the same procedures described above for determining microscopic counts of *L. monocytogenes* within PMNL. Differences in the number of intracellular listeriae were statistically determined by Student's *t* test (16).

RESULTS

Properties of milk before heating. The somatic cell counts, standard plate counts, numbers of *L. monocytogenes* determined by plating and microscopic counting procedures, fat and total solids contents, and pH values of milk from *L. monocytogenes*-infected cows before heat treatment are shown in Table 1. Somatic cell counts ranged from 4.5×10^5 to 2.4×10^6 cells per ml. Standard plate counts ranged from 5.1×10^5 to 3.8×10^7 CFU/ml, with milk of all 12 trials exceeding the 1.0×10^5 CFU/ml standard for grade A raw milk from individual producers (10).

L. monocytogenes was detected in only 4 of the 12 milk samples by directly plating dilutions of the milk onto MLA, and it was detected in two additional samples when the milk was sonicated or homogenized before plating onto MLA. The numbers of *L. monocytogenes* determined by the direct plating procedure ranged from 3.0×10^2 to 1.9×10^4 CFU/ml. Generally about two- to fivefold more *L. monocytogenes* cells were detected in samples that were sonicated or homogenized before plating. Overall, slightly higher counts were obtained from sonicated than from homogenized samples (data not shown). Because of the large number of background colonies developing on MLA from milk of trials 1 through 4, it was not possible to detect and confirm individual colonies of *L. monocytogenes* at $\leq 10^4$ CFU/ml. Hence, we were not able to determine an exact *L. monocytogenes* plate count for these samples.

Milk samples from individual quarters of infected cows were enumerated for *L. monocytogenes* at six different times during the course of the heating trials. The numbers of *L. monocytogenes* ranged from <10 to 10^5 CFU/ml, with counts generally between 10^2 and 10^4 CFU/ml in most quarter samples (data not shown).

The presence of *L. monocytogenes* within PMNL was confirmed microscopically (Fig. 1), with numbers of intracellular listeriae determined by a direct microscopic count-

TABLE 1. Properties of milk from *L. monocytogenes*-infected cows before heat treatment

Heat treatment trial no.	Somatic cell count (cells/ml)	Standard plate count (CFU/ml)	*L. monocytogenes* plate count (CFU/ml)		Direct microscopic count of *L. monocytogenes* within PMNL			Fat (%)	Total solids (%)	pH
			Directly plated	Sonicated or homogenized before plating	No. of *L. monocytogenes* in PMNL/ml	Avg no. of *L. monocytogenes* in PMNL	Maximum no. of *L. monocytogenes*/ PMNL			
1	1.9×10^6	7.4×10^6	$<10^4$	$<10^4$	2.1×10^4	4.7	19	3.40	11.6	6.6
2	1.5×10^6	1.1×10^7	$<10^4$	$<10^4$	2.0×10^4	2.8	10	3.20	11.7	6.6
3	1.3×10^6	2.0×10^7	$<10^4$	$<10^4$	2.8×10^4	3.3	14	3.20	11.2	6.5
4	2.3×10^6	9.4×10^6	$<10^4$	$<10^4$	4.8×10^4	6.3	14	ND[a]	11.4	6.5
5	1.8×10^6	5.0×10^5	5.0×10^3	1.2×10^4	8.0×10^3	4.5	11	3.45	ND	6.6
6	1.3×10^6	1.5×10^6	1.9×10^4	3.6×10^4	4.8×10^4	6.5	19	3.70	11.8	6.5
7	5.8×10^5	2.5×10^7	$<10^2$	2.0×10^3	2.1×10^4	9.2	26	3.25	10.8	6.7
8	8.1×10^5	6.9×10^6	$<10^2$	1.0×10^2	2.6×10^4	7.3	18	3.40	11.7	ND
9	4.5×10^5	2.7×10^6	$<10^2$	$<10^2$	1.4×10^3	2.7	10	3.53	11.4	6.7
10	2.4×10^6	5.5×10^6	$<10^2$	5.0×10^2	$<1.2 \times 10^2$	0	0	3.48	12.0	6.6
11	1.1×10^6	1.7×10^7	5.0×10^2	1.0×10^3	1.6×10^3	1.5	5	3.30	11.0	6.5
12	5.4×10^5	3.8×10^7	3.0×10^2	1.8×10^3	4.4×10^3	2.7	7	3.35	10.7	6.2

[a] ND, Not determined.

ing procedure ranging from $<1.2 \times 10^2$ to 4.8×10^4 cells per ml of milk. The organism was detected in PMNL in milk samples from 11 of the 12 pasteurization trials. The average number of *L. monocytogenes* within a PMNL ranged from 0 to 9.2, with a maximum of 26 listeriae within a PMNL.

The fat content of the milk samples was typical of milk from Holstein cows, ranging from 3.2 to 3.7%. The total solids content was relatively consistent, ranging from 10.5 to 12.0%, as were the pH values, which ranged from pH 6.5 to 6.7 in 10 of 11 trials.

Isolation of *L. monocytogenes* from milk after heat treatment. The temperature and time of each pasteurization treatment and the procedures by which *L. monocytogenes* was isolated from milk after pasteurization are shown in Table 2. *L. monocytogenes* was isolated from milk in six of the nine trials in which the heat treatment was 71.7 to 73.9°C, but the organism was not detected in any of the milk heated at 76.4 to 77.8°C. Interestingly, in only one instance (trial 1, sample 2) was the organism isolated from the same milk sample by all three enrichment procedures. All other isolations from a single sample were made by only one enrichment procedure. Overall, *L. monocytogenes* was isolated from four samples by cold enrichment, from four samples by a selective enrichment procedure (7), and from two samples by the FDA procedure. No single enrichment procedure was better than another or entirely reliable for detecting surviving listeriae. However, since this study was done, the FDA procedure has been modified to replace the 48-h sampling time with a 7-day sampling time. This change may result in higher isolation rates of thermally stressed *L. monocytogenes* than was observed in this study.

No surviving listeriae were detected by either direct plating procedure, and there was little background growth of bacteria on these plates to confound the isolation of *L. monocytogenes* colonies.

Effect of storage on PMNL with intracellular *L. monocytogenes*. Milk from *L. monocytogenes*-infected cows was held at 4°C to determine whether listeriae grew within PMNL during refrigerated storage. During the first 2 days of cold storage there was no significant difference in the number of intracellular *L. monocytogenes* (Table 3). However, at day 3 the organism was no longer detected within PMNL, and by day 4 the PMNL had decomposed to cellular debris, probably as a result of autolytic enzyme activity. The death and subsequent degeneration of PMNL (predominantly at day 3) started with nuclear pyknosis and shrinkage of the cytoplasm followed by disruption of the cellular membrane and karyolysis of pyknotic nuclei. Once initiated, the degradation of PMNL occurred within hours.

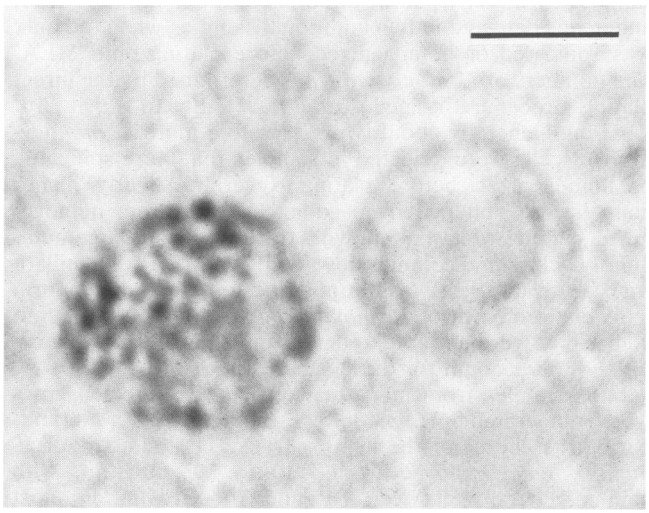

FIG. 1. Cells of *L. monocytogenes* (dark areas in microphotograph) within PMNL in milk from *L. monocytogenes*-infected cows (peroxidase stain, not counterstained; glycol methacrylate section, 2 μM). Bar = 5 μM.

DISCUSSION

L. monocytogenes within PMNL of milk from *L. monocytogenes*-infected cows survived a minimum heat treatment of 72.2°F for 16.4 s in a high-temperature, short-time pasteurizer. These results differ from those of a study by Bradshaw et al. (3) from which the conclusion was made that heating raw milk at 71.7°C for 15 s should be adequate to destroy *L. monocytogenes* in whole milk. There were major differences in the experimental approaches used in these two studies which may explain the disparate results. Two principal differences were (i) the methods used to detect surviv-

TABLE 2. Isolation of *L. monocytogenes* from milk after heat treatment

Trial no.	Pasteurization temp and time	Sample no.	Isolation procedure(s)[a] detecting surviving *L. monocytogenes*
1	162–164.5°F	1	FDA, 48 h, direct + KOH
	72.2–73.6°C	2	FDA, 48 h, direct + KOH
	16.4 s	2	SEP, 5 day
			Cold, 8 week
2	161–163.8°F	1	LmNI[b]
	71.7–73.2°C	2	SEP, 1 day
	16.4 s	3	SEP, 3 day
3	162–164.2°C	1	LmNI
	72.2–73.4°C	2	LmNI
	16.4 s	3	SEP, 3 day
4	162–164°F	1	LmNI
	72.2–73.3°C	2	Cold, 4 week
	16.4 s	3	LmNI
5	161.5–164.2°F	1	LmNI
	71.9–73.4°C	2	LmNI
	16.4 s	3	Cold, 3 and 4 week
6	161.8–163.8°F	1	LmNI
	72.1–73.2°C	2	LmNI
	16.4 s	3	LmNI
7	161–164°F	1	LmNI
	71.7–73.3°C	2	LmNI
	16.4 s	3	LmNI
8	163–165°F	1	LmNI
	72.8–73.9°C	2	LmNI
	16.4 s	3	LmNI
9	169.8–172°F	1	LmNI
	76.6–77.8°C	2	LmNI
	15.4 s	3	LmNI
10	169.8–171.8°F	1	LmNI
	76.6–77.7°C	2	LmNI
	15.4 s	3	LmNI
11	161.8–163.6°F	1	Cold, 6 week
	72.1–73.1°C	2	LmNI
	16.4 s	3	LmNI
12	169.5–171.5°F	1	LmNI
	76.4–77.5°C	2	LmNI
	15.4 s	3	LmNI

[a] FDA, FDA enrichment procedure; SEP, selective enrichment procedure of Doyle and Schoeni (7); Cold, cold enrichment procedure.
[b] LmNI, *L. monocytogenes* not isolated.

ing listeriae and (ii) the intraleukocytic position of *L. monocytogenes* in the milk.

In our study, the heat-treated milk was assayed for *L. monocytogenes* by five different procedures, including two direct plating methods and three enrichment procedures. The direct plating procedures are restrictive in that only a small amount (0.1 ml) of milk can be applied to the surface of each agar plate. In contrast, 25 ml of milk was added to the enrichment broth for each enrichment procedure. Hence, substantially more sample was assayed by enrichment. Furthermore, enrichment procedures may be more conductive to resuscitating injured *L. monocytogenes* cells than direct plating methods. Bradshaw et al. (3) used a direct plating method for detecting heat-treated listeriae, and this method may have been less sensitive than enrichment procedures for recovering heat-injured cells. We did not detect, by either of two direct plating methods, *L. monocytogenes* on any agar plates to which heat-treated milk was directly applied.

The presence of *L. monocytogenes* within PMNL may also be an important factor affecting the survival of the organism in milk heated at 72.2°C for 16.4 s. The organism is a facultative intracellular parasite that may exist within PMNL in milk from *L. monocytogenes*-infected cows (Fig. 1). Indeed, in 11 of 12 trials *L. monocytogenes* at levels of 1.4×10^3 to 4.8×10^4 CFU/ml was observed within PMNL of milk used in our study. In contrast, the listeriae used in the study of Bradshaw et al. (3) were grown in broth culture, diluted in phosphate-buffered water, and added to raw milk to yield approximately 10^5 *L. monocytogenes* cells per ml. Under these conditions, few, if any, *Listeria* cells would be protected from pasteurization by the intraleukocytic position of the organism (9), although the mechanism for such a protective effect has not been identified. Perhaps listeriae growing within PMNL are in a physiologically different state from either listeriae freely suspended in milk or listeriae that are simply phagocytized by macrophages but are not growing within them. It is well documented that bacteria in the stationary phase of growth are generally more heat resistant than bacteria in the logarithmic phase of growth, which are in a different physiologic state than stationary-phase cells. Similarly, because of physiologic differences, listeriae growing within PMNL in milk may be more heat resistant than listeriae that are simply engulfed by phagocytes or are freely suspended in milk.

If the intracellular nature of *L. monocytogenes* in PMNL in milk is indeed a factor in the thermal resistance of the organism, then the degradation of PMNL and release of listeriae into milk after holding milk for 3 to 4 days at refrigeration temperature should increase the sensitivity of the bacterium to heat. Although not necessarily practical, holding milk for several days before processing may be a means of obtaining *L. monocytogenes*-free milk with a heat treatment of 71.7°C for 15 s or less.

Pasteurization studies by Donker-Voet (6) used milk from a cow naturally infected with *L. monocytogenes*. However, to obtain enough milk to run through the pasteurizer, the milk had to be accumulated and stored in an icebox at 4°C for a week. Holding milk under those conditions likely resulted in degradation of PMNL and release of intracellular listeriae into the milk, thereby resulting in increased thermal sensitivity of the organism. This may at least partially explain why listeriae did not survive a heat treatment of 66.4°C or higher for 15 s in her studies.

TABLE 3. Change in number of *L. monocytogenes* within PMNL of unpasteurized milk during storage at 4°C

Day	No. of *L. monocytogenes* in PMNL/ml	No. of *L. monocytogenes*/PMNL	
		Avg[a]	Maximum
0	1.8×10^4	4.2 ± 2.2^b	7
1	4.4×10^4	9.2 ± 6.6^b	20
2	2.7×10^4	8.7 ± 9.4^b	24
3	<1.8	0	0
4	<1[c]	0	0

[a] Mean ± standard deviation.
[b] Means with the same superscript are not significantly different ($P > 0.05$).
[c] PMNL decomposed to cellular debris.

The numbers of *L. monocytogenes* cultured from milk used in the present study were similar to those in milk of the "carrier" dairy cow studied by Donker-Voet (6). The naturally infected animal shed *L. monocytogenes* in almost pure culture at between 1×10^3 and 2×10^4 listeriae per ml of milk from all four quarters. Interestingly, although the animal had a mastitic condition as indicated by abnormally high numbers of leukocytes (between 1.4×10^6 and 5.0×10^6 leukocytes per ml) in the milk, the milk appeared completely normal (6).

Similarly, the milk used in our study appeared normal in visual and olfactory aspects. However, the standard plate count of milk of all our pasteurization trials was above the standard for grade A raw milk. This was probably a reflection of inefficient cooling of milk after removal from cows. Although the milk was put into cleaned and sanitized 37.9-liter milk cans immediately after the cows were milked, there were no special cooling facilities at the isolation facilities where the infected cows were housed. Hence, the milk was at times held for 1 to 2 h without refrigeration after milking and in transport from the isolation facility to a walk-in refrigerator (4°C) where the milk was stored before each pasteurization trial.

Although *L. monocytogenes* was isolated from milk heated at 72.2°C for 16.4 s, the organism was not detected in the few trials of milk heated at 76.4 to 77.8°C for 15.4 s. This provides some indication that the latter heat treatment may be sufficient to eliminate viable listeriae from milk. Additional studies are needed to determine what intermediate heat treatments can be used to destroy listeriae in PMNL in milk.

ACKNOWLEDGMENTS

We thank Ralston B. Read, Jr., Roger W. Dickerson, Jr. (FDA), Everett Johnson (Wisconsin Department of Agriculture), and Michael Ely (Wisconsin Department of Agriculture) for their efforts in inspecting and standardizing the instrumentation of the pasteurizer. We also thank N. R. Gandhi (Auro Tech, Inc.) for assistance with the pasteurizer.

This work was supported by the National Cheese Institute, College of Agricultural and Life Sciences, University of Wisconsin–Madison, and by contributions to the Food Research Institute.

LITERATURE CITED

1. **Bearns, R. E., and K. F. Girard.** 1958. The effect of pasteurization on *Listeria monocytogenes*. Can. J. Microbiol. **4**:55–61.
2. **Beery, J. T., M. P. Doyle, and J. L. Schoeni.** 1985. Colonization of chicken ceca by *Escherichia coli* associated with hemorrhagic colitis. Appl. Environ. Microbiol. **49**:310–315.
3. **Bradshaw, J. G., J. T. Peeler, J. J. Corwin, J. M. Hunt, J. T. Tierney, E. P. Larkin, and R. M. Twedt.** 1985. Thermal resistance of *Listeria monocytogenes* in milk. J. Food Prot. **48**:743–745.
4. **Brinn, N. T., and J. P. Pickett.** 1979. Glycol methacrylate for routine, special stains, histochemistry, enzyme histochemistry and immunochemistry: a simplified cold method for surgical biopsy tissue. J. Histotechnol. **2**:125–130.
5. **Case, R. A., R. L. Bradley, Jr., and R. R. Williams.** 1985. Chemical and physical methods, p. 327–404. *In* G. H. Richardson (ed.), Standard methods for the examination of dairy products, 15th ed. American Public Health Association, Washington, D.C.
6. **Donker-Voet, J.** 1963. My view on the epidemiology of *Listeria* infections, p. 133–139. *In* M. L. Gray (ed.), Second symposium on listeric infection. Montana State College, Bozeman.
7. **Doyle, M. P., and J. L. Schoeni.** 1986. Selective-enrichment procedure for isolation of *Listeria monocytogenes* from fecal and biologic specimens. Appl. Environ. Microbiol. **51**:1127–1129.
8. **Doyle, M. P., and J. L. Schoeni.** 1987. Comparison of procedures for isolation of *Listeria monocytogenes* in soft, surface-ripened cheese. J. Food Prot. **50**:4–6.
9. **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.
10. **Food and Drug Administration.** 1978. Grade A pasteurized milk ordinance. 1978 recommendations. Public Health Service publication 229 (Rev. 1983). U.S. Government Printing Office, Washington, D.C.
11. **Food and Drug Administration.** 1980. Milk laboratory evaluation form. Form FDA 2400d (Rev. 9/80). U.S. Government Printing Office, Washington, D.C.
12. **Isobe, Y., S. T. Chen, P. K. Nakane, and W. R. Brown.** 1977. Studies on translocation of immunoglobulins across intestinal epithelium. I. Improvements in the peroxidase-labeled antibody method for application to study of human intestinal mucosa. Acta Histochem. Cytochem. **10**:161–173.
13. **Kaufman, O. W.** 1985. Direct microscopic somatic cell count, p. 49–54. *In* Laboratory examination of dairy products. FDA course manual. Food and Drug Administration, Cincinnati, Ohio.
14. **Lillie, R. D., and H. M. Fullmer.** 1976. Histopathologic technic and practical histochemistry, 4th ed., p. 635–636. McGraw-Hill Book Co., New York.
15. **Messer, J. W., H. M. Behney, and L. O. Leudecke.** 1985. Microbiological count mehods, p. 133–150. *In* G. H. Richardson (ed.), Standard methods for the examination of diary products, 15th ed. American Public Health Association, Washington, D.C.
16. **Ryan, T. J., Jr., B. L. Joiner, and B. F. Ryan.** 1976. Minitab student handbook. Duxbury Press, North Seituate, Mass.