Comparison of Heat Resistance of *Listeria monocytogenes* in Milk as Determined by Two Methods

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**ABSTRACT**

The thermal resistance of 3 strains of *Listeria monocytogenes* was compared using test tube versus sealed tube methods of thermal inactivation. All *L. monocytogenes* strains were rapidly inactivated in milk when survival was measured using sealed tube thermal inactivation methods. Calculated D_{62°C} values ranged between 0.1-0.4 min for the three strains tested. In contrast, total inactivation of *L. monocytogenes* populations using test tube methods of thermal inactivation could not be accomplished within 30 min at 62°C. Extensive tailing of survivor curves was consistently observed. When an initial population of $5 \times 10^6$ *L. monocytogenes*/ml was heated at 72, 82, or 92°C, consistent survival of a population of $10^2$-$10^3$ *L. monocytogenes*/ml after 30 min was observed. The results prove that the test tube method for measuring thermal resistance of *L. monocytogenes* is inaccurate. Reports of extraordinary heat resistance based upon this method are correspondingly inaccurate. *L. monocytogenes* cells, dispersed freely in milk, will not survive pasteurization.

*L. monocytogenes* is a gram-positive, non-sporeforming, aerobic to facultatively anaerobic, rod-shaped bacterium that is pathogenic to man and animals. Between June and August 1983, 49 patients in Massachusetts were hospitalized with septicemia or meningitis caused by *L. monocytogenes*. Fourteen deaths resulted from this outbreak (5). Investigations conducted by the Centers for Disease Control (CDC) determined that onset of illness was associated with consumption of whole or 2% pasteurized milk. At the factory where the incriminated milk was processed, neither evidence of improper pasteurization nor a source of contamination after pasteurization was identified. Thus the mechanisms of entry or survival of *L. monocytogenes* in the incriminated milk remain unclear.

A central question emerging from this outbreak concerns the ability or inability of *L. monocytogenes* to survive pasteurization. Data concerning the heat resistance of *L. monocytogenes* are conflicting (1,2,8). Part of the conflict arises from lack of documentation of methods used to measure thermal resistance. Beams and Girard (1), using the holding technique of pasteurization (61.7°C, 35 min), determined that survival occurred when population levels exceeded $5 \times 10^3$ *L. monocytogenes*/ml. Bradshaw et al. (2) using sealed borosilicate glass tubes, determined that *L. monocytogenes* was unable to survive pasteurization. This study was designed to compare the heat resistance of *L. monocytogenes* in milk using test tube versus sealed tube methods of thermal inactivation.

**MATERIALS AND METHODS**

*Bacterial cultures*

Strains of *L. monocytogenes* used in this study consisted of serotypes most frequently isolated from raw milk (5). These strains were *L. monocytogenes* ATCC 19115 (serotype 4b), obtained from the American Type Culture Collection, Rockville, MD; and F5027 (serotype 1a) and F5069 (serotype 4b), both raw milk isolates, which were obtained from Dr. Robert Weaver, CDC, Atlanta, GA. All cultures were maintained at 4°C on tryptose-phosphate agar (TPA, Difco Laboratories, Detroit, MI) slants.

*Heating menstrua*

Sterile whole milk or 11% non-fat milk solids (NFMS) were used as heating menstrua in this study. Whole (3.5% fat) milk was obtained from the dairy factory at the University of Vermont, and autoclaved at 121°C for 12 min to insure sterilization. Eleven percent NFMS (ICN Nutritional Biochemicals, Cleveland, OH) were rehydrated in distilled H2O and sterilized at 121°C for 12 min. Following sterilization, milk was immediately cooled in an ice water bath to minimize caramelization. Milk was stored at room temperature in the absence of light before use.

*Thermal resistance studies*

Cells evaluated for thermal resistance were propagated in tryptose-phosphate broth (TPB) or sterile whole milk for 12 h at 37°C before use. At this phase, cells were present at population levels ranging between $5.0 \times 10^8-1.5 \times 10^9$ colony forming units (CFU)/ml. Thermal resistance was measured using two different methods. For the test tube method of inactivation, the method of Beams and Girard (1) was used with the following modifications. A 0.1-ml portion of culture grown in TPB or whole milk was inoculated into tubes containing 10 ml of sterile 11% NFMS or whole milk which had been heated to 62°C in
a constantly circulating 3:1 water/ethylene glycol bath. Upon inoculation, initial populations of *L. monocytogenes* were between $10^6$-$10^7$ per ml. A constant temperature immersion circulator (Haake Model D8, Fisher Scientific Co., Medford, MA) was used to establish and maintain a 62°C temperature to within +/− 0.5°C. This temperature was constantly monitored by means of a metal probe connected to a digital readout thermometer in a control tube containing 10 ml of sterile whole milk or 11% NFMS. The surface of the milk in tubes was kept 4 cm below the water level in the bath. Duplicate tubes were used at each heating interval. Tubes were removed from the water bath at fixed intervals and immediately placed in an ice water bath. Two replicate trials were conducted for each strain.

For the sealed tube method of inactivation, we modified the procedure of Bradshaw et al. (2). Cells were either propagated in TPB and added to sterile whole milk, or grown directly in sterile whole milk. A 1.5-ml portion of whole milk containing approximately $10^6$-$10^7$ *L. monocytogenes* per ml was transferred to 2-ml glass reaction vials (Hewlett-Packard, Mt. View, CA). Vials were crimp-sealed with metal caps containing teflon lined seals. Triplicate vials were used at each heating interval. Times to reach reaction temperatures were established as described above using a control vial containing 1.5 ml of sterile whole milk. Vials were removed from the water bath at fixed intervals and immediately placed in an ice water bath. Two replicate trials were conducted for each strain.

**Enumeration of bacteria**

Viable counts of surviving cell populations were determined in duplicate for each vial or test tube at each heating interval according to methods described in *Standard Methods for the Examination of Dairy Products* (3). The medium used to enumerate bacteria was TPA to which 0.05% esculin and 0.05% ferric citrate (TPA-FE) were added. Addition of esculin and ferric citrate to TPA aided in visualization of colonies produced by surviving cells on plates. These components were added to tempered TPA immediately before plating. TPA-FE plates were incubated for 48 h at 37°C before enumeration.

**Biochemical confirmation**

Surviving cell populations were confirmed as *L. monocytogenes* biochemically, by gram stain, and by motility at 22°C. For biochemical confirmation, we developed a procedure which utilized the Minitek (BBL Microbiology Systems, Cockeysville, MD) minituarized biochemical identification system. Single colony isolates from surviving cell populations on TPA-FE were propagated in TPB at 37°C for 24 h. A 4.5-ml portion of culture was added to sterile polypropylene culture tubes and centrifuged for 5 min at 3000 × g using a Savant Hi Speed centrifuge equipped with an HSC rotor (Savant Instruments, Farmingdale, NY). Cells were washed once in sterile phosphate buffered saline (PBS) solution, re centrifuged, and the resulting pellet was resuspended in *Listeria* identification medium to an optical density of 0.35 at 650 nm on a spectrophotometer (Bausch and Lomb Spectronic 20). *Listeria* identification medium consisted of 20 g of tryptone (Difco), 5 g of NaCl and 2.5 g of disodium phosphate per liter. Resuspended cells were transferred in 0.05-ml portions to microtiter plates containing the following test discs: arabinose, citrate, dextrose, esculin, galactose, lactose, malto se, mannitol, melibiose, rhamnose, salicin, sucrose, trehalose, urea and xylose. Plates were incubated anaerobically at 37°C for 48 h using the BBL Gas-Pak system and CO₂-H₂ generator envelopes. Results were compared with typical reactions for *L. monocytogenes* according to Bergey's *Manual for Determinative Bacteriology* (9) (Table 1). Cells were gram stained by the Hucker method (4), while motility was confirmed by inoculating motility test medium (Difco) and incubating tubes at 22°C for 48 h.

**Determination of D-values**

Rates for thermal inactivation for *L. monocytogenes* were determined graphically by plotting the log₁₀ cfu/ml of surviving cell populations versus heating times as described previously (2). A best fit line was drawn through the data points. For results using the sealed tube method of inactivation, an estimate of the D-value was obtained from the slope of the best-fit line. Because of the non-linearity of results obtained using the test tube procedure, D-values were not calculated. All curves represent the average of two replicate thermal inactivation trials.

**RESULTS**

Initial trials using $10^6$-$10^7$ CFU/ml of *L. monocytogenes* F5069, F5027 or 19115 grown in TPB and heated in 11% NFMS at 62°C (143°F) using the test tube method indicated that complete inactivation could not be accomplished within 30 min at 62°C. Figure 1 typifies results observed in these trials. Beginning with a population of $10^6$-$10^7$ *L. monocytogenes/ml, populations declined rapidly during the first 5 min of inactivation, after which time a stable population of approximately $10^4$ *L. monocytogenes/ml persisted. Additional inactivation trials were conducted using cells grown and heated in sterile whole milk. As in previous

<table>
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*a/n/a = Results not available.  
+ = Ferments carbohydrate within 48 h at 37°C.  
− = Does not ferment carbohydrate within 48 h at 37°C.  
+/− = Partial fermentation of carbohydrate within 48 h at 37°C.  
d = Reactions differ.*
trials, surviving cell populations were observed long beyond schedules established for milk pasteurization (data not shown). Colonies from surviving cells recovered following inactivation by the test tube method were biochemically confirmed as *L. monocytogenes*, thus eliminating the possibility that survivors could be due to non-*Listeria* contaminants.

Results obtained using the sealed tube method of thermal inactivation were different from those obtained using the test tube method. Thermal inactivation profiles were linear throughout the course of inactivation (Fig. 2). $D_{62°C}$ values obtained for different serotypes of *L. monocytogenes* ranged from 0.1 to 0.4 min. Duplicate vials for each inactivation trial were prepared for the 30-min heating interval. Half of the vials were plated immediately following treatment; the remaining vials were stored at 4°C for 1 month, then plated on TPA-FE. No surviving cell populations were ever found through this procedure.

Inactivation trials using the test tube procedure were conducted with *L. monocytogenes* at 72, 82 and 92°C. Figure 3 depicts results obtained for *L. monocytogenes* F5069. Even though the temperatures of inactivation are different, thermal resistance profiles exhibit similar patterns. Using the test tube method of inactivation, survivor curves constructed for *L. monocytogenes* F5069 heated at 72, 82, and 92°C all showed a consistent 3-4 log cycle drop in cells, followed by a 1-2 log cycle increase, then a further decrease to a stable population of approximately $10^{2}-10^{3}$ surviving cells. Similar results were obtained for *L. monocytogenes* F5027 (data not shown). Using the sealed tube method of inactivation at these same temperatures, inactivation was so rapid that no surviving cell populations could ever be measured.

**DISCUSSION**

In this report, we have compared two methods for thermal inactivation of *L. monocytogenes*. It is apparent from the data that survival at pasteurization temperatures depends on the method used to inactivate cells and is not a biological phenomenon. Using the sealed tube method of inactivation, *L. monocytogenes* was easily inactivated at pasteurization temperatures. However, when identical cell populations were heated using the test tube method of inactivation, survival was consistently observed, regardless of whether a heating temperature of 62, 72, 82 or 92°C was employed.
The tailing observed for populations of *L. monocytogenes* using the test tube method of inactivation could be explained in two ways. As the first explanation, condensate and splashed cells could collect in the cap of the test tube above the level of the water bath, and drip back into the heating menstrua. Tubes would have various levels of survivors depending on the amount of condensate in the cap. Eventually, an essentially constant, low number of survivors would be present. Since the cap is above the level of the water bath and not exposed to thermal inactivation temperatures, this would explain why elevation of the inactivation temperature from 72 to 92°C had no influence on survivor curves when the test tube method of inactivation was employed.

As an alternate explanation, upon initial mixing of *Listeria* with their resuspension menstrua, cells could coat the walls of the test tubes. The only cell populations exposed to the inactivation temperatures would be those populations which were below the level of water in the water bath. Cells coating the walls of the test tube above the level of the water bath would never be exposed to the temperature of inactivation, and since a constant, finite amount of surface area for this coating is available, a stable, constant, low number of survivors could be enumerated following inactivation. This latter explanation seems the most likely.

Beams and Girard (1) studied the thermal resistance of six strains of *L. monocytogenes*. All cultures were grown on tryptose agar slants at 37°C for 18-24 h before thermal inactivation. Growth from the agar slants was diluted in sterile distilled water to yield suspensions containing $5 \times 10^5$ *L. monocytogenes* per ml. Serial dilutions of this suspension were then transferred to sterile skim milk. Initial populations of *L. monocytogenes* in studies by Beams and Girard ranged from $5 \times 10^1$-$5 \times 10^7$/ml. Survivors after heating at 61.7°C for 35 min were only observed when populations exceeded $5 \times 10^2$ organisms per ml. In the procedure for thermal inactivation used by Beams and Girard, 20 × 150 mm test tubes were placed in a water bath, keeping the milk surface 3-4 cm below the water level. Tubes were then connected to a shaker to insure distribution of the bacteria in the milk samples. As with each of the situations which we have described above, cells coating the glass walls of test tubes would not be exposed to the 61.7°C temperature of the water bath, resulting in bacterial survival after 35 min of heating. Although the procedure for the test tube method of inactivation used in our studies was modified slightly from that used by Beams and Girard, we believe the survival we have observed during extended heating of populations of *L. monocytogenes* by the test tube method is for similar reasons.

Provisions have been made in the Pasteurized Milk Ordinance (PMO) (6) to eliminate the potential for problems which we have observed in these studies from occurring in practice. Although high-temperature short-time (HTST) pasteurization is widely used in the dairy industry, many small manufacturing facilities continue to rely on vat pasteurization to eliminate pathogenic organisms from milk. During the filling of milk vats before pasteurization, splashing of milk on vat surfaces above the level of milk may occur. During vat pasteurization, simply heating the milk vat without consideration of these splashed particles may result in survival of pathogenic organisms associated with these particles. To eliminate the potential for such problems during batch pasteurization, it is imperative that airspace heaters be used to insure adequate heating of every particle of milk present in a milk vat. This provision is part of the PMO. Failure to use airspace heaters, or improper operation of such devices, could result in the survival of pathogenic organisms such as *L. monocytogenes*.

Tierney et al. (10) have reported errors when the heat resistance of viruses is determined by test tube methodology. For the same reasons discussed by these authors, we have found the test tube method to be an inaccurate procedure for determining the heat resistance of *L. monocytogenes*. Work by Bradshaw et al. (2), using sealed tube methodology for determining the heat resistance of *L. monocytogenes*, has shown that this organism is unable to survive pasteurization. Our heat inactivation data for *L. monocytogenes* using the sealed tube method is consistent with the findings of Bradshaw et al. These observations are particularly significant considering recent assertions that *L. monocytogenes* can survive pasteurization. Fleming et al. (5) and Ho et al. (7) have published articles citing the work of Beams and Girard as potential evidence that *L. monocytogenes* is capable of surviving pasteurization. The conclusions of Beams and Girard are most likely the result of the methodology employed in their studies and not due to the intrinsic heat resistance of *L. monocytogenes*. Authors citing the work of Beams and Girard (1) as evidence that *L. monocytogenes* possesses extraordinary heat resistance should do so with caution.

**ACKNOWLEDGMENTS**

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**REFERENCES**

REFERENCES


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