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Heat Inactivation of *Mycobacterium paratuberculosis* in Raw Milk: Are Current Pasteurization Conditions Effective?

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Currently, it is not known whether commercial pasteurization effectively kills *Mycobacterium paratuberculosis* in contaminated raw milk. Results from holder test tube experiments indicated that a residual population of viable bacteria remained after treatment at 65, 72, 74, or 76°C for 0 to 30 min. Use of a laboratory-scale pasteurizer unit demonstrated that treatment of raw milk at 72°C for 15 s effectively killed all *M. paratuberculosis*.

Recent evidence suggests that the etiological agent of Crohn's disease in humans, a severe inflammatory enteritis involving the terminal ileum, may be mycobacterial and could be *Mycobacterium paratuberculosis* (2, 4, 8). Current concerns regarding a possible relationship between Crohn's disease and *M. paratuberculosis* have been stimulated by the recent finding by researchers in the United Kingdom that *M. paratuberculosis* DNA could be detected in pasteurized milk samples purchased from retail markets (10). There is no definitive evidence to date that viable *M. paratuberculosis* is present in retail pasteurized dairy products. The present studies were conducted to determine the optimal time and temperature conditions for effective killing of *M. paratuberculosis* in experimentally inoculated raw milk. We also compared two methods of heat inactivation, the holder test tube method, which has commonly been used by researchers in the past to determine thermal death curves for *M. paratuberculosis* and other bacteria (3, 5, 9), and the lab-scale pasteurizer method, which simulates the high-temperature, short-time (HTST) conditions (72°C, 15 s) of an industrial pasteurizer unit.

The following two strains of *M. paratuberculosis* were utilized in the pasteurization studies: strain 19698 (American Type Culture Collection, Rockville, Md.) and strain Ben (American Type Culture Collection). Strain 19698 is a laboratory strain of *M. paratuberculosis* originally isolated from ileal tissue of a cow with clinical Johne's disease but has been passaged innumerable times. Strain Ben was isolated from human intestinal biopsy tissue from a patient with Crohn's disease. The bacteria were grown in Middlebrook 7H9 medium supplemented with 2 mg of mycobactin J (Allied Monitor, Fayette, Mo.) per liter until they reached a concentration of 10^8 to 10^9 cells per ml (A_{540} = 1.15). The bacterial suspensions were centrifuged (10,000 × *g*, 15 min) and washed twice in phosphate-buffered saline (PBS) (pH 7.4). The pellets were resuspended to a concentration of 10^9 CFU/ml in PBS, and aliquots were placed in sterile, snap-cap tubes (12 by 75 mm) and frozen at −80°C until used in experiments. Prior to use in experiments, bacterial suspensions were thawed at room temperature and sonicated (35 W, 15 s) to disperse clumps.

Milk from an on-site herd of healthy Holstein cows was used to conduct the experiments in this study. Fecal samples from the cows were cultured twice within a 6-month period for *M. paratuberculosis* before initiation of pasteurization experiments and once again during the 1-year experimental period to ensure that they were free of *M. paratuberculosis*. Milk was obtained from individual weigh jars at the time of milking, transported to the laboratory, and stored at 4°C overnight.

Five milliliters of raw milk obtained from *M. paratuberculosis*-free cows was dispensed into sterile, polystyrene, snap-cap tubes (13 by 100 mm; Falcon, Becton-Dickinson, Lincoln Park, N.J.) for experiments in which the holder test tube method was used. Tubes were placed in a shaking water bath (Bellco, Vineland, N.J.) set at the desired temperature (65, 72, 74, or 76°C) with the water level several inches above the meniscus of the milk in the tubes. One tube, serving as a temperature control, contained a thermometer submerged in the milk; the thermometer was inserted through a modified air-tight cap. When the milk reached the desired temperature, experimental tubes were inoculated with 10^8 CFU of *M. paratuberculosis* per ml (500 μl of a 10^9-CFU/ml stock preparation in 5-ml of milk). Aliquots (200 μl) were removed from tubes at each time point (0, 0.25, 0.50, 1, 5, 15, and 30 min) and held on ice. Aliquots were either plated directly or serially diluted (1:10 dilutions from 10^8 to 10^1), sonicated (35 W, 15 s), and plated (100 μl/slant) onto Herrold's egg yolk medium (HEYM) (National Animal Disease Center, Ames, Iowa). The bacteria were sonicated to disperse clumps that may have formed during processing, which resulted in more accurate quantitation of CFU of bacteria on agar slants. A control standard curve for serial dilutions of the bacterial stock preparations was prepared simultaneously to monitor the viability of the cultures. Results were determined after 4, 8, and 12 weeks of incubation at 37°C. Two experiments were conducted with each strain of bacteria tested for each temperature variable.

The laboratory-scale pasteurizer unit utilized in the next set of experiments (Armfield, London, England) is an accurate miniature version of industrial pasteurizers used for the HTST heating process. Briefly, this unit consists of a heater, a cooler, and a regenerator. Raw milk is placed in a feed tank and is then pumped to the regenerator for heat exchange. It then passes into the heating section (holding tube), where it is brought up to the pasteurizing temperature. The holding tube is designed to allow heating of the fluid at the set temperature for 15 s. Variation of the holding time is not possible with this pasteurizer unit. Any product not at the required temperature after it passes through the holding tube is diverted back to the feed tank by a diverter valve through the action of the tem-

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TABLE 1. Heat inactivation of *M. paratuberculosis* in raw milk as determined by the holder test tube method

Strain	Temp (°C)	Level of *M. paratuberculosis* (log_{10} CFU/ml)[b] at:						
		Zero time	0.25 min	0.5 min	1 min	5 min	15 min	30 min
19698	NH[a]	7.5 ± 0.5 (7–8)	7.5 ± 0.5 (7–8)	7.5 ± 0.5 (7–8)	7.5 ± 0.5 (7–8)	7.5 ± 0.5 (7–8)	7.0 ± 0	7.0 ± 0
	65	7.0 ± 0	7.0 ± 0	7.5 ± 0.5 (7–8)	6.5 ± 0.5 (6–7)	2.5 ± 0.5 (2–3)	2.0 ± 1 (1–3)	2.0 ± 1 (1–3)
	72	7.5 ± 0.5 (7–8)	6.5 ± 0.5 (6–7	5.5 ± 0.5 (5–6)	3.0 ± 0	1.5 ± 0.5 (1–2)	1.5 ± 0.5 (1–2)	1.5 ± 0.5 (1–2)
	74	7.5 ± 0.5 (7–8)	7.5 ± 0.5 (7–8)	7.0 ± 1 (6–8)	7.0 ± 0	1.5 ± 0.5 (1–2)	2.5 ± 0.5 (2–3)	2.5 ± 0.5 (2–3)
	76	7.5 ± 0.5 (7–8)	7.0 ± 0	7.0 ± 0	5.5 ± 0.5 (5–6)	2.0 ± 0	1.5 ± 0.5 (1–2)	1.0 ± 0
Ben	NH	6.5 ± 0.5 (6–7)	6.5 ± 0.5 (6–7)	6.0 ± 0	6.5 ± 0.5 (6–7)	6.0 ± 0	7.0 ± 1 (6–8)	6.5 ± 0.5 (6–7)
	65	7.0 ± 0	6.5 ± 0.5 (6–7)	6.5 ± 0.5 (6–7)	4.5 ± 0.5 (4–5)	1.5 ± 0.5 (1–2)	1.0 ± 0	1.0 ± 0
	72	6.0 ± 1 (5–7)	3.0 ± 2 (1–5)	3.0 ± 1 (2–4)	1.5 ± 0.5 (1–2)	1.5 ± 0.5 (1–2)	1.0 ± 0	1.0 ± 0
	74	7.0 ± 1 (6–8)	3.5 ± 0.5 (3–4)	2.0 ± 0	1.5 ± 0.5 (1–2)	1.5 ± 0.5 (1–2)	1.0 ± 0	1.5 ± 0.5 (1–2)
	76	4.5 ± 1.5 (3–6)	1.5 ± 0.5 (1–2)	1.5 ± 0.5 (1–2)	1.5 ± 0.5 (1–2)	1.0 ± 0	1.5 ± 0.5 (1–2)	2.5 ± 1.5 (1–4)

[a] NH, nonheated raw milk.
[b] The values are the means ± standard errors of the means from two experiments conducted with each strain of *M. paratuberculosis* at each temperature; the numbers in parentheses are the ranges of values obtained in the experiments. A standard error of the mean of 0 means that there was no difference in the means in the two experiments. A mean of 1.0 log_{10} CFU/ml was the lower limit of detection in these experiments.

perature controller. Heating is accomplished by using water (at a high flow rate) which is only 6 to 8°C hotter than the pasteurizing temperature. The water is heated by an immersion heater in a closed system also controlled by the temperature controller. The product then passes back through the regenerator and the cooling section for storage or output. The temperature of the final product should be <10°C after it passes through the cooler.

In these experiments, raw milk (1 to 2 liters) was inoculated with two concentrations of *M. paratuberculosis* (10^4 and 10^6 cells per ml) and mixed thoroughly prior to introduction into the holding vessel. The sensitivity of bacterial detection, as determined by previous experiments, allowed us to decrease the bacterial concentrations of the inocula in these experiments. The flow rate of the pasteurizer unit was set to achieve conditions of 72°C for 15 s. Additional experiments were conducted to determine the effects of heat treatment at 55, 60, 65, 70, and 75°C for 15 s on bacterial survival in milk. In each experiment, milk samples (50 ml) were obtained from the output tube at the beginning, middle, and end of each run. The milk samples were placed on ice and then diluted 1:10 in PBS. Diluted aliquots were sonicated, serially diluted (1:10) in PBS, and plated onto HEYM (one slant per dilution). Dilutions were then frozen at −20°C until DNA extraction was performed. Two experiments were conducted with each strain of bacteria tested for each temperature variable. Standard curves were generated by serial dilution of stock bacterial cultures and plating onto HEYM for each experiment. Results were determined after 4, 8, and 12 weeks of incubation at 37°C.

Results from heat inactivation experiments in which the holder test tube method was used are presented in Table 1. Heat treatment for 1 min at 65°C had no effect on the number of viable *M. paratuberculosis* 19698 CFU recovered; however, by 5 min a 5-log_{10} reduction was observed. Increasing the incubation temperature to 72°C concomitantly increased the rate of bacterial inactivation and there was a 4-log_{10} reduction in viable bacteria after 1 min of incubation. However, bacteria were not totally inactivated until after 15 min of incubation at 72°C. Raising the experimental incubation temperature to either 74 or 76°C did not reduce the amount of time required to inactivate *M. paratuberculosis* 19698 in raw milk compared to previous experiments. Similar experiments conducted with *M. paratuberculosis* Ben, an isolate from a human with Crohn's disease, showed that this strain was inactivated within 5 min at 65°C and within 1 min at 72°C. Further increases in temperature did not significantly alter the results and there was total inactivation within 1 min at either 74 or 76°C.

Results from *M. paratuberculosis* heat inactivation experiments when the lab-scale pasteurizer method was used indicated that both strains of *M. paratuberculosis* tested, 19698 and Ben, at a concentration of either 10^4 or 10^6 CFU/ml were effectively inactivated after treatment at 72°C for 15 s (data not shown). Culturing aliquots of milk obtained during the beginning, middle, or end of sample expulsion after 15 s of heat treatment did not affect the results.

The results of heat inactivation of *M. paratuberculosis* in raw milk with the lab-scale pasteurizer are shown in Table 2. Treatment of raw milk inoculated with 10^6 CFU of *M. paratuberculosis* 19698 per ml for 15 s at 55 and 60°C reduced the numbers of viable bacteria recovered to 5.5 and 4.5 log_{10} CFU/ml, respectively (Table 2). Slightly higher numbers of *M. paratuberculosis* Ben remained viable after similar experimental treatments (Table 2). At temperatures of 65, 70, and 75°C no viable bacteria were recovered after 15 s of heat treatment regardless of the strain of *M. paratuberculosis* tested.

If *M. paratuberculosis* is indeed the causative agent of Crohn's disease, then possible modes of transmission of the organism from animals to humans should be considered. Dairy products processed from contaminated milk sources may be

TABLE 2. Heat inactivation of *M. paratuberculosis* as determined by the laboratory-scale pasteurizer method resulting from treatment at different temperatures for 15 s

Temp (°C)	Level of *M. paratuberculosis* (log_{10} CFU/ml)	
	Strain 19698	Strain Ben
NH[a]	5.5 ± 0.5 (5–6)[b]	6.0 ± 0
55	5.5 ± 0.5 (5–6)	6.0 ± 0
60	4.5 ± 0.5 (4–5)	5.0 ± 0
65	1.0 ± 0	1.0 ± 0
70	1.0 ± 0	1.0 ± 0
75	1.0 ± 0	1.0 ± 0

[a] NH, nonheated raw milk.
[b] The values are means ± standard errors of the means from two experiments conducted with each strain of *M. paratuberculosis* at each temperature; the values in parentheses are the ranges of values obtained in the experiments. A standard error of the mean of 0 means that there was no difference in the means in the two experiments. A mean of 1.0 log_{10} CFU/ml was the lower limit of detection in these experiments.

the logical source of human infection. Although fecal contamination of the udder may account for a portion of subsequent contamination of milk from *M. paratuberculosis*-infected cows, it has also been demonstrated that animals infected with *M. paratuberculosis* shed the organism directly into their milk (1, 6, 11–13).

Due to the potential association between Crohn's disease and *M. paratuberculosis*, a number of studies have recently been initiated to determine if pasteurization conditions currently utilized by the dairy industry kill *M. paratuberculosis* in milk. The methods used to evaluate thermal inactivation of bacteria in milk vary widely, but generally some type of test tube model is used. Our results obtained with a test tube method demonstrate that there is a rapid decline in viable numbers within the first 5 min of heat treatment, followed by a plateau or tailing effect caused by residual survivors. Similar findings were reported in a previous study in which heat treatment of raw milk inoculated with *M. paratuberculosis* under holder pasteurization conditions (63°C, 30 min) resulted in a 9% survival rate for both animal strains tested and an average survival rate of 32% for two human strains (3). A test tube method simulating HTST pasteurization conditions (72°C, 15 s) reduced the survival rate to 5% for animal strains of *M. paratuberculosis*. The survival rate of human strains was significantly increased compared to pretreatment colony counts and was attributed to a destruction of bacillary clumps during heat treatment of inoculated milk samples followed by rapid cooling on ice. More recently, Grant et al. (7) evaluated the effectiveness of the holder and HTST pasteurization methods for inactivation of *M. paratuberculosis* in raw milk and demonstrated that the survival rate of *M. paratuberculosis* was $\leq$1% regardless of the strain or method of pasteurization used. The thermal death curve was concave, with rapid initial killing of the bacterium followed by a significant tailing effect, resulting in low numbers of survivors after heat treatment. These experiments were conducted with a laboratory-scale pasteurizer unit designed to emulate heat exchanger models used by industry; however, unlike industry units, the milk remained static during heat treatment.

Experiments in which the test tube method was used in our laboratory confirmed the ineffectiveness of this method for total inactivation of *M. paratuberculosis* inocula. Therefore, previous studies in which the test tube method was used to perform mycobacterial heat inactivation studies should be interpreted carefully. The major difference between the test tube method and the lab-scale pasteurizer method was the static versus active flow of milk during heat treatment. Studies conducted in our laboratory with the lab-scale industrial pasteurizer unit have demonstrated that turbulent flow of milk during pasteurization is essential for complete killing of contaminating *M. paratuberculosis*. This seems rational since organisms may clump more readily in a static environment than in a nonstatic environment and may protect themselves from heat penetration. Results from these studies indicate that transmission of viable *M. paratuberculosis* from animals to humans via pasteurized dairy products is unlikely and minimize the potential threat of this organism as a zoonotic agent of Crohn's disease.

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