Efficacy of Pasteurization Conditions for the Inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in Milk

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis,* the causative agent of a chronic enteritis in ruminants (Johne's disease), has been linked to Crohn's disease in humans. This microorganism is shed by infected animals primarily in the feces but is also shed in the milk at much lower levels. Therefore, dairy products from infected animals may be one mode of transmission of this animal pathogen. This study was designed to evaluate the effectiveness of the holder and high-temperature short-time pasteurization standards on the destruction of *M. paratuberculosis.* One hundred eighty experiments were conducted in this study using a slug-flow pasteurizer unit and a laboratory scale pasteurizer unit. Ultrahigh-temperature milk was inoculated at two concentrations, 10^8 and 10^5 CFU/ml, with three different field strains of *M. paratuberculosis.* Five different time-temperature combinations were evaluated: 62.7°C for 30 min, 65.5°C for 16 s, 71.7°C for 15 s, 71.7°C for 20 s, and 74.4°C for 15 s. Three replicates of each experiment were run for the pasteurizer unit, time-temperature combination, and strain of *M. paratuberculosis.* Treatment of milk regardless of bacterial strain or pasteurizer unit resulted in an average 5.0- and 7.7-log kill for the low and high concentrations of inoculum, respectively. Milk treated for cheese production (65.5°C for 16 s) resulted in a much lower and more variable kill. Results from this study indicate that the current U.S. minimum standards for batch and high-temperature short-time pasteurization of grade A milk significantly reduced the survivability of *M. paratuberculosis,* but some bacteria survived subpasteurization heat treatment of milk used for cheese manufacture.

A causative link between the consumption of raw milk and the incidence of human illness was recognized in the 1900s. The threat of bovine tuberculosis to the public was a strong impetus for the establishment of commercial standards for the heat treatment of milk *(11).* The first milk pasteurization ordinance was published in 1924 and stated that conditions of temperatures not less than 142°F (61.1°C) for 30 min in approved equipment were optimal for the destruction of contaminating pathogens. North and Park *(11)* established that these conditions provided an adequate margin of safety for the destruction of *Mycobacterium tuberculosis* in milk. However, *Coxiella burnetti* proved to be more heat resistant than *M. tuberculosis,* and studies conducted with this organism resulted in an increase in the official U.S. pasteurization standards to 62.8°C with a holding time of 30 min *(7).*

The introduction of high-temperature short-time (HTST) pasteurization took place in 1933. The methodology required heat treatment of milk at 71.7°C for 15 s, a regimen that was determined after numerous studies evaluating the effects of HTST pasteurization on the survivability of such pathogens as *M. tuberculosis, Mycobacterium bovis, Brucella,* and *Streptococcus (21).* HTST pasteurization currently is the primary method for heat treatment of milk in dairy processing plants.

The advent of commercial pasteurization resulted in a significant decline in milkborne human illness, and cur-

rently less than 1% of human foodborne illness is attributed to milk products. In general, consumer surveys indicate that Americans feel that the food supply in the United States is safe (www.ific.org/foodinsight/1999). However, most of these surveys are based upon the degree of acute illness caused by milk products. Little is known about the relationship of potential pathogens in milk and chronic diseases of humans. A documented relationship between *M. paratuberculosis* and Johne's disease in dairy cattle and a suggested relationship with Crohn's disease in humans prompted researchers to evaluate the heat resistance of *M. paratuberculosis* in milk. Crohn's disease is a chronic, progressive, debilitating enteritis in humans whose clinical signs mimic some of those noted in cattle infected with *M. paratuberculosis.* Because cattle shed *M. paratuberculosis* into their milk, the question of whether this microorganism could survive the pasteurization process has been addressed. However, the results of studies evaluating the survival of *M. paratuberculosis* after heat treatment are very conflicting. Results of a previous study conducted by the U.S. Department of Agriculture suggested that HTST pasteurization was effective in inactivating *M. paratuberculosis* in raw milk *(15),* and studies conducted within the UK demonstrated the recovery of viable *M. paratuberculosis* from retail-ready pasteurized milk *(4).* To date, there is no definitive answer to the question of whether *M. paratuberculosis* is completely destroyed during pasteurization, but the majority of the studies conducted have demonstrated a significant log kill during the process *(3, 8, 12, 15).* The present study was conducted to evaluate a broader range of

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TABLE 1. *Experimental time and temperature combinations utilized to evaluate destruction of* Mycobacterium paratuberculosis *in UHT milk*

Method	Temperature (°C)	Time	Description
1	62.7	30 min	Conditions for holder treatment
2	65.6	16 s	Conditions for milk used for cheese production
3	71.7	15 s	Conditions for HTST treatment in United States
4	71.7	20 s	Experimental conditions for HTST treatment
5	74.4	15 s	Experimental conditions for HTST treatment

pasteurization conditions for the processing of raw milk for fluid milk and a range of subpasteurization conditions applied to milk used in the manufacture of cheese to determine the efficacy of these methods in the destruction of *M. paratuberculosis.*

MATERIALS AND METHODS

Pasteurizer units. A two-phase slug-flow heat exchanger (Moffett Center, Summitt Argo, Ill.) was utilized in this study *(17).* This unit allows precisely defined residence times by using air to compartmentalize the liquid sample flowing through a capillary tube. The air also breaks up the laminar flow so that come-up times are reduced, and the fastest particle velocity is near that of the median for particle velocity.

A laboratory-scale HTST unit was also employed in this study (Armfield, London, UK). This unit had previously been used to perform heat inactivation experiments with *M. paratuberculosis (15).* Modification to the unit included employment of adjustable coiled tubing as the holding tube to allow evaluation of different residence times and turbulent flow of the product during heat treatment. Previously, the instrument was outfitted with a holding tube capable of only a 15-s residence time *(15).* Stainless steel tubing was used for experiments with short-term residence times, and Tygon tubing was utilized for the experiments with extended hold times of 30 min.

Strain selection. Three bovine isolates of *M. paratuberculosis* were utilized in the experiments: strains 167, 5007, and 6112. All three isolates were obtained from the ileum of infected cows that were necropsied in the late stage of clinical disease at the National Animal Disease Center (Ames, Iowa). Isolates were propagated from the original culture in flasks containing Middlebrook 7H9 medium (M7H9, pH 5.9) (Difco, Becton Dickinson, Sparks, Md.) containing 2 mg/liter mycobactin J (Allied Monitor, Fayette, Mo.) and 1% oleic albumin complex (Difco, Becton Dickinson). Cultures were monitored for growth at 39°C and were harvested when they reached the log phase of growth (Abs_{540nm} = 0.4) by pelleting at $10,000 \times g$ for 20 min, decanting the supernatant, and washing the pellets twice with phosphate-buffered saline (PBS; 0.1 M, pH 7.2). The pellets were resuspended in 10 ml of PBS, and the concentration of bacteria was adjusted to 10^6 CFU/ml using a growth curve monitoring turbidity at Abs_{540nm}. One hundred microliters of bacterial suspension (10^5 CFU) was inoculated onto agar slants of Herrold's egg yolk medium (HEYM; National Animal Disease Center) containing 50 μg/ml vancomycin, 50 μg/ml nalidixic acid, and 100 μg/ml amphotericin B (Sigma Chemical Co., St. Louis, Mo.). Slants were incubated at 37°C until utilized in an experiment, with a maximum shelf time of 6 months.

Inoculum preparation. For each experiment the appropriate bacterial strain was harvested from these agar slants by adding 10 ml of PBS per slant and inverting for 30 min on a rotator to free the bacterial cells from the agar. The bacterial solution was then transferred to a sterile 50-ml conical tube and centrifuged at $1,800 \times g$ for 20 min at room temperature. The pellet was resuspended in PBS and kept on ice until used in the experiment. Bacterial suspensions were added to the milk to achieve high and low concentrations of inoculum. High concentrations of inoculum were approximately 10^8 CFU/ml ($6.7 \times 10^8 \pm 1.8 \times 10^8$) and low concentrations averaged 10^5 CFU/ml ($3.2 \times 10^5 \pm 1.8 \times 10^5$). Bacterial concentrations for each experiment were verified by inoculating in duplicate onto HEYM slants after 10-fold serial dilutions were performed. Results were recorded as CFU per slant after 4, 8, and 12 weeks of incubation.

Milk. Experiments were conducted using ultrahigh-temperature (UHT) whole homogenized milk (Parmalat, Wallington, N.J.). Each milk lot was plated onto blood agar plates before use to determine sterility. UHT milk was used in this study to avoid problems with contamination during the lengthy incubation required for growth of *M. paratuberculosis.* Using aseptic milk also precluded use of a decontamination protocol that might have reduced the recovery of viable heat-injured organisms after heat treatment. The aseptic milk had physiochemical characteristics similar to those of raw milk, such as pH, fat, protein, and sugar content; therefore, we expected that heat inactivation characteristics would be comparable.

Experimental protocol. Three liters of UHT milk was used for each experimental run, with experiments run concurrently on the slug-flow and HTST units. An aliquot of milk was obtained before the addition of *M. paratuberculosis* inoculum as a negative control for culture and PCR analyses. Immediately after inoculation of the milk with the appropriate concentration of bacteria, another aliquot of milk was taken for use as a positive experimental control. Two liters of inoculated milk was used for the HTST unit, and 1 liter was used for the slug-flow unit.

Heat treatment. Experiments were conducted to evaluate effects of minimum time-temperature combinations utilized by U.S. dairy processing plants on the survival of *M. paratuberculosis* (Table 1). All heat treatments were evaluated using both the slug-flow and HTST pasteurizer units. Different holding tubes were utilized to allow different residence times for heating of the product. Each time-temperature experiment was replicated three times and included evaluation of each strain of *M. paratuberculosis* (167, 5007, and 6112) at two inoculum concentrations (10^8 and 10^5 CFU/ml) for a total of 90 experiments (5 treatments × 3 bacterial strains × 2 inoculum concentrations × 3 replicates) on each unit (Table 2). Upon completion of each heat treatment, 150 ml of milk was collected into 50-ml sterile conical tubes and immediately placed on ice.

TABLE 2. *Strain evaluation for each experiment for the slug-flow and HTST pasteurizer units*

Mycobacterium paratuberculosis strain	Inoculum (CFU/ml)	No. of replicates	No. of runs for each strain	No. of runs for each time-temperature combination	Total experiments for each unit
167	10^8	3	6	18	90
	10^5	3			
5007	10^8	3	6		
	10^5	3			
6112	10^8	3	6		
	10^5	3			

Culturing of milk for *M. paratuberculosis*. Culturing of *M. paratuberculosis* was performed with three different media (Fig. 1). Two 50-ml aliquots of the negative and positive controls and test samples from both slug-flow and HTST units from each experiment were centrifuged at $1,172 \times g$ for 30 min at 4°C. The fluid portion of each sample was discarded, and the pellet was resuspended in 1 ml of PBS. This suspension was further diluted 10-fold in PBS. The original suspension and the diluted suspension of each sample (200 μl of each) were inoculated in duplicate onto HEYM. Samples were incubated at 37°C, and the number of CFU from both slants was averaged and recorded at 4, 6, 8, 26, and 52 weeks. Final results for each sample were presented as CFU per milliliter after dilution factors were applied to raw CFU data. Colonies were confirmed as *M. paratuberculosis* by PCR, subculture, plating onto blood agar, and acid-fast stain. A suspect colony was picked using a sterile disposable loop and placed into 1 ml of Tris-EDTA buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0), and PCR analysis was performed. The suspect colony was subcultured by transfering it into 5 ml of M7H9 medium using a sterile plastic loop. Growth was monitored by turbidity changes in the medium during incubation of the samples for 8 weeks at 37°C. When changes in the medium were observed, aliquots of the sample were removed and plated onto blood agar to verify that the organism was not a contaminant. Aliquots were also dried onto glass slides and examined after Ziehl-Neelsen staining for the presence of acid-fast bacteria *(12)*.

Sample suspensions and their dilutions (500 μl) and controls were inoculated into BACTEC medium (Becton Dickinson) containing 0.1 ml of mycobactin J, 1 ml of 50% egg yolk suspension (Difco, Becton Dickinson), and no antibiotics, 0.1 ml of PANTA antibiotic supplement (Becton Dickinson), or 0.4 ml of vancomycin (50 μg/ml). Negative and positive controls were included for each experiment and consisted of UHT milk alone and UHT milk spiked with the appropriate concentration of *M. paratuberculosis*. Samples were incubated at 37°C, and growth was monitored each week for 8 weeks. A growth index of more than 30 was indicative of bacterial respiration and growth *(1)*. Acid-fast staining was performed to confirm growth in vials with positive signals.

Detection thresholds for recovery of *M. paratuberculosis* from experimentally inoculated samples were determined prior to this study for both HEYM and BACTEC medium. Sensitivity of detection was approximately 10 CFU/ml and <10 CFU/ml for HEYM and BACTEC medium, respectively.

Dubos broth preenrichment cultures. In an attempt to improve the sensitivity of detection of sublethally injured *M. paratuberculosis* after heat treatment, a preenrichment protocol was employed for a replicate set of samples from each experiment *(6)* (Fig. 1). A 50-ml sample of milk was centrifuged at $1,140 \times g$ for 30 min at 4°C. The fluid portion was discarded, and the pellet was resuspended in 1 ml of 0.85% NaCl. This suspension was
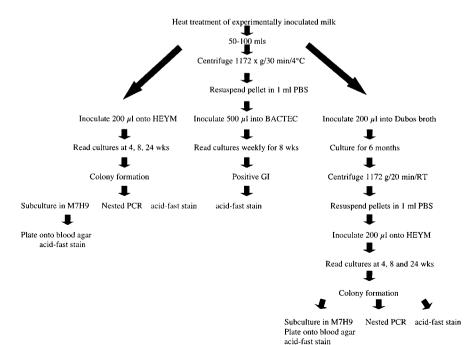
FIGURE 1. *Flow diagram of culture methods after heat treatment of UHT milk spiked with* Mycobacterium paratuberculosis.

further diluted 10-fold in 0.85% NaCl. Each sample and dilution was inoculated (200 μl) into tubes containing Dubos broth (Difco, Becton Dickinson) with 10% fetal calf serum (Hyclone, Logan, Utah), 2 mg/liter mycobactin J, and 5% PACT antibiotic solution (polymyxin-B, carbenicillin, trimethoprim lactate, and amphotericin B; Sigma) in triplicate. Samples were incubated at 37°C for 6 months. Triplicate samples were then pooled into a single 15-ml conical tube and centrifuged at 1,850 × g for 20 min at room temperature. The supernatant was decanted, and the pellet was resuspended in 0.85% NaCl. Aliquots of the suspension were then plated onto blood agar plates (Becton Dickinson) to check for contamination and onto glass slides for Ziehl-Neelsen staining *(12)*. Samples that were not contaminated were immediately inoculated onto HEYM slants and into BACTEC vials to assess the presence of viable *M. paratuberculosis*. Samples that demonstrated the presence of a contaminant on the blood agar plate were subjected to a decontamination protocol that consisted of the addition of 1 ml of a solution containing 4% sodium hydroxide, 0.5 g *N*-acetyl-L-cysteine (NALC), and 2.9% sodium citrate to the sample pellet. Samples were agitated for 30 min, and then one drop of 5% Tween 80 was added. Samples were centrifuged at 1,850 × g for 20 min at room temperature, the supernatant was discarded, and pellets were resuspended in 2 ml of 0.85% NaCl. Samples were then inoculated onto HEYM and into BACTEC medium as previously described.

PCR analyses. Confirmation of colonies on agar slants was performed by PCR analysis. Agar slants were flooded with 1 ml of sterile 1 mM Tris–0.05 mM EDTA buffer (pH 7.6), and slants were scraped. The solution was then decanted into a sterile microfuge tube, and the tubes were placed in a boiling water bath for 10 min to release the DNA from the bacteria. After cooling to room temperature, tubes were briefly centrifuged at 14,000 rpm in a microfuge to pellet the bacterial debris.

DNA was amplified using a nested PCR protocol as previously described *(5)*. For the first amplification reaction, forward and reverse primers (5′-GTTCGGGGCCGTCGCTTAGG-3′ and 5′-GAGGTCGATCGCCCACGTGA-3′) were used to amplify a 400-bp region of the insertion element IS900. A second amplification reaction further amplified the product of the first reaction using internal forward and reverse primers (5′-GCTTA-GGCTTCGAATTGCC-3′ and 5′-CTCCGTAACCGTCATTGTCC-3′) and resulted in a final product of 194 bp. After amplification, DNA was electrophoresed in a 4% agarose gel (Reliant Gel Systems, FMC Bioproducts, Rockland, Maine) containing ethidium bromide in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA), and bands were visualized using a UV transilluminator (Bio-Rad, Hercules, Calif.). A positive control DNA sample (*M. paratuberculosis* DNA) was included in each PCR run and on each gel for assay verification. A negative control consisting of buffer only was run each time to verify lack of cross-contamination of samples.

RESULTS

Results of all experiments evaluating survival of *M. paratuberculosis* after five different heat treatment regimens using the slug-flow pasteurizer unit and the HTST pasteurizer unit are shown in Tables 3 and 4, respectively. Survivors were identified by a colony (or colonies) present on HEYM, the successful propagation of the colony by subculture in MH79 medium, and a positive acid-fast stain and PCR product with *M. paratuberculosis*-specific primers. Therefore, conditions for recognition of positive growth

were quite stringent. There were several experiments in which survivors were not detected on HEYM but a positive signal was noted in the BACTEC vials. These results were included in the tables as being indicative of viable *M. paratuberculosis* after heat treatment. More survivors were noted in experiments in which the higher concentration of inoculum was utilized to spike the milk. Survivors were apparent by at least one culture method (HEYM or BACTEC) in 17 of the 45 (38%) experiments conducted with the slug-flow unit and 12 of the 45 (27%) experiments with the HTST pasteurizer unit when milk was inoculated with 10^8 CFU/ml. Fewer survivors were found in experiments in which milk was spiked with low concentrations of *M. paratuberculosis* after processing with either the slug-flow unit (9 of 45) or the HTST unit (8 of 45). Fewer survivors also were found in experiments in which milk was treated with the HTST unit than in experiments with the slug-flow unit regardless of inoculum concentration (42 versus 58%). The HEYM and BACTEC culture results were closely correlated in this study, with both methods having similar sensitivity for detection of viable *M. paratuberculosis* after heat treatment of milk regardless of the unit used.

Survivors were noted infrequently for all treatments except for the 65.5°C–16 s method. When treated at 62.7°C for 30 min to simulate the holder method, all experimental replicates demonstrated high lethality for *M. paratuberculosis,* averaging a 7.3-log kill. A low number of survivors was noted in three slug-flow experiments with strain 5007 when the initial inoculum was 10^8 CFU/ml, but the average kill was still 7 log of bacteria. Other experimental regimens that demonstrated efficient destruction of *M. paratuberculosis* were treatment of milk at 71.7°C for 15 s, 71.7°C for 20 s, and 74.4°C for 15 s, regardless of pasteurizer unit (Tables 3 and 4). Only one experiment of the three replicates conducted for any bacterial strain at either inoculum concentration resulted in any survivors on HEYM for these heat-treatment regimens, indicating that the experimental regimens were highly effective. Kill of bacteria for these experimental regimens collectively averaged 4.9 ± 0.037 log for the lower concentration of inoculum and 7.9 ± 0.074 log for the higher concentration. Complete kill of *M. paratuberculosis* regardless of strain was observed for the lower concentration of inoculum for the HTST unit, and an average 8-log kill was observed for the higher concentration for all three replicates for all three strains of bacteria for these three temperature-time combinations.

In contrast, a significant number of surviving *M. paratuberculosis* was noted after treatment of UHT milk at 65.5° for 16 s, regardless of pasteurizer unit, inoculum concentration, or bacterial strain (Tables 3 and 4). Heat treatment of milk containing bacteria at 10^8 CFU/ml with the HTST and slug-flow units resulted in a average of 5.0- and 3.7-log kill of *M. paratuberculosis,* respectively, across strains. After heat treatment of milk containing the lower concentration of inoculum (10^5 CFU/ml), results were more variable, ranging from a 2- to 5-log kill with the HTST unit and 1- to 4-log kill with the slug-flow unit.

Preenrichment cultures of *M. paratuberculosis* did not yield any useful information. Contamination of the Dubos

Table 3. *Results of heat treatment of UHT milk spiked with* Mycobacterium paratuberculosis *using a slug-flow pasteurizer unit*

Heat treatment	Bacterial strain	Inoculum (CFU/ml)	No. of replicate experiments with survivors	HEYM (CFU/ml)	Average log kill	No. of BACTEC cultures with survivors
62.7°C, 30 min	167	10^8	1/3	1.1×10^3	7	1/3
		10^5	0/3	<10	>5	0/3
	5007	10^8	3/3	7.5×10^2	6	0/3
		10^5	0/3	<10	5	0/3
	6112	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
65.5°C, 16 s	167	10^8	3/3	6.0×10^4	4	3/3
		10^5	1/3	6.7×10^2	4	3/3
	5007	10^8	2/2	7.0×10^4	4	2/2
		10^5	3/3	7.9×10^4	1	3/3
	6112	10^8	3/3	6.6×10^5	3	3/3
		10^5	2/3	2.5×10^5	<1	2/3
71.7°C, 15 s	167	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
	5007	10^8	0/3	<10	>8	0/3
		10^5	1/3	3.9×10^2	4	1/3
	6112	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
71.7°C, 20 s	167	10^8	0/3	<10	>8	2/3
		10^5	0/3	<10	>5	0/3
	5007	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
	6112	10^8	0/3	<10	>8	2/3
		10^5	0/3	<10	>5	0/3
74.4°C, 15 s	167	10^8	1/3	6.7×10^3	7	1/3
		10^5	0/3	<10	>5	0/3
	5007	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
	6112	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3

broth medium with *Pseudomonas alcaligens* was a significant problem throughout the study, occurring in every experimental regimen, and despite our best efforts to control the contamination by heat and filter sterilization of media components we were unable to salvage any useful data. We had little success when we attempted further optimization of the preenrichment protocol by evaluating other antibiotics or combinations of antibiotics because some of the antibiotics seemed to have inhibitory effects on *M. paratuberculosis* in addition to *P. alcaligens.* We also performed experiments to assess the efficacy of two different decontamination protocols, NALC and 0.9% hexadecylpyridinium chloride (HPC) and several antibiotics or combinations of antibiotics, PACT, cefotaximine, ceftriaxone, and sulfamethoxadole. We determined that NALC was superior to HPC as a decontaminant for *P. alcaligens.* We also found that PACT alone or PACT in combination with cefotaximine or ceftriaxone were equally effective in reducing *P. alcaligens* without affecting recovery of *M. paratuberculosis* in the first 2 months of incubation. However, extended incubation of Dubos medium resulted in *P. alcaligens* contamination regardless of decontamination or antibiotics. Although not adequately tested, a shorter preenrichment procedure may be more effective in the recovery of heat-in-

jured *M. paratuberculosis* than the 6-month incubation that was attempted in this study. This discovery was not made until we were well into the primary study and a large number of heat-treatment experiments had already been performed. Therefore, we were unable to include any modified Dubos preenrichment data in this report. We did not encounter any problems with *P. alcaligens* contamination in our HEYM or BACTEC medium cultures.

DISCUSSION

Because of a suggested association between *M. paratuberculosis* and Crohn's disease, several modes of transmission of this pathogen from animals to humans have been examined. Although environmental contamination with fecal matter from infected animals is undoubtedly one mode of transmission, human ingestion of *M. paratuberculosis* may also occur through infected dairy products. Infected dairy cattle shed viable *M. paratuberculosis* into their milk albeit at low concentrations *(20).* More recently, quantitation of *M. paratuberculosis* in the milk of naturally infected cows during the periparturient period confirmed that shedding concentrations are relatively low *(15).* Infected cows shed the highest number of organisms (50 CFU/ml of milk) in colostral milk within 1 to 2 days after calving, with con-

TABLE 4. *Results of heat treatment of UHT milk spiked with* Mycobacterium paratuberculosis *using an HTST pasteurizer unit*

Heat treatment	Bacterial strain	Inoculum (CFU/ml)	No. of replicate experiments with survivors	HEYM (CFU/ml)	Average log kill	No. of BACTEC cultures with survivors
62.7°C, 30 min	167	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
	5007	10^8	1/3	2.7×10^3	6	0/3
		10^5	0/3	<10	>5	0/3
	6112	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
65.5°C, 16 s	167	10^8	3/3	1.0×10^3	5	3/3
		10^5	0/3	<10	>5	3/3
	5007	10^8	3/3	7.9×10^2	6	2/3
		10^5	3/3	1.4×10^4	3	3/3
	6112	10^8	3/3	2.0×10^4	5	3/3
		10^5	2/3	1.0×10^4	2	2/3
71.7°C, 15 s	167	10^8	0/3	<10	>8	1/3
		10^5	0/3	<10	>5	0/3
	5007	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
	6112	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
71.7°C, 20 s	167	10^8	0/3	<10	>8	1/3
		10^5	0/3	<10	>5	0/3
	5007	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
	6112	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
74.4°C, 15 s	167	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
	5007	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3
	6112	10^8	0/3	<10	>8	0/3
		10^5	0/3	<10	>5	0/3

centrations dropping to less than 1 CFU/ml by 5 days after calving. However, fecal matter from infected cows may be a significant contributor to contamination of the milk rather than actual shedding of the organism directly into the milk *(10)*. Because infected cows may shed up to 10^8 CFU/g of feces, exogenous contamination of the milk supply may account for a portion of *M. paratuberculosis* present in milk. The contamination of milk with *M. paratuberculosis* can be controlled only with improved management and control of the disease within herds.

In a study published in 1996, viable *M. paratuberculosis* cells were identified in pasteurized milk purchased from retail markets, raising concerns about the effectiveness of current pasteurization conditions in the inactivation this microorganism *(9)*. This finding stimulated a number of studies to evaluate the destruction of *M. paratuberculosis* in milk by the holder method or HTST method of pasteurization. Grant et al. *(3)* demonstrated a survival rate of ≤1% when *M. paratuberculosis* inoculated into raw milk was subjected to either the holder or HTST method of pasteurization. There was significant tailing after an initial rapid kill of the bacterium, resulting in low numbers of survivors after heat treatment. Sung and Collins *(18)* reported D-values (time to kill 1 log of bacteria) of 11.7, 21.8, 47.8,

and 228.8 s at 71, 68, 65, and 62°C, respectively, for clinical cow strains in milk when using a test tube model, indicating that *M. paratuberculosis* would survive at these temperatures. Stabel et al. *(16)* also noted a tailing effect when inoculated raw milk was processed by the holder method using a test tube model, but *M. paratuberculosis* was inactivated to nondetectable concentrations (<10 CFU/ml) when subjected to HTST conditions using a laboratory-scale pasteurizer unit. Keswani and Frank *(8)* obtained D-values of 1.6 to 2.9 min at 63°C using a capillary tube method. They also noted a 5-log reduction in *M. paratuberculosis* after capillary tubes were subjected to HTST conditions, indicating that adequate killing of *M. paratuberculosis* was obtained after either holder or HTST pasteurization treatment. In a study in Canada in which raw milk was spiked with 10^3, 10^5, and 10^7 CFU of *M. paratuberculosis* per ml *(2)*, minimum kills of 5 log and 7 log were achieved with methods simulating HTST and batch pasteurization, respectively.

The significant log reduction in viable *M. paratuberculosis* after heat treatment noted in the Canadian study is in agreement with the data presented here. We were able to demonstrate a high degree of inactivation of *M. paratuberculosis* using pasteurization conditions that simulated cur-

rent minimum standards for the holder and HTST methods in the United States. Our results from experiments with the HTST unit after milk was inoculated with the higher concentration of bacteria produced an average 8-log kill and an estimated D_{72}-value of 1.83 s. The holder method was equally effective in reducing the number of viable *M. paratuberculosis* but required more time to destroy the bacteria because of the lower temperature (average D_{63}-value of 240 s across strains). Of all the experimental regimens, only one run at 62.7° for 30 min had surviving bacteria, and this occurred in only one of three replicates with strain 5007 at the higher concentration of inoculum. These data indicate clearly that conditions for pasteurization of milk in the United States can result in a significant inactivation of *M. paratuberculosis.*

The HTST pasteurizer unit was more effective than the slug-flow unit in the destruction of *M. paratuberculosis* for most experimental regimens in this study. Only relative comparisons between the two units can be made because the slug-flow unit is designed to give precise results under given conditions. We utilized data from both units to determine whether precise time in residence for heating the milk was effective in reducing the number of bacteria in the treated milk. However, the HTST unit was designed to simulate equipment used in a dairy processing plant to pasteurize milk for human consumption, with turbulent flow of the product during processing. Thus far, only two studies have been published using a pilot-scale or commercial pasteurizer to evaluate survival of *M. paratuberculosis* in raw milk. Pearce et al. *(13)* inoculated raw milk with human and bovine isolates of *M. paratuberculosis* and demonstrated a mean D_{72}-value of <2.03 s, indicating that a >7-log kill would occur within the 15-s treatment period. In contrast, viable *M. paratuberculosis* cells were cultured from 6.9% of pasteurized milk samples processed using a commercial-scale pasteurizer unit at 73°C for 15 or 25 s *(4)*. Survivors were not quantitated in this study, so the log reduction could not be ascertained; however, the authors concluded that HTST pasteurization did have a significant impact on the viability of *M. paratuberculosis.*

Survival of *M. paratuberculosis* after treatment of milk at 65.5°C for 16 s was observed using both pasteurization units for all three strains of bacteria and at both high and low concentrations of inoculum. However, cheese processing involves various factors that may affect microbial survival, such as salt content, pH, and ripening period. Sung and Collins *(19)* revealed that pH played a significant role in the survival of *M. paratuberculosis* in a Hispanic-style soft cheese, with lower pH associated with decreased *D*-values. However, results of a more recent study indicated that the effects of pH were probably short lived, with the lowest pH achieved within 24 h of manufacture and increasing to 5.7 and 5.8, respectively, in hard and semihard Swiss cheeses after 120 days of ripening *(14)*. Another important factor influencing the inactivation of *M. paratuberculosis* in cheese was the temperature applied during cheese manufacture *(14)*. Curds for hard cheese were treated at 53°C for 45 min and those for semihard cheese were held at 44°C for 10 min in this study. The authors concluded

that the cooking process may have resulted in sublethal injury to *M. paratuberculosis,* leading to reduced survivability during the ripening period. Both of these studies indicated that between 10^3 and 10^4 *M. paratuberculosis* cells per g of cheese would be inactivated during the cheese manufacturing process. These data would suggest that an additive effect of heat treatment of milk or curds for cheese production combined with other factors involved in cheese ripening may achieve an inactivation of ≥7 log.

In the present study, we achieved effective inactivation of *M. paratuberculosis* in UHT milk at very high inoculum concentrations using the minimum pasteurization treatments recommended in the pasteurized milk ordinance for grade A milk processing (http://www.cfsan.fda.gov/~ear/pmo01-4.html). Survivors were noted in 1.4% (1 of 72) of experiments conducted with the HTST unit and 8.3% (6 of 72) of experiments conducted with the slug-flow unit. Increasing the time or temperature beyond standard pasteurization conditions did not impact our results. Only in experiments in which milk was treated at subpasteurization conditions for cheese production were survivors noted on a frequent basis (28 of 35 experiments). Increasing the time or temperature beyond minimum U.S. pasteurization standards did not impact our results. Because natural shedding rates of *M. paratuberculosis* in the milk of infected cows are comparatively low, these results indicate that *M. paratuberculosis* would be effectively inactivated by current pasteurization practices in the United States.

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