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Inactivation of *Mycobacterium paratuberculosis* in Cows' Milk at Pasteurization Temperatures

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The thermal inactivation of 11 strains of *Mycobacterium paratuberculosis* at pasteurization temperatures was investigated. Cows' milk inoculated with *M. paratuberculosis* at two levels (10^7 and 10^4 CFU/ml) was pasteurized in the laboratory by (i) a standard holder method (63.5°C for 30 min) and (ii) a high-temperature, short-time (HTST) method (71.7°C for 15 s). Additional heating times of 5, 10, 15, 20, and 40 min at 63.5°C were included to enable the construction of a thermal death curve for the organism. Viability after pasteurization was assessed by culture on Herrold's egg yolk medium containing mycobactin J (HEYM) and in BACTEC Middlebrook 12B radiometric medium supplemented with mycobactin J and sterile egg yolk emulsion. Confirmation of acid-fast survivors of pasteurization as viable *M. paratuberculosis* cells was achieved by subculture on HEYM to indicate viability coupled with PCR using *M. paratuberculosis*-specific IS*900* primers. When milk was initially inoculated with 10^6 to 10^7 CFU of *M. paratuberculosis* per ml, *M. paratuberculosis* cells were isolated from 27 of 28 (96%) and 29 of 34 (85%) pasteurized milk samples heat treated by the holder and HTST methods, respectively. Correspondingly, when 10^3 to 10^4 CFU of *M. paratuberculosis* per ml of milk were present before heat treatment, *M. paratuberculosis* cells were isolated from 14 of 28 (50%) and 19 of 33 (58%) pasteurized milk samples heat treated by the holder and HTST methods, respectively. The thermal death curve for *M. paratuberculosis* was concave in shape, exhibiting a rapid initial death rate followed by significant "tailing." Results indicate that when large numbers of *M. paratuberculosis* cells are present in milk, the organism may not be completely inactivated by heat treatments simulating holder and HTST pasteurization under laboratory conditions.

Mycobacterium paratuberculosis is a gram-positive, acid-fast bacillus which is the etiological agent of paratuberculosis, commonly known as Johne's disease (JD), in cattle and other ruminants. It is a slowly growing fastidious organism which generally requires the presence of mycobactin (an iron-chelating compound produced by other mycobacteria) in order to proliferate in vitro. Primary isolation can take as long as 4 months to show visible colony formation on solid media. It has been proposed that infection of newborn calves is the main cause of JD in cattle and that infection occurs by ingestion of colostrum and milk from paratuberculous cows (4).

M. paratuberculosis has also been implicated in Crohn's disease (CD) in humans. CD and JD have some clinical features in common: both are chronic granulomatous diseases of the gut affecting nutrient absorption and frequently affect the young. Reviews of the evidence for a possible role of *M. paratuberculosis* in CD have concluded that the case for *M. paratuberculosis* as the etiological agent is inconclusive (1, 29). If it is implicated, then milk is a possible vehicle of transmission of the organism to humans (13, 29), since detectable quantities of *M. paratuberculosis* cells in milk from both clinically affected cattle (28) and asymptomatic carriers have been reported (27).

Milk is involved in the transmission of JD to young calves, and it has been shown that the ingestion of a few organisms can result in disease (3). If the same is also true for CD, then the effectiveness of milk pasteurization is crucial. A study by Chiodini and Hermon-Taylor (2) presented evidence to show that *M. paratuberculosis* in milk is not completely inactivated by pasteurization. A further study involving PCR techniques has detected the DNA of *M. paratuberculosis* in 6% (21 of 336 samples) of retail pasteurized whole-milk samples in southern England and south Wales (24), although viable *M. paratuberculosis* cells have yet to be cultured from these samples.

Milk pasteurization was introduced in the mid-nineteenth century as a public health measure in order to destroy the most heat-resistant non-spore-forming human pathogens (*M. tuberculosis* and *Coxiella burnetti* [16]) likely to be present in raw milk. In the United Kingdom, milk is required to be pasteurized commercially by a high-temperature, short-time (HTST) process whereby milk is heated to at least 71.7°C and held at this temperature for 15 s (9). However, in order to study the effect of pasteurization in the laboratory, it is common practice to treat milk samples by a standard holder method, in which the milk is heated to and held at 63.5°C for 30 min (an equivalent time-temperature combination in terms of total process lethality [16]). Franklin (11) suggested that results obtained with the holder method may not be directly comparable to those obtained with commercial pasteurization, since the heating and cooling profiles are not identical. Therefore, in order to more closely simulate HTST pasteurization of milk at 71.7°C under laboratory conditions, Franklin developed a simple laboratory-scale HTST pasteurizing unit (12). This pasteurizing unit consisted of two stainless steel plates (50 by 25 cm) sealed by a rubber gasket, with an internal capacity of 300 ml. The unit was designed to operate on the principle of a heat exchanger. Milk introduced into the gap between the plates, via an inlet funnel, was heated up or cooled down by placing the entire unit in a circulating heating or cooling bath. The heating and cooling curves of this pasteurizing unit were shown to approximate closely those of commercial HTST milk pasteurizing plants, and the end product, as measured by residual

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TABLE 1. *M. paratuberculosis* strains studied

Strain	Origin	Source
ATCC 19698	Bovine	American Type Culture Collection, Rockville, Md.
ATCC 43015 (Linda)	Human	American Type Culture Collection, Rockville, Md.
NCTC 8578	Bovine	National Collection of Type Cultures, Colindale, London, United Kingdom
B1	Bovine	Veterinary Sciences Division, Department of Agriculture for Northern Ireland, Belfast, United Kingdom
B2	Bovine	Veterinary Sciences Division, Department of Agriculture for Northern Ireland, Belfast, United Kingdom
B4	Bovine	Veterinary Sciences Division, Department of Agriculture for Northern Ireland, Belfast, United Kingdom
M8	Bovine	Moredun Research Institute, Edinburgh, United Kingdom
M9	Caprine	Moredun Research Institute, Edinburgh, United Kingdom
USDA 1038	Bovine	National Animal Disease Center, Ames, Iowa
USDA 1113	Bovine	National Animal Disease Center, Ames, Iowa
DVL 943	Bovine	National Veterinary Laboratory, Ministry of Agriculture, Copenhagen, Denmark

phosphatase, was found to be similar. The results obtained with this apparatus can therefore be directly related to the results obtained with commercial pasteurization.

This study was undertaken to investigate the thermal inactivation of a number of strains of *M. paratuberculosis* of bovine, caprine, and human origin in milk at pasteurization temperatures. The standard holder method of pasteurization was employed to provide information on the thermal death curve of this organism at 63.5°C. Laboratory-scale HTST pasteurizing units, similar to those developed by Franklin (12), were employed to determine whether *M. paratuberculosis* can survive HTST pasteurization at 71.7°C for 15 s.

MATERIALS AND METHODS

Organisms studied. Three reference strains and eight field isolates of *M. paratuberculosis* were used in this study (Table 1). One *Mycobacterium bovis* field isolate (T/94/163C, obtained from Veterinary Sciences Division, Department of Agriculture for Northern Ireland, Stormont, Belfast, United Kingdom) was also included as a control to check the efficacy of the pasteurization treatments.

Raw-milk samples. The milk from a total of four healthy Friesian cows was used during the course of this study. On each milking occasion, milk was aseptically drawn from a single cow after the udder had been thoroughly washed with a warm disinfectant solution (1:100 dilution of Hibicet hospital concentrate [Zeneca Ltd., Macclesfield, England] in warm water), rinsed with sterile distilled water, and dried with a sterile udder cloth. The first few discharges of milk from each teat were discarded before milk samples were hand milked into 300-ml sterile bottles. The milk samples were transported to the laboratory on ice and then refrigerated overnight at 4°C. Prior to use, the raw-milk samples were routinely tested for the presence of antibiotics by using Delvotest P ampoules (Gist-Brocades, Delft, Holland) according to the manufacturer's instructions, and a total viable count at 30°C was determined by a standard pour plate method (8).

Preparation of *M. paratuberculosis* inoculum. *M. paratuberculosis* cells were washed from slopes of Herrold's egg yolk medium (HEYM [20]) with approximately 10 ml of phosphate-buffered saline (PBS; ICN Biomedicals Ltd., Thame, United Kingdom), centrifuged (2,400 × *g*, 20 min), and resuspended in PBS to yield a standard suspension containing approximately 10^9 *M. paratuberculosis* cells per ml (determined by nephelometry). Appropriate aliquots of this standard suspension were added to raw milk to yield inoculated milk containing either 10^4 or 10^7 CFU of *M. paratuberculosis* per ml for use in pasteurization trials.

Pasteurization treatments. The inoculated milk was pasteurized in the laboratory by two methods as follows. **(i) Holder method.** Aliquots (5 ml) of inoculated milk were heated in stoppered glass test tubes (150 by 15 mm) by complete immersion in a Grant FE50 water bath (Grant Instruments Ltd., Cambridge, United Kingdom) operating at 63.5 ± 0.5°C. Milk temperature during heating was monitored by means of a thermocouple connected to a Kane-May 1242 recording thermometer (Kane-May Instruments, Welwyn Garden City, United Kingdom). The tubes were agitated until the pasteurization temperature of 63.5°C was attained (90-s "come-up" period) but not during subsequent holding at this temperature for 5, 10, 15, 20, 30, or 40 min. After the appropriate holding times, the tubes were transferred to an ice-water bath and cooled to <10°C prior to sampling.

(ii) HTST method. The time necessary for milk in the HTST pasteurizing units to attain the temperature of 71.7°C was determined for three of the available pasteurizing units by inserting a thermocouple through the rubber gasket below the inlet funnel and recording the temperature of uninoculated milk every 10 s during the heating and cooling phases. Inoculated milk was then HTST pasteurized as follows. The pasteurizing unit was placed in a water bath (Grant type SB3; Grant Instruments Ltd.) operating at 72 ± 0.1°C and allowed to equilibrate to temperature. Inoculated milk (250 ml) was poured into the unit via the inlet funnel. The milk was heated for a total of 70 s (55 s to attain pasteurization temperature plus 15 s holding time), after which the entire apparatus was transferred to a cold-water bath attached to an LKB Bromma 2219 Multitemp II thermostatic circulator (LKB Produkter AB, Bromma, Sweden) running at 6°C. Once cooled, the heat-treated milk was transferred via the outlet spout to a sterile container and held at 4°C until sampled.

Each strain of *M. paratuberculosis* was inoculated into milk and pasteurized by both holder and HTST methods on three separate occasions for each inoculum level (10^4 and 10^7 CFU/ml). Eleven strains were tested in total. In addition, milk inoculated with *M. bovis* T/94/163C (10^4 and 10^7 CFU/ml) was subjected to holder pasteurization at 63.5°C on three separate occasions. After each pasteurization treatment, a subsample of milk was subjected to the phosphatase test (15) to check that proper pasteurization had occurred.

Assessment of viability. Initial inoculum levels achieved and the number of *M. paratuberculosis* cells surviving heat treatment were estimated as follows. Milk samples were serially diluted in Maximum Recovery Diluent (Unipath Ltd., Basingstoke, United Kingdom) as necessary. For each milk sample, aliquots (100 μl per HEYM slope and 500 μl per BACTEC bottle) of a range of appropriate dilutions were inoculated onto HEYM slopes containing mycobactin J (2 μg/ml; Rhone Merieux Ltd., Essex, United Kingdom) and into sealed glass bottles containing 4 ml of BACTEC Middlebrook 12B radiometric medium (Becton Dickinson UK Ltd., Oxford, United Kingdom) supplemented with mycobactin J (2 μg/ml) and 0.5 ml of sterile egg yolk emulsion (Oxoid) (5). For all milk samples, just one slope per dilution was inoculated, except in the case of undiluted milk samples which had received a pasteurization treatment (i.e., 30 min at 63.5°C or 71.7°C for 15 s) when five HEYM slopes were inoculated for the sole purpose of increasing the likelihood of detecting small numbers of *M. paratuberculosis* cells (6). Both media were incubated at 37°C for up to 18 weeks. HEYM slopes were visually examined periodically for the presence of colonies, which was taken as a positive sign of growth (18). By assuming that growth on a HEYM slope at a particular dilution indicated that at least 1 CFU of *M. paratuberculosis* was present, and taking into account any dilution factor, an estimate of the number of *M. paratuberculosis* cells present per ml of milk was obtained. It is recognized that this method of enumerating *M. paratuberculosis* cells is imprecise and underestimates the number of viable organisms present in the milk, but it was chosen in preference to an actual colony count because of the inherent difficulties associated with enumerating colonies on an agar slope. Growth in BACTEC medium was measured every 2 weeks with a BACTEC 460 instrument (Johnston Laboratories, Towson, Md.). A growth index reading greater than 30 was considered presumptively positive. Growth detected in either medium was confirmed as that of acid-fast bacteria by the Ziehl-Neelsen staining procedure (19).

PCR confirmation of survivors. Suspect *M. paratuberculosis* cultures which had survived any of the pasteurization treatments were subcultured to fresh HEYM slopes and incubated at 37°C for several weeks to verify viability. One colony from each HEYM slope was suspended in 50 μl of sterile deionized water and vortexed for 1 min, and the suspension was heated to 100°C for 15 min to release the DNA. For PCR amplification, 5 μl of this suspension was added to 45-μl aliquots of PCR mix (consisting of 50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 1.75 mM $MgCl_2$, 150 μM [each] deoxynucleotide triphosphate, 60 pmol of [each] oligonucleotide primers P90 and P91 [26], and 1.25 U of *Taq* DNA polymerase [Promega]) and overlaid with 2 drops of light mineral oil (Sigma Chemical Co. Ltd). PCR amplification was carried out on a thermal cycler (LEP Scientific). The amplification cycle consisted of an initial denaturation of target DNA at 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 2 min, and primer extension at 72°C for 2 min. The final cycle included extension at 72°C for 7 min and refrigeration. Primers P90 and P91, previously described by Moss et al. (26), were selected to amplify a unique 400-bp fragment of the 5′ region of the insertion element IS*900*, which is specific to *M. paratuberculosis*. Negative controls for both sample prep-

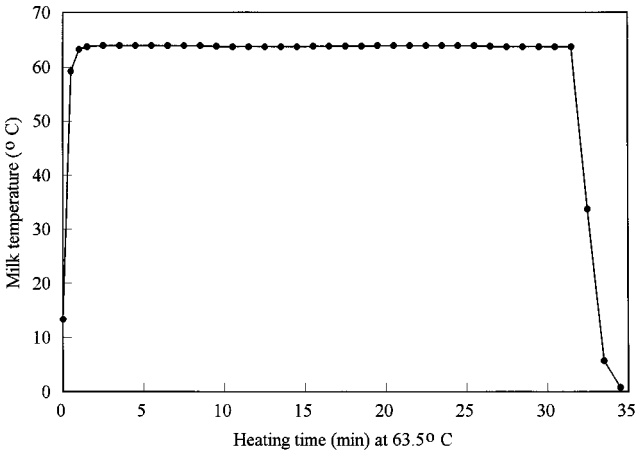

FIG. 1. Heating-cooling curve for holder pasteurization at 63.5°C.

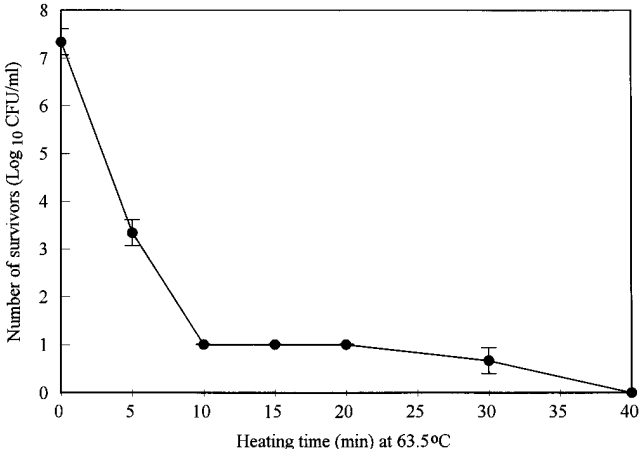

FIG. 2. Thermal death curve for *M. paratuberculosis* ATCC 19698 in milk at 63.5°C. Each point represents the mean of three replicates (± standard error). Some error bars are contained within the symbols.

aration procedure and PCR reagents and a positive PCR control (DNA from a confirmed *M. paratuberculosis* culture) were run in parallel with each series of PCR samples.

IS*900* PCR products were visualized on 2% agarose gels stained with ethidium bromide (0.5 μg/ml), and the sizes of products amplified were determined by utilizing the commercially available molecular weight markers, ϕX174 replicative form DNA-*Hae*III fragments (Gibco BRL).

RESULTS

Properties of milk before heat treatment. The total viable count of the raw milk prior to inoculation ranged from 4×10^1 to 1.2×10^4 CFU/ml, with the majority (80%) of milk samples containing $\leq 10^3$ CFU/ml. All raw-milk samples contained <0.003 IU of penicillin per ml.

Effect of pasteurization by holder method. The heating and cooling profiles for holder pasteurization are shown in Fig. 1. Acid-fast survivors were detected in 27 of 28 (96%) and 14 of 28 (50%) milk samples pasteurized by the holder method when *M. paratuberculosis* was initially present at levels of ca. 10^7 and 10^4 CFU/ml, respectively. All pasteurized milk samples tested negative by the phosphatase test, indicating that proper pasteurization had occurred. Growth was observed both on HEYM and in BACTEC medium after pasteurization, although in general growth was detected earlier by the BACTEC system. It was noticeable that heat-treated *M. paratuberculosis* cells required a longer incubation period than unheated controls before growth was detected by either method, which suggests the existence of sublethally injured cells following pasteurization. A thermal death curve for strain ATCC 19698, typical of all 11 strains studied, is shown in Fig. 2. Results suggest that the majority of the *M. paratuberculosis* population was killed within 5 to 10 min at 63.5°C, but a few cells (ca. 10 CFU/ml [Table 2]) survived heating at 63.5°C for 30 min. In contrast, the *M. bovis* strain tested was inactivated within 20 min at 63.5°C when 10^7 CFU/ml were present initially (Fig. 3) and was inactivated within 10 min at 63.5°C when 10^4 CFU/ml were present.

Effect of pasteurization by the HTST method. Heating and cooling profiles for three HTST pasteurizing units are shown in Fig. 4. These indicate a come-up time at 71.7°C of 55 s, which is in agreement with Franklin's original work (12). Acid-fast survivors were detected in 29 of 34 (85%) and 19 of 33 (55%) milk samples pasteurized by the HTST method when *M. paratuberculosis* was initially present at levels of ca. 10^7 and 10^4 CFU/ml, respectively. All HTST-pasteurized milk samples tested negative by the phosphatase test, indicating that proper pasteurization had occurred. A 10^5- to 10^6-fold reduction in numbers of *M. paratuberculosis* cells was achieved by HTST pasteurization when 10^7 CFU/ml were present initially (Table 3). When 10^4 CFU/ml were present initially, a 10^2- to 10^3-fold reduction was observed.

Confirmation of survivors as viable *M. paratuberculosis* cells. Acid-fast survivors were isolated from milk samples pasteurized by both the holder and HTST methods. Confirmation that these survivors were *M. paratuberculosis* cells was obtained by IS*900*-based PCR (Fig. 5).

DISCUSSION

Although to date there is no conclusive proof, *M. paratuberculosis* may be implicated in the etiology of CD in humans. Interest in the thermal inactivation of *M. paratuberculosis* cells in milk at pasteurization temperatures has arisen in recent years as a consequence of the suggestion that milk may be a vehicle by which *M. paratuberculosis* could be transmitted to humans (13). The present study was undertaken to determine whether *M. paratuberculosis* is able to survive milk pasteurization. Viable *M. paratuberculosis* cells were isolated from milk samples pasteurized by both the holder and HTST methods of pasteurization, which suggests that these heat treatments may not be sufficient to eliminate *M. paratuberculosis* from milk if the organism is present in high numbers. Similar findings were reported by Chiodini and Hermon-Taylor (2), who carried out a limited study of the heat resistance of four strains of *M. paratuberculosis* (including strains ATCC 19698 and ATCC 43015) in milk under conditions simulating pasteurization. These researchers reported 5 to 9% survival of *M. paratuberculosis* after holder pasteurization (63°C, 30 min) and 3 to 5% survival after HTST pasteurization (72°C, 15 s) for the two bovine strains tested and concluded that heat treatments simulating pasteurization were not completely effective against *M. paratuberculosis*. Results of the present study indicate survival of *M. paratuberculosis* after pasteurization at levels (≤1%) much lower than those suggested by Chiodini and Hermon-Taylor (2). Neither the present study nor Chiodini and Hermon-Taylor's study (2) employed commercial HTST pasteurization equipment or a pilot-scale version of such. Consequently, the findings of both studies cannot be considered to indicate resistance of *M. paratuberculosis* to commercial HTST pasteurization. In the case of the present study, we

TABLE 2. Effect of holder pasteurization (63.5°C for 30 min) on strains of *M. paratuberculosis*

Strain	No. of *M. paratuberculosis* cells (log_{10} CFU/ml ± SD)[a] at indicated inoculum level			
	10^7 CFU/ml of milk		10^4 CFU/ml of milk	
	Prepasteurization	Postpasteurization	Prepasteurization	Postpasteurization
ATCC 19698	7.3 ± 0.6	1.0 ± 0.0	4.0 ± 0.0	1.3 ± 0.6
M8	7.0 ± 0.0	1.0 ± 0.0	4.0 ± 0.0	0.5 ± 0.7
B4	6.3 ± 0.6	1.0 ± 0.0	3.7 ± 0.6	0.3 ± 0.6
NCTC 8578	6.7 ± 1.2	1.0 ± 0.0	4.0 ± 0.0	0.3 ± 0.6
B1	6.0 ± 0.0	0.7 ± 0.6	3.0 ± 1.2	1.0 ± 0.0
B2	6.0 ± 0.0	1.0 ± 0.0	3.0 ± 0.0	1.0 ± 0.0
DVL 943	7.0 ± 0.0	1.0 ± 0.0	3.7 ± 0.6	0.3 ± 0.6
USDA 1038	6.6 ± 0.7	1.0 ± 0.0	4.0 ± 0.0	ND[b]
USDA 1113	6.6 ± 0.7	1.0 ± 0.0	3.0 ± 0.0	0.5 ± 0.7
ATCC 43015	6.0 ± 0.0	1.0 ± 0.0	3.7 ± 0.6	0.3 ± 0.6
M9	6.3 ± 1.2	1.0 ± 0.0	3.3 ± 0.6	0.7 ± 0.6

[a] Mean number of *M. paratuberculosis* cells in triplicate milk samples.
[b] ND, no survivors detected in any of three pasteurized milk samples.

are satisfied that the heating and cooling profiles of the heat treatments employed closely simulate the temperature profiles of commercial-batch and HTST pasteurization processes published recently (14). The main difference between the HTST pasteurizing apparatus used in the present study and a commercial HTST pasteurizer is that the milk was static during heating in the laboratory, whereas turbulent flow would occur in a commercial HTST pasteurizer. It is possible that greater thermal inactivation would be achieved under turbulent-flow conditions.

The number of *M. paratuberculosis* cells which might naturally be encountered in cows' milk has not been firmly established. To our knowledge, only two studies of the incidence of the organism in the milk of cattle with JD have been reported. Taylor et al. (28) found detectable quantities of *M. paratuberculosis* cells in the milk of cows showing clinical signs of JD, but no titer was reported. A more recent study by Sweeney et al. (27) reported an *M. paratuberculosis* titer of 2 to 8 CFU per 50 ml of milk from subclinically affected animals. This latter figure suggests a low incidence of the organism in milk of asymptomatic cows, which are thought to outnumber symptomatic cattle in infected herds (4). However, opportunity for fecal contamination during the milking process exists if due care is not taken by milking personnel, and as the concentration of *M. paratuberculosis* cells in the feces of a cow with JD may exceed 10^8 CFU/g (3), this low natural level could be significantly augmented. Inoculum levels of ca. 10^4 and 10^7 CFU of *M. paratuberculosis* cells per ml of milk were employed in the present study. The higher inoculum level (10^7) could be considered excessive relative to the natural field situation but was included in order to obtain a valid thermal death curve for the organism, as it has been suggested that thermal death information derived from survivor curves which traverse only 4 or 5 log_{20} cycles could be misleading (25). The lower inoculum level of 10^4 CFU/ml is certainly more realistic than 10^7 CFU/ml and is not inconceivable if fecal contamination were to occur. However, even this lower inoculum level probably still represents a worst-case situation.

The thermal death curve obtained for *M. paratuberculosis* was concave in shape, exhibiting a rapid initial death rate followed by significant "tailing," which indicated survival at low levels after pasteurization. Previous studies of the thermal inactivation of *Mycobacterium avium-Mycobacterium intracellulare* (21, 22) and *M. bovis* (23) have yielded linear thermal death curves. It has been reported that the methodology employed in studies to determine whether a particular organism survives pasteurization can have a significant effect on the

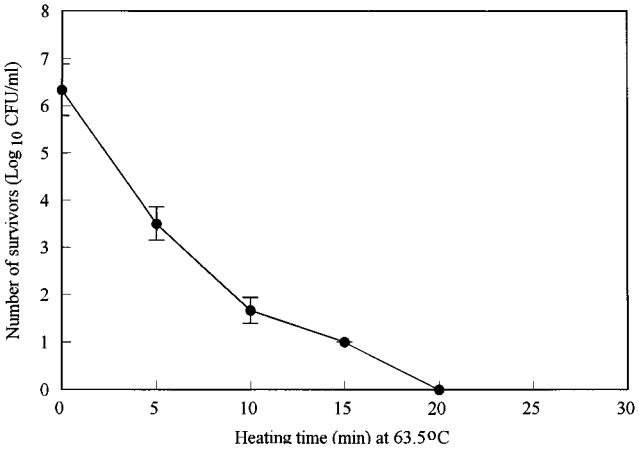

FIG. 3. Thermal death curve for *M. bovis* T/94/163C in milk at 63.5°C. Each point represents the mean of three replicates (± standard error). Some error bars are contained within the symbols.

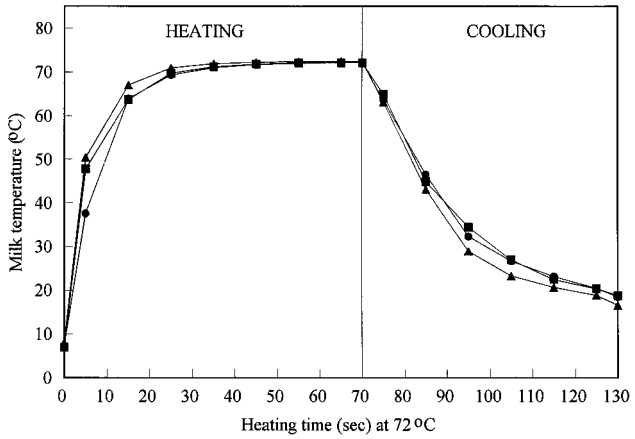

FIG. 4. Heating and cooling curves of three HTST pasteurizing units in a water bath at 72°C.

TABLE 3. Effect of HTST pasteurization (71.7°C for 15 s) on strains of *M. paratuberculosis*

Strain	No. of *M. paratuberculosis* cells (log_{10} CFU/ml ± SD)[a] at indicated inoculum level			
	10^7 CFU/ml of milk		10^4 CFU/ml of milk	
	Prepasteurization	Postpasteurization	Prepasteurization	Postpasteurization
ATCC 19698	7.3 ± 0.6	2.7 ± 0.6	4.0 ± 0.0	2.0 ± 0.0
M8	7.0 ± 0.0	2.7 ± 0.6	4.0 ± 0.0	1.7 ± 0.6
B4	7.0 ± 0.0	1.7 ± 1.2	3.7 ± 0.6	1.3 ± 1.2
NCTC 8578	6.7 ± 0.6	1.7 ± 1.2	4.0 ± 0.0	1.0 ± 1.2
B1	7.0 ± 0.0	1.7 ± 0.6	3.3 ± 0.6	0.3 ± 0.6
B2	6.5 ± 0.7	2.0 ± 1.4	3.7 ± 0.6	1.0 ± 0.0
DVL 943	7.0 ± 0.0	1.0 ± 1.7	4.0 ± 0.0	ND[b]
USDA 1038	7.0 ± 0.0	1.0 ± 0.0	3.0 ± 0.0	0.5 ± 0.7
USDA 1113	7.0 ± 0.0	1.0 ± 0.0	3.5 ± 0.7	ND
ATCC 43015	6.3 ± 1.2	0.7 ± 0.6	3.0 ± 1.4	0.5 ± 0.7
M9	5.7 ± 1.2	0.3 ± 0.6	3.3 ± 0.6	0.7 ± 0.6

[a] Mean number of *M. paratuberculosis* cells in triplicate milk samples.
[b] ND, no survivors detected in any of three pasteurized milk samples.

shape of resulting thermal death curves. A study by Donnelly et al. (7) compared a test tube method and a sealed-tube method to assess the heat inactivation of *Listeria monocytogenes* in milk at 62°C. When the test tube method, in which test tubes containing inoculated milk (10 ml) were placed in a water bath at 62°C so that the surface of the milk was 4 cm below the water level in the bath, was used, concave thermal death curves were obtained for *L. monocytogenes*. These exhibited an initial 10^3- to 10^4-fold kill within 5 min, which was followed by a further small decrease to a stable population of 10^2 to 10^3 surviving cells which persisted beyond heating times established for milk pasteurization. This rapid initial kill and this long tail were also observed when inoculated milk was heated at 72, 82, and 92°C. In contrast, when a sealed-tube method, in which inoculated milk (1.5 ml) was heated in 2-ml glass reaction vials (crimp sealed with metal caps containing teflon-lined seals) totally immersed in a water bath at 62°C, was employed, Donnelly et al. (7) obtained linear thermal death curves for *L. monocytogenes* and complete inactivation by pasteurization. The method employed in the present study to pasteurize milk at 63.5°C for 30 min could be considered to be a combination of the two methods compared by Donnelly et al. (7), viz., inoculated milk was heated in stoppered test tubes which were totally immersed, not just standing in, a water bath at 63.5°C. The fact that the test tubes were completely immersed in water should have ensured that all cells were exposed to thermal inactivation temperatures throughout heating even if milk was splashed on the sides of the tube. However, the resulting thermal death curves for *M. paratuberculosis* were not linear as expected but were similar to those of *L. monocytogenes* obtained by Donnelly et al. (7) by the test tube method. This indicates that tailing of the thermal death curve of *M. paratuberculosis* is not an artifact of the heating method employed.

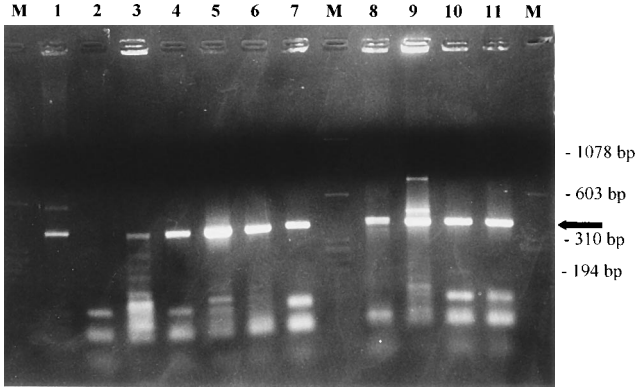

FIG. 5. Gel electrophoresis analysis of PCR products obtained from DNA of survivors of holder and HTST pasteurization with IS*900*-derived primers. The 400-bp PCR product specific for *M. paratuberculosis* is indicated (arrow). Lanes 1 and 2, PCR positive and negative controls, respectively; lanes 3 to 7, survivors of HTST pasteurization (strains ATCC 19698, B2, NCTC 8578, M8, and ATCC 43015, respectively); lanes 8 to 11, survivors of holder pasteurization (strains B1, M9, ATCC 19698, and B4, respectively). M, molecular weight markers ϕX174 replicative-form DNA-*Hae*III.

In the past, tailing of thermal death curves has frequently been attributed to the presence of a particularly heat-resistant fraction of the population under study (25). Bacterial cells are known to be more heat resistant while in the stationary phase of growth and less heat resistant in the logarithmic phase (16). As the suspension of *M. paratuberculosis* cells used to inoculate raw milk in the present study was prepared from cells washed from a slope rather than from a broth culture, it is conceivable that cells at different stages of growth, and hence with different heat sensitivities, may have been present. This may partially explain the tailing observed. It has also been suggested that tailing of thermal death curves might result from clumping of bacteria or spores during heating (10, 25). If this were the case, then the initial rapid drop in numbers of *M. paratuberculosis* cells observed may not represent a true kill but, instead, aggregation of cells into clumps, each clump of cells rather than each individual cell giving rise to a colony on HEYM. Clumping is a characteristic of *M. paratuberculosis*, and it is possible that clumping of the cells was favored during heating or cooling because, firstly, the milk was not agitated once the pasteurization temperature had been attained, and secondly, natural agglutinins present in milk may encourage clumping (17). We speculate that clumping of cells is primarily responsible for tailing of the thermal death curve of *M. paratuberculosis*, and we are currently undertaking further studies to provide evidence in support of this hypothesis.

Our results indicate that heat treatments simulating pasteurization under laboratory conditions are not sufficient to completely eliminate viable *M. paratuberculosis* cells from milk when the organism is present in large numbers (10^4 to 10^7 CFU/ml). The high inoculum levels of *M. paratuberculosis* employed in this study were chosen to represent a worst-case

scenario, and in reality, such high levels are probably unlikely to be encountered in milk which is to be commercially pasteurized. It must be emphasized that the results of this study are not sufficient to establish resistance of *M. paratuberculosis* to commercial pasteurization. Only if the findings of this study are corroborated by additional studies employing continuous-flow HTST equipment might possible changes to current pasteurization criteria need to be considered. The number of *M. paratuberculosis* cells which might be encountered in naturally infected milk which is to be pasteurized must also be established.

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