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Effect of Pasteurization on Survival of *Mycobacterium paratuberculosis* in Milk

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ABSTRACT

Mycobacterium paratuberculosis (Mptb) is the causative agent of Johne's disease of ruminant animals including cattle, goats, and sheep. It has been suggested that this organism is associated with Crohn's disease in humans, and milk is a potential source of human exposure to this organism. A total of 18, including 7 regular batch and 11 high temperature short time (HTST) pasteurization experiments, were conducted in this study. Raw milk or ultra-high temperature pasteurized milk samples were spiked at levels of 10^3, 10^5, and 10^7 cfu of Mptb/ml. *Escherichia coli* and *Mycobacterium bovis* BCG strains at 10^7 cfu/ml were used as controls. Pasteurization experiments were conducted using time and temperature standards specified in the Canadian National Dairy Code: regular batch pasteurization method: 63°C for 30 min, and HTST method: 72°C for 15 s. The death curve of this organism was assessed at 63°C. No survivors were detected after 15 min. Each spiked sample was cultured in Middlebrook 7H9 culture broth and Middlebrook 7H11 agar slants. Samples selected from 15 experiments were also subjected to BACTEC culture procedure. Survival of Mptb was confirmed by IS*900*-based PCR of colonies recovered on slants. No survivors were detected from any of the slants or broths corresponding to the seven regular batch pasteurization trials. Mptb survivors were detected in two of the 11 HTST experiments. One was by both slant and broth culture for the sample spiked to 10^7 cfu/ml of Mptb, while the other was detected by BACTEC for the sample spiked to 10^5 cfu/ml. These results indicate that Mptb may survive HTST pasteurization when present at $\geq 10^5$ cfu/ml in milk. A total of 710 retail milk samples collected from retail store and dairy plants in southwest Ontario were tested by nested IS*900* PCR for the presence of Mptb. Fifteen percent of these samples (n = 110) were positive. However, no survivors were isolated from the broth and agar cultures of 44 PCR positive and 200 PCR negative retail milk samples. The lack of recovery of live Mptb from the retail milk samples tested may be due to either the absence of live Mptb in the retail milk samples tested or the presence of low number of viable Mptb which were undetected by the culture method used in this study.
(**Key words:** *Mycobacterium paratuberculosis*, pasteurization, milk, polymerase chain reaction)

Abbreviation key: CD = Crohn's disease, **JD** = Johne's disease, **Mptb** = *Mycobacterium paratuberculosis*.

INTRODUCTION

Mycobacterium paratuberculosis (**Mptb**) is the etiological agent of Johne's disease (**JD**) or paratuberculosis, a chronic progressive disease principally involving the lower small intestine and affecting ruminants and other animals (Chiodini and van Kruiningen, 1985; Beard et al., 2001). Although the causal link has not been proven, Mptb is speculated by some to be a potential etiologic agent implicated in Crohn's disease (**CD**) in humans (Chiodini, 1989; Sanderson et al., 1992; Dell'Isola et al., 1994; Hermon-Taylor et al., 1998). Evidence in support of a role of Mptb in CD includes similarity in pathological features of CD and JD (Chiodini, 1989). The bacterium was cultured or its DNA has been identified from tissue biopsy of patients with CD (Sanderson et al., 1992; Hermon-Taylor et al., 1998; Schwartz et al., 2000). Recently, Mptb bacterium was cultured from the breast milk of a patient with active CD (Naser et al., 2000). However, many studies have failed to detect the bacterium or its DNA in biopsies from CD patients (Chiba et al., 1998; Kanazawa et al., 1999).

The prevalence of Mptb in Ontario dairy cattle has been estimated to be 6.1% (McNab et al., 1991). The prevalence of Mptb was 2.6% in New Brunswick, Nova Scotia, and Prince Edward Island. At the herd level,

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16.7% of herds in these provinces had at least two Mptb-positive cows (VanLeeuwen et al., 2001). Infection in most cases remains subclinical for several years before the development of clinical symptoms including diarrhea, weight loss, and decreased production of milk. Infected animals shed organisms in feces and milk, with those in the late subclinical and clinical phases shedding much higher numbers. Milk is involved in the transmission of JD to young calves (Sweeney, 1996). Detectable quantities of this organism have been found in milk from both clinically affected cattle (Taylor et al., 1981) and asymptomatic carriers (Sweeney et al., 1992; Streeter et al., 1995). Consequently, raw milk is suggested as a potential vehicle of transmission of Mptb to humans (Kubica, 1984; Hermon-Taylor et al., 2000).

There are reports that Mptb bacteria were inactivated by the current HTST pasteurization methods (Stabel et al., 1997; Keswani and Frank, 1998). On the other hand, a number of studies demonstrated that Mptb was not completely inactivated by pasteurization (Chiodini and Hermon-Taylor, 1993; Meylan et al., 1996; Grant et al., 1996, 1998a, 2002; Stabel et al., 1997; Sung and Collins, 1998; (batch pasteurization method). The ability of Mptb to survive commercial pasteurization process remains controversial. A study on the detection of Mptb in retail pasteurized whole milk in southern England and South Wales showed that 7% of the 312 samples tested were positive for Mptb by PCR, and 50% of the PCR-positive samples and 16% of the PCR negative samples tested positive after 13 to 40 mo of incubation (Millar et al., 1996). A recent study in the United Kingdom provided the evidence that Mptb bacteria in naturally infected milk are capable of surviving commercial HTST pasteurization if they are present in raw milk in sufficient numbers (Grant et al., 2002). The objectives of the current study are 1) to determine the survival of Mptb in raw milk after laboratory simulated batch pasteurization or HTST pasteurization, and 2) to determine the prevalence of Mptb in retail milk supply in Ontario by IS*900* PCR and culture.

MATERIALS AND METHODS

Milk Samples

Raw milk collected from dairy farms in Ontario was used for all the pasteurization and death curve experiments. To study whether retail milk was contaminated with Mptb, a total of 710 pasteurized milk samples, including 31 samples from ultra-filtered milk, were collected from outlets in southwestern Ontario. Of these, 175 samples were from dairy plants in Ontario, and 535 samples were purchased from local retail stores. All samples were screened for the presence of Mptb DNA using IS*900* PCR-assays. All PCR-positive samples and a selection of PCR-negative samples were screened for the presence of live Mptb by culture on the Middlebrook broth and agar and by BACTEC 12B radiometric culture.

Bacterial Strains, Growth Media, and Preparation of Bacterial Suspension

Mptb ATCC 19698 was used in this study to spike the raw milk samples. Whenever indicated, *Escherichia coli* (ATCC 25922), and *M. bovis* BCG laboratory strains were used as controls. Mptb was grown in Middlebrook 7H9 broth and Middlebrook 7H11 agar slants. The basic 7H9 broth contained, per liter, 4.7 g of Difco Middlebrook broth and 0.25 g Tyloxapol (Sigma). The basic 7H11 agar contained, per liter, 21 g of Difco Middlebrook 7H11 agar and 100 g of egg yolk (from about six large-size eggs). To each liter of the basic broth or agar media was added, 5 ml Difco glycerol, 2 mg of Mycobactin J, 100,000 u of penicillin G, 50 mg of chloramphenical, and 100 ml of Difco OADC. *Escherichia coli* were grown on basic 7H11 agar slants without supplements. *Mycobacerium bovis* BCG was cultured on 7H11 agar slants containing OADC only. To prepare working dilutions of the bacteria, Mptb ATCC 19698 cells were harvested from mid- to late-logarithmic growth culture (generally 2-to-3 month old cultures) from either Middlebrook 7H9 broth culture or the 7H11 agar slants.

Colonies growing on the slants were scraped and suspended in PBS (pH 7.2). Bacteria were harvested by centrifugation at 2400× *g* for 20 min. The bacterial cell pellets were suspended in PBS to yield standard bacterial suspension of approximately 10^9 cfu/ml, using McFarland standards. To disrupt aggregates of the Mptb cells, the suspension was passed several times through a 22G needle before the suspension was standardized.

HTST Simulation and Batch Pasteurization Procedures

Pasteurization experiments were conducted as described by Grant et al. (1996). Pasteurization conditions used in this study were 63°C for 30 min for the regular batch method, and 72°C for 15 s for the HTST method as specified in the Canadian National Dairy Code (Anonymous, 1997). The cfu/ml of Mptb used for spiking milk was determined as previously described (Odumeru et al., 2001). Serial dilutions (1:10) of bacterial suspension corresponding to McFarland 10^9 cfu/ml were prepared, and 100 μl was plated on 7H11 Middlebrook agar slants. The slants were incubated at 37°C until colonies

were visible after around 3 mo. In this study, a suspension of Mptb cells corresponding to McFarland 10^9 cfu/ml was determined to contain 2.3 x 10^8 cfu/ml (n = 18) based on plate count results.

For each experimental trial, seven screw cap tubes (13 x 105 mm) each containing 2 ml of raw milk were used. Tubes 1 to 3 were spiked with 20 μl of 10^5, 10^7, and 10^9 cfu/ml Mptb, respectively, to give final concentrations of 10^3, 10^5 and 10^7 cfu/ml. Tubes 4 and 5 were spiked with 10^7 cfu/ml of *M. bovis* and *E. coli*, respectively. Tube 6 was spiked with saline. A thermometer was placed in tube 7 through a rubber plug and used to monitor the temperature of the milk throughout the duration of the experiment. The tubes were held on a rack and placed in ice water before being placed in a water bath incubator (Julabo SW-20C incubator, Johns Scientific, Inc., Toronto, Canada). All tubes were completely immersed in the water, and the bath was agitated at 100 rpm/min. Once the contents of the tubes, as indicated by the thermometer in tube 7, achieved the desired temperature (63°C for regular batch method and 72°C for HTST method), the tubes were held at that temperature for the required time (30 min for batch method and 15 s for HTST method). Subsequently, the tubes were transferred to ice water and cooled to ≤ 4°C. Pasteurized milk samples were cultured as described below under section on Culture of Mptb from Heat-Treated and Laboratory-Pasteurized Milk.

Death Curve

Death curve experiments were conducted using the batch pasteurization procedure. For these experiments, raw milk samples were inoculated with Mptb to a final concentration of 10^7 cfu/ml as described above and placed in 63°C water bath. Controls included raw milk samples spiked with *E. coli* or *M. bovis* and an unseeded milk sample. A temperature monitoring tube was also included. Once the temperature of the milk samples reached 63°C, the samples spiked with Mptb were removed one tube at a time at 0, 5, 10, 15, 20, 30 and 40 min incubation and immediately placed in ice water. The control samples, spiked with *E. coli* and *M. bovis*, and the blank control were all removed after 30 min incubation. These samples were cultured onto Middlebrook 7H11 agar slant, into Middlebrook 7H9 broth, and incubated at 37°C.

Culture of Mptb from Heat-treated and Laboratory-Pasteurized Milk

In this study, a total of 18 pasteurization experiments (seven batch and 11 HTST) and three death curve experiments were performed. All samples were inoculated on Middlebrook 7H11 agar slants and 7H9 broths, and IS*900* PCR was employed to confirm the presence of Mptb cells. Five Middlebrook 7H11 slants and five Middlebrook 7H9 broths were inoculated for each milk sample spiked with Mptb in the first seven pasteurization experiments and three death curve experiments. Three slants and three broths were used to culture each milk sample for the 11 pasteurization experiments.

A 100-μl milk sample was inoculated onto each 7H11 agar slant and into 10 ml of 7H9 broth. The 7H11 agar slant without antibiotics and mycobactin J was used for *E. coli*, and 7H11 agar slant with OADC enrichment only was used for *M. bovis*. All slants and broths were incubated at 37°C for at least 5 to 6 mo. Samples from 3-mo-old broth cultures were plated onto slants and colonies of surviving Mptb enumerated. Colonies growing on the slants were subjected to PCR to detect the presence of IS*900* DNA.

Preparation of Template DNA for PCR

Template DNA was prepared from 1 ml of each milk sample tested. To determine the efficiency of template DNA preparation from milk, each experimental run also included positive and negative control samples. The positive control sample was spiked to the level of 100 cfu/ml of Mptb organisms, which was the limit of detection of a reliably repeatable nested PCR assay as evaluated in our laboratory (unpublished data). The negative control was spiked with *M. phlei* to about 10^7 cfu/ml in water.

One-milliliter milk samples were centrifuged using a Beckman J2-M1 centrifuge with JA 18.1 rotor at 42,000×*g* (18,000 rpm) for 1.5 h. The whey fraction was discarded, and the pellet and cream fractions were pooled for further testing. The pooled pellet and cream were transferred to a 2.0-ml screw cap tube containing 0.8 ml of lysis buffer (10 mm Tris-Cl, 10 mm EDTA, 0.5% Tween 20, 0.5% Triton X-100, 1 m guanidinium thiocyanate, 0.3 m sodium acetate, pH 7.5). To facilitate breakage and release of DNA from the Mptb bacterial cells contained in the milk pellet and cream fractions, two methods were employed, a freeze-thaw method and mechanical grinding using the beadbeater (BioSpec, Bartlesvillen, OK) (Odumeru et al., 2001).

In the freeze-thaw method, tubes containing the milk pellets or cream were subjected to five rounds of 2 min in liquid nitrogen followed by 2.5 min in hot water (90°C). The samples were then incubated for 20 min in a boiling water bath and incubated for at least 5 min in ice water. The samples were centrifuged for 5 min at 14,000 rpm using a microcentrifuge. Then 0.65 ml of the supernatant was transferred to a fresh 1.5-ml microcentrifuge tube containing 0.65 ml of 100%

isopropanol. The contents were mixed by inverting the tube several times and incubated at room temperature (21 to 25°C) for 10 min. After centrifugation at 14,000 rpm for 10 min, the supernatants were carefully aspirated. A 0.5-ml volume of 75% isopropanol was added to the pellet, and the mixture was incubated for 10 min at room temperature. Following centrifugation, the supernatant was discarded. The pellets obtained contained the template or chromosomal DNA. The DNA was suspended in 70 μl of water by being incubated at 95°C for 3 min. Template DNA was used for PCR immediately after preparation or stored at -20°C. In this study, the freeze-thaw method was used for Mptb DNA preparation from the first 500 milk samples.

In the course of this study, we developed a bead-beater cell disruption method, which was shown to be very effective in breaking up Mptb cells and releasing bacterial DNA (Odumeru et al., 2001). The bead-beater method was employed for the last 210 milk samples tested. The pooled pellet and cream were transferred to a 2.0-ml screw cap tube containing about 0.6 g of 0.1 mm zirconia/silica beads and 0.8 ml of lysis buffer. The tubes were placed in the Mini-Beadbeater, and contents subjected to mechanical grinding at 5000 rpm for 5 min. The samples were incubated for 20 min in a boiling water bath and the DNA extracted by isopropanol precipitation as described in the freeze-thaw protocol.

Nested PCR for Detection of Mptb-Specific IS*900* DNA

Template DNA prepared from milk by the freeze-thaw or bead-beater protocols was subjected to IS*900* PCR using a modification of protocol described by Millar et al. (1996). All assays were performed using GeneAmp system thermocycler (Perkin Elmer, Shelton, CT). For each PCR experiment, we also included a positive sample containing purified Mptb DNA and three negative control samples containing *M. phlei* DNA. These samples were used as internal controls of the IS*900* assay for the PCR reaction. The PCR reaction mix (50 μl) contained 0.2 mm each of the four dNTP, 0.2 μm each of the two primers P90$^+$and P91$^+$, 1.5 mm $MgCl_2$, 1 U of *Taq* polymerase (Roche, Montreal, Canada), and 20 μl of template DNA. The thermocycler was programmed for one cycle at 94°C x 5 min and 40 cycles of 93°C x 2 min, 58°C x 1 min, and 72°C x 3 min, with a final extension at 72°C x 10 min. A product of 413 bp was obtained from this first round of PCR. In the nested PCR reaction, the above PCR conditions were used with following exceptions: 1) primers P25 and P26 (Millar et al., 1996), generating an internal 219-bp product from the first round PCR reaction, were used; 2) 1 μl of the first PCR product served as template in a 15-μl reaction; and 3) programmed for 30 cycles. The nested PCR products were separated using a 1.6% agarose gel at 5 V/cm x 30 min, stained with ethidium bromide, and visualized using the Gel Doc 1000 system (Bio-Rad, Hercules, CA).

Precautions Taken to Avoid Contamination of Samples Used for PCR Reactions

Stringent precautions were taken to avoid cross-contamination of individual samples with extraneous DNA. The PCR work was conducted in five separate laboratories. PCR premix was prepared in an access-restricted room where all the stock reagents were stored. DNA was not allowed in this room. Pasteurization experiments and template DNA preparation were conducted in laboratory with a class 2 bio-containment cabinet. Direct PCR was set up in the third laboratory. Nested PCR was set up in the fourth, and gel was run in the fifth. In other words, the sample flow was unidirectional, with no processed samples brought back to a previous laboratory once removed. The PCR thermocyclers were located in a separate room away from the main laboratory area. Laboratory coats were changed between rooms, and gloves were changed often to prevent cross-contamination. All clean pipette tips, tubes, and gels were packed separately, all contact with soiled labware was stringently controlled, and all materials were separately sterilized. Working bench and tube racks were routinely washed and decontaminated by ELIMINase (Fisher, Canada) after use daily.

Detection of Mptb from Retail Milk Samples

A total of 710 retail milk samples were screened for contamination with Mptb DNA and the live bacteria. Triplicates were subjected to IS*900* PCR from one DNA preparation of each sample. In the first set of 500 retail milk samples, samples in which two or three of the subsamples were PCR-positive were subjected to culture on Middlebrook agar and broth for recovery of the live Mptb bacteria. This was accomplished by inoculating pellet and cream fractions obtained by centrifugation of 1 ml of milk into 10 ml of broth, while same fractions from 0.5 ml of milk were used to inoculate slants. At least two broths and two slants were inoculated for each selected sample. Five negative samples were also cultured for comparison. A total of 208 samples were cultured from the last 210 milk samples. In an effort to enhance the recovery from milk of any contaminating Mptb bacteria from these 208 samples, pellet and cream were obtained from 5 ml of milk and pooled after centrifugation. One-fifth of the pooled sample was used for DNA template preparation for PCR,

Table 1. Summary of pasteurization experiments.

Type of method	Batch (63°C, 30 min)			HTST (72°C, 15s)		
Spiking level (cfu/ml)	10^3	10^5	10^7	10^3	10^5	10^7
Number of experiments	7	7	7	11	11	11
With survivors recognized	0	0	0	0	1[1]	1[2]

[1]Survivors were detected by BACTEC test, not by culture on slants and in broths.

[2]*Mycobacterium paratuberculosis* survivors detected both in broth culture and on slants. Numbers of survivors on all 5 inoculated slants were 11, 10, 11, 7, and 4, respectively.

and the remaining sample was inoculated in 10 ml of Middlebrook 7H9 broth. After 2 to 3 mo of incubation, 5 ml of each broth sample was centrifuged, and the pellets were used for DNA preparation and subjected to IS*900* PCR as described above. To confirm the presence of viable bacteria, all PCR-positive samples were plated on 7H11 agar slants, incubated for 4 to 6 mo and IS*900* PCR performed on the isolated single colonies.

Detection of Live Mptb by BACTEC Radiometric Culture

BACTEC 12B radiometric culture system was used to detect the presence of live Mptb cells in samples from all three death curve experiments, four of the first seven pasteurization experiments, all of the last 11 pasteurization experiments, and 31 PCR-positive and four PCR-negative retail milk samples. All of these resulted in a total of 114 samples plus controls.

For these assays, pooled pellet and cream fractions of milk samples cultured in Middlebrook broth for 1 wk to 19 mo were used. One milliliter of the broth enrichment cultures was centrifuged for 30 min, and the pellet suspended in 0.2 ml of 7H9 broth and injected into each BACTEC 12B bottle.

The BACTEC 12B radiometric mycobacteria culture bottles contained 4 ml of a modified 7H9 broth supplemented with [^{14}C]-labeled fatty acid substrate (Becton, Dickinson, Mississauga, ON, Canada). Immediately before inoculation with the samples, the bottles were flushed with CO_2, and the supplements containing Mycobactin J and PANTA PLUS antibiotic cocktail were added. To prepare the supplements, mycobactin J (0.45 mg dissolved in 450 μl 100% ethanol) was added to a bottle containing 5 ml of BACTEC PANTA PLUS media (Becton, Dickinson) and mixed. One hundred microliters of the PANTA PLUS-Mycobactin J mixture was injected to each BACTEC 12B bottle. Vials were inoculated with 0.2 ml of sample, and control vials contained 0.2 ml each of 7H9 broth contained heat-killed bacteria or a known cfu/ml of viable Mptb. The BACTEC bottles were packed in Safety-pack containers and sent to the laboratory of M. Behr (McGill University) for testing using the BACTEC 460 system. Live (myco)bacterial cells in the sample utilize the [^{14}C]-labeled fatty acid, releasing [^{14}C]-labeled CO_2 into the gas phase above the culture media. In the BACTEC 460 system, the gas phase from each vial is automatically sampled, and small increases in released CO_2 are detected. Due to the sensitivity of the assay, and since metabolic activity is not dependent on cell division, viable bacteria can be detected before detection of visible colonies on agar (Sung and Collins, 1998). For this project, all BACTEC culture samples were incubated and growth screened over a 16-wk period.

RESULTS AND DISCUSSION

Survivors after Pasteurization of Mptb-Seeded Milk

Live Mptb survivors were detected within 3 mo of incubation at 37°C on both Middlebrook agar slants and broth cultures in one of the 11 HTST pasteurization experiments initially spiked with 10^7 Mptb per milliliter (Table 1). Another sample, which had been spiked with 10^5 of Mptb per milliliter prior to pasteurization and showed no survivors on agar slants and broth cultures, showed a positive result by BACTEC after 4 wk of incubation. There were no Mptb survivors found on any of the slants, broth cultures, and BACTEC tubes from the regular batch experiments. The batch-pasteurized milk samples remained negative for viable Mptb after 12 to 15 mo of incubation in Middlebrook 7H9 broth followed by plating on Middlebrook 7H11 agar slants and incubation at 37°C for 10 to 12 mo. Total incubation time was 22 to 27 mo.

Mptb cells surviving pasteurization have been reported in a number of publications (Chiodini and Hermon-Taylor, 1993; Grant et al., 1996, 1998a, 1998b, 1999, 2002; Hope et al., 1996; Stabel et al., 1997 [batch pasteurization method]; Sung and Collins, 1998). Other studies found no live Mptb after pasteurization (Stabel et al., 1997 [HTST method]; Keswani and Frank, 1998). Among these, Grant et al. (2002) for the first time reported that naturally infected milk is capable of surviving small-scale commercial HTST pasteurization, although this study may not be entirely representative of large-scale dairy processing operations. Our results

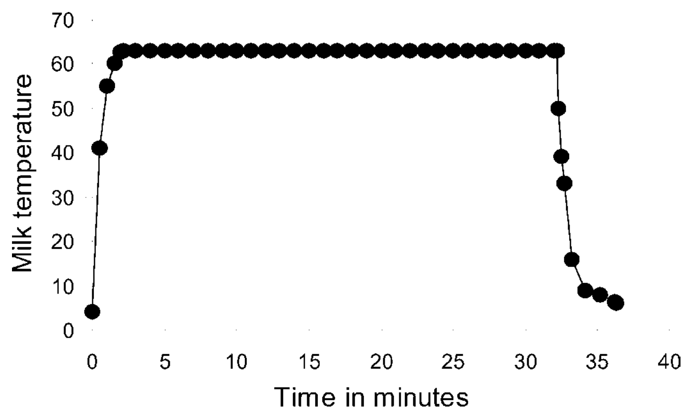

Figure 1. Heating and cooling curve of regular batch pasteurization at 63°C.

confirmed the work of Grant et al. (1999), that each of the heat treatments achieved a substantial (5 to 6 $\log_{10}$) reduction in numbers of viable Mptb. It also suggests that in milk heavily contaminated with Mptb, small numbers of the bacterium could survive the HTST pasteurization procedure. Surviving organisms other than Mptb were detected in some of the slants, broth cultures, and BACTEC vials, after incubation. Most of these contaminant organisms grew much faster than Mptb and subsequently overgrew the whole slant within a couple of weeks. If low levels of Mptb organisms survived the pasteurization process, the survivors may not be easily detected among other milk contaminants. In this situation, the use of decontamination and immunomagnetic beads for enhanced capture and enrichment of Mptb from milk and combined with decontamination of the sample will significantly enhance the isolation of (Grant et al., 1998b).

It was observed that if the HTST pasteurization was simulated using a single water bath and the temperature of the water bath was set to exactly 72°C, it took about 90 s of incubation for the milk to heat up to 70°C and another 2 min to reach the pasteurization temperature of 72°C for 15 s. Under this experimental condition, the time required to reach the holding temperature was several times longer than the holding time. When the water bath was set to temperatures of 73 to 75°C, the time required to reach the holding temperature was reduced to around 80 to 120 s prior to holding for 15 s at 72°C. In an effort to further reduce the time required to bring the sample to pasteurization temperature, two water baths were used. One water bath set to 75°C was used to heat the samples, while a second water bath set to 72°C was used for the 15-s holding. This method simulated the heating and holding chambers of commercial pasteurizer. The two-bath method was employed in three of the 11 HTST experiments. The heating and cooling curves for regular batch pasteurization and HTST method are shown in Figures 1 through 3. In an on-farm pasteurization experiment, a temperature of 60°C was reached after 40 min of heating time, and another 20 min was required to reach to the target temperature of 65.5°C before the milk was held for 30 min (Stabel, 2001). The time required to reach the holding temperature plays an important role in pasteurization. Unfortunately, it is not standardized in most pasteurization procedures published. No information is available on how much effect it has on the survival of Mptb for different types of commercial pasteurization. This should be considered in the interpretation of the results of pasteurization experiments simulated in laboratories.

Death Curve of Mptb at 63°C

The results of death curve determination experiments are shown in Table 2. Mptb colonies on agar slants were counted after 3 mo of incubation and confirmed by IS*900* PCR. These results indicate that while the majority of the Mptb organisms lost their viability in the first 5 to 10 min, survivors were detected up to the first 10 min of pasteurization by both culture methods and BACTEC test. Similar results were obtained when the broth cultures of pasteurized milk samples were plated out on agar slants (Table 2). This is in agreement with the results of other researchers (Grant et al., 1996). However, no survivors were recovered on either agar slants or broth culture after 15 min of pasteurization at 63°C in our study, whereas Grant et al. (1996) reported that a small number of Mptb survived after 30 min of pasteurization.

Detection of Mptb by PCR and Culture in Retail Milk

The IS*900* PCR results of the 710 retail milk samples tested are presented in Table 3. Of the 710 samples

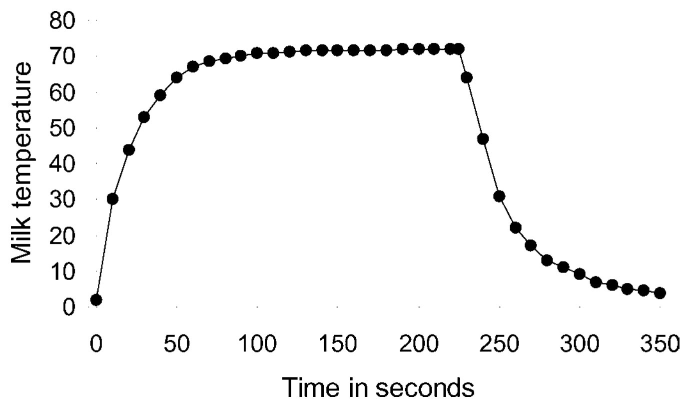

Figure 2. Heating and cooling curve of HTST method simulated in one water bath set at 72°C.

Table 2. Results of death curve determination experiment of *Mycobacterium paratuberculosis*.

Holding time (min)	0	5	10	15	20	30	40
Survivor(s) on slants[1]	10000	60	1	0	0	0	0
Survivor(s) in broth	Yes	Yes	Yes	No	No	No	No

[1]Average of three experiments.

tested, 110 (15.5%) were positive for Mptb DNA by PCR. None of the 31 filtered milk samples tested in this study was positive by PCR. However, this sample size is too small to draw any conclusions from this result.

Twenty-one of the 36 slant cultures selected from the first 500 retail milk samples and 10 of the 35 BACTEC samples tested demonstrated growth of bacteria other than Mptb. Contamination was also recognized in some of the 244 broth cultures. Although a few broth cultures were positive in nested IS*900* PCR, the positive results were not reproducible. We determined that the DNA, which produced signal in PCR, was from dead bacteria instead of the multiplication of viable Mptb organisms in broth. The lack of recovery of live Mptb from the retail milk samples tested may, therefore, be due to either the absence of live Mptb in the retail milk samples tested, or the presence of a very low number of viable Mptb per milliliter of milk, since the detection limit of a reliably reproducible nested PCR procedure was 100 cfu/ml. Levels lower than 100 cfu/ml could not be guaranteed to be reproducible in all the triplicates in nested PCR. The detection of low numbers of viable Mptb was further complicated because of contamination with other fast growing bacteria.

Streeter et al. (1995) screened 126 subclinically normal cows of a single herd in central Ohio with high prevalence of Mptb infection. Thirty-six cows were determined to be Mptb shedders in feces, while Mptb was isolated from colostrum of eight and milk of three animals. It is more efficacious to detect Mptb contamination from individual animals than from herds due to the dilution of contaminated milk in a huge volume of normal milk. This dilution makes Mptb almost unrecoverable by regular culture method; subsequently failure in recovery of viable Mptb from milk does not mean that viable Mptb organisms did not exist in milk.

In conclusion, viable Mptb organisms were not recovered from raw milk seeded with Mptb organisms and pasteurized in the laboratory by batch pasteurization method, while two of the 11 HTST experiments spiked with 10^5 to 10^7 cfu/ml of Mptb produced survivors. It should be noted that the HTST heat treatment employed in this study is a simulation of HTST pasteurization, and further studies using pilot scale HTST equipment are required to confirm Mptb survival data reported in this study. Of the 710 retail milk samples tested, 15.5% of the retail milk samples examined tested positive for Mptb DNA in the IS*900* PCR method. No viable Mptb organisms were recovered from the 12-to-30 mo cultures of 44 PCR-positive and 200 PCR-negative retail milk samples. It is possible that the culture methods used were not sensitive enough to recover low levels of Mptb. For a complete inactivation of Mptb in milk, it was reported that longer holding time in HTST heat treatment (25 s) is more likely to achieve complete inactivation of Mptb in milk than a higher pasteurization temperature (90(C, Grant et al., 1999). In this study, small numbers of live bacteria were recovered from Mptb-seeded milk samples heat-treated by the HTST method but not from the samples by the batch protocol. These observations suggest that more investigations are needed to determine the optimal HTST procedure in dairy processing operations.

Table 3. Results of screening of retail milk samples using nested PCR.[1]

N	Negative	1 positive	2 positive	3 positive
710	600 84.5%	73 10.3%	32 4.5%	5 0.7%

[1]Nested PCR was conducted in triplicate for each sample. 1 positive, one of three PCR reactions was positive out of the triplicates. 2 positive, two of three PCR reactions tubes were positive. 3 positive, all three PCR reactions were positive.

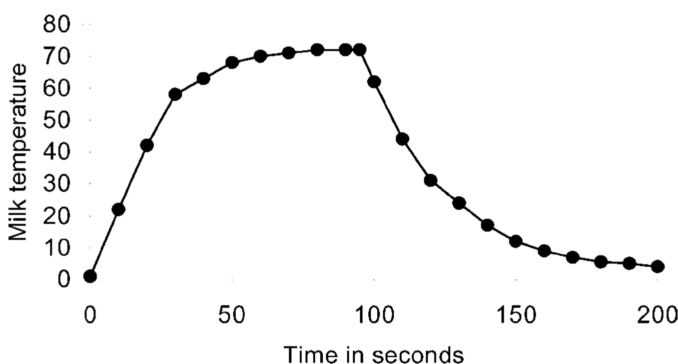

Figure 3. Heating and cooling curve of HTST method at 72°C simulated in two water baths, the first one for heating-up (75°C) and the second one for holding (72°C).

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