Applied and Environmental Microbiology, Mar. 2010, p. 1777–1782 Vol. 76, No. 6
0099-2240/10/$12.00 doi:10.1128/AEM.02625-09


# Rapid Assessment of the Viability of *Mycobacterium avium* subsp. *paratuberculosis* Cells after Heat Treatment, Using an Optimized Phage Amplification Assay[▽]

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Received 28 October 2009/Accepted 13 January 2010

**Thermal inactivation experiments were carried out to assess the utility of a recently optimized phage amplification assay to accurately enumerate viable *Mycobacterium avium* subsp. *paratuberculosis* cells in milk. Ultra-heat-treated (UHT) whole milk was spiked with large numbers of *M. avium* subsp. *paratuberculosis* organisms ($10^6$ to $10^7$ CFU/ml) and dispensed in 100-μl aliquots in thin-walled 200-μl PCR tubes. A Primus 96 advanced thermal cycler (Peqlab, Erlangen, Germany) was used to achieve the following time and temperature treatments: (i) 63°C for 3, 6, and 9 min; (ii) 68°C for 20, 40, and 60 s; and (iii) 72°C for 5, 10, 15, and 25 s. After thermal stress, the number of surviving *M. avium* subsp. *paratuberculosis* cells was assessed by both phage amplification assay and culture on Herrold's egg yolk medium (HEYM). A high correlation between PFU/ml and CFU/ml counts was observed for both unheated ($r^2 = 0.943$) and heated ($r^2 = 0.971$) *M. avium* subsp. *paratuberculosis* cells. *D* and *z* values obtained using the two types of counts were not significantly different ($P > 0.05$). The $D_{68°C}$, mean $D_{63°C}$, and $D_{72°C}$ for four *M. avium* subsp. *paratuberculosis* strains were 81.8, 9.8, and 4.2 s, respectively, yielding a mean *z* value of 6.9°C. Complete inactivation of $10^6$ to $10^7$ CFU of *M. avium* subsp. *paratuberculosis*/ml milk was not observed for any of the time-temperature combinations studied; 5.2- to 6.6-$\log_{10}$ reductions in numbers were achieved depending on the temperature and time. Nonlinear thermal inactivation kinetics were consistently observed for this bacterium. This study confirms that the optimized phage assay can be employed in place of conventional culture on HEYM to speed up the acquisition of results (48 h instead of a minimum of 6 weeks) for inactivation experiments involving *M. avium* subsp. *paratuberculosis*-spiked samples.**

Due to the possible association of *Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease in cattle, with Crohn's disease in humans, the consumption of milk and dairy products contaminated with this pathogenic bacterium has been suggested as a possible source of infection for humans (18). So far, the presence of viable *M. avium* subsp. *paratuberculosis* cells has been reported for pasteurized cows' milk (6, 14, 23) and various cheeses (1, 4, 19). However, the rapid detection of viable *M. avium* subsp. *paratuberculosis* cells in food remains problematic. Culture is considered the gold standard method of demonstrating the viability of *M. avium* subsp. *paratuberculosis* cells, yet this approach is far from perfect and is not really appropriate for risk assessment purposes. First, *M. avium* subsp. *paratuberculosis* is a fastidious, slow-growing bacterium requiring a long incubation period before producing visible colonies (4 to 6 weeks minimum). Second, there is no selective growth medium for *M. avium* subsp. *paratuberculosis*, and chemical decontamination is required before plating samples on solid Herrold's egg yolk medium (HEYM). This decontamination step, which aims to inactivate the competitive microflora, is often not totally effective, and cultures can be overgrown quickly by non-acid-fast bacteria during incubation. Third, the decontamination step has been demonstrated to have adverse effects on *M. avium* subsp. *paratuberculosis* viability (5). This extends the time required for primary isolation (to up to 20 weeks) and undoubtedly underestimates the number of cells originally present in the sample.

Recently, we reported an optimization of the conditions of a commercially available phage amplification assay involving D29 mycobacteriophage (FASTPlaqueTB assay; Biotec Laboratories, Ipswich, United Kingdom) to permit accurate enumeration of *M. avium* subsp. *paratuberculosis* cells in milk (7). The main advantage of using phage amplification to detect *M. avium* subsp. *paratuberculosis* is that the number of viable cells can be estimated quickly, within 24 to 48 h, based on the count of plaques produced when D29-infected cells burst on a lawn of *M. smegmatis* indicator cells in an agar plate. Moreover, there is no need to carry out chemical decontamination of the sample before the phage assay because the D29 phage will infect only viable mycobacterial cells, and thus the detection sensitivity of the test is enhanced. For these reasons, the optimized phage amplification method may be used to speed up the acquisition of results during inactivation experiments involving samples artificially spiked with *M. avium* subsp. *paratuberculosis*.

So far, the optimized phage amplification assay has been applied for the detection of viable *M. avium* subsp. *paratuberculosis* cells in spiked broth and milk samples. However, the performance of the test in assessing the viability of *M. avium* subsp. *paratuberculosis* cells subjected to physical or chem-

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▽ Published ahead of print on 22 January 2010.

ical treatments, which are likely to comprise mixtures of viable cells, injured/stressed cells, and dead cells, still needed to be investigated. For this reason, thermal inactivation experiments were carried out in order to assess the utility of this optimized phage assay for use instead of conventional culture for research involving artificially spiked milk samples. The main objectives of this study were to evaluate the correlation between colony and plaque counts for heat-treated *M. avium* subsp. *paratuberculosis* and to demonstrate a quicker acquisition of accurate results than that obtainable by culture.

## MATERIALS AND METHODS

**Bacterial strains.** Two type strains (ATCC 19698 and NCTC 8578) and two milk isolates (796PSS and 806R [14]) of *M. avium* subsp. *paratuberculosis* were included in this study. *M. avium* subsp. *paratuberculosis* strains were grown in a shaker incubator for 4 to 6 weeks at 37°C to stationary phase in Middlebrook 7H9 broth containing 10% oleic acid-albumin-dextrose-catalase (OADC) supplement (both from Difco) and 2 μg/ml mycobactin J (Synbiotics Europe SAS, Lyon, France). Before being used to spike milk for heat inactivation experiments, broth cultures were declumped by being vortexed (three times for 2 min) with five 3-mm sterile glass beads (VWR International Ltd., England). *Mycobacterium smegmatis* mc$^2$ 155 (originally a gift from Ruth McNerney, London School of Hygiene and Tropical Medicine [LSHTM]) was cultured to stationary phase (2 to 3 days at 37°C) in Middlebrook 7H9 broth containing 10% OADC for use as sensor cells in the optimized phage assay.

**Heat treatments.** Ultra-heat-treated (UHT) milk purchased from a local supermarket was spiked with $10^6$ to $10^7$ CFU of *M. avium* subsp. *paratuberculosis* per ml and dispensed in 100-μl aliquots into strips of thin-walled 200-μl PCR tubes. A Primus 96 advanced thermal cycler (Peqlab, Erlangen, Germany) was programmed to achieve the following time and temperature treatments: (i) 63°C for 3, 6, and 9 min; (ii) 68°C for 20, 40, and 60 s; and (iii) 72°C for 5, 10, 15, and 25 s. In order to mimic as closely as possible the temperature profile of the laboratory-scale high-temperature, short-time (HTST) pasteurizer (8), each heat treatment included (i) equilibration of the spiked milk at 8°C for 2 min, (ii) heating at a rate of 1.2°C per s, (iii) holding for the desired time, and (iv) cooling on ice. The maximum cooling rate achievable on the thermal cycler was too low compared to that obtained with Franklin HTST plates, so when the thermal cycler display indicated that the holding time was complete, milk samples were immediately transferred to an ice bath for 2 min to facilitate more rapid cooling. Heating experiments were replicated twice with each of the four *M. avium* subsp. *paratuberculosis* strains at each of the three temperatures, 63, 68, and 72°C.

**Enumeration of *M. avium* subsp. *paratuberculosis* cells.** Viable *M. avium* subsp. *paratuberculosis* cells were enumerated by both the optimized phage assay and a conventional culture method, as described below. For unheated samples and samples heated for the first heating time at each temperature, 100 μl of sample was appropriately diluted in 7H9 broth plus 10% OADC. For longer heating times at each temperature, 10 100-μl samples that had been heated simultaneously were combined to give a sample volume of 1 ml for testing to improve detection sensitivity. Before being processed through the phage assay, all milk samples (heated and unheated) were centrifuged (16,000 × *g* for 15 min) to remove milk components known to be inhibitory to the phage assay. The milk pellet was washed once in phosphate-buffered saline, pH 7.4, and resuspended initially in 1 ml 7H9 broth plus 10% OADC. A 100-μl portion of each sample (or appropriate dilutions in 7H9 broth plus 10% OADC, depending on heating time and the estimated number of survivors) was spread on HEYM containing 2 μg/ml mycobactin J for colony count (CFU/ml) determination. The remaining sample (900 μl), with the addition of $CaCl_2$ to a final concentration of 2 mM, was incubated overnight at 37°C and processed through the optimized phage assay as described by Foddai et al. (7). Briefly, 100 μl D29 mycobacteriophage ($10^8$ PFU/ml) (originally a gift from Ruth McNerney, LSHTM) was added to the sample before incubation at 37°C for 2 h. Viricide (100 μl of 10 mM ferrous ammonium sulfate) was then added, and the sample was mixed thoroughly and incubated for 5 min at room temperature to inactivate extraneous phage before the addition of 5 ml 7H9 broth plus 10% OADC plus 2 mM $CaCl_2$. The sample was returned to the incubator at 37°C for a further 1.5 h before being plated with 1 ml *M. smegmatis* mc$^2$ 155 culture ($10^8$ CFU/ml) and 5 ml molten 7H9 agar. Plaque counts (PFU/ml) were available the next day. Corresponding colony counts (CFU/ml) were obtained after incubating HEYM plates (wrapped in Duraseal laboratory sealing film) at 37°C for 4 to 6 weeks.

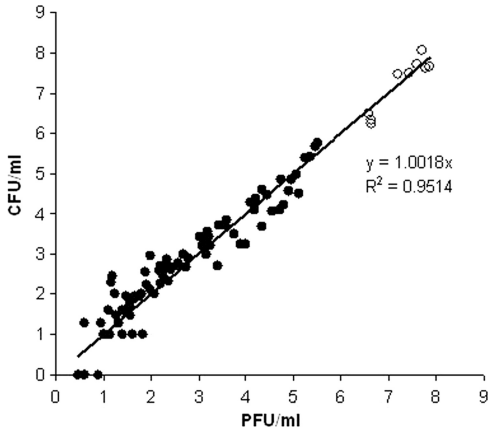


FIG. 1. Relationship between colony and plaque counts for unheated (empty circles) and heated (solid circles) *M. avium* subsp. *paratuberculosis* cells.

**Determination of thermal *D* and *z* values.** *D* values (decimal reduction time; the time required at a particular temperature to inactivate a 1-$\log_{10}$ concentration of bacteria) for all four *M. avium* subsp. *paratuberculosis* strains, at 63, 68, and 72°C, were calculated from the slope of the regression line obtained by plotting the $\log_{10}$ survivors/ml versus the time of heat exposure at the three test temperatures. *z* values (the temperature change [°C] required to reduce or increase the *D* value by 1 $\log_{10}$) for the *M. avium* subsp. *paratuberculosis* strains were determined by plotting $\log_{10}$ *D* values versus the heating temperatures.

**Statistical analysis.** The correlation between plaque and colony counts produced from the heated and unheated samples was assessed using linear regression analysis (Microsoft Excel). The statistical significance of differences between colony and plaque counts was assessed using a paired *t* test (GraphPad Instat 3; GraphPad Prism, La Jolla, CA); differences with *P* values of $<0.05$ were considered significant.

## RESULTS

**Comparison of plaque and colony counts.** Plaque counts (PFU/ml) obtained for samples processed through the phage assay were available within 48 h. This allowed for rapid assessment and enumeration of surviving *M. avium* subsp. *paratuberculosis* cells rather than having to wait 4 to 6 weeks for the corresponding colony count (CFU/ml) by conventional culture on HEYM. The optimized phage assay was able to detect smaller numbers of viable cells due to the larger sample volume tested than that used for culture (900 μl and 100 μl, respectively). For 7 of 100 samples, no colonies were observed on HEYM, whereas corresponding PFU counts indicated the presence of between 1 and 10 viable *M. avium* subsp. *paratuberculosis* cells per ml in these samples.

The relationship between the PFU and CFU counts produced from both heated and unheated cells is shown in Fig. 1. A high correlation between plaque (PFU/ml) and colony (CFU/ml) counts obtained for samples before ($r^2 = 0.943$) and after ($r^2 = 0.971$) thermal stress was observed during this study. There was found to be no significant difference between corresponding PFU and CFU counts produced by either unheated or heated *M. avium* subsp. *paratuberculosis* cells (paired *t* test; $P = 0.47$ and $P = 0.73$, respectively). This proved that the heat treatment did not affect the sensitivity of *M. avium* subsp. *paratuberculosis* cells to infection by mycobacteriophage D29.

TABLE 1. Thermal *D* and *z* values for four strains of *M. avium* subsp. *paratuberculosis* in milk, calculated from plaque (PFU) and colony (CFU) counts[a]

| Strain | Count type | Mean *D* value (s) at: | | | Mean *z* value (°C) |
|---|---|---|---|---|---|
| | | 63°C | 68°C | 72°C | |
| NCTC 8578 | PFU | 79.9 | 8.6 | 4.4 | 7.1 |
| | CFU | 74.8 | 8.6 | 2.9 | 6.3 |
| ATCC 19698 | PFU | 83.5 | 9.8 | 3.0 | 6.2 |
| | CFU | 70.5 | 10.1 | 4.6 | 7.5 |
| 806R | PFU | 78.5 | 10.5 | 4.7 | 7.3 |
| | CFU | 74.8 | 10.6 | 4.9 | 7.5 |
| 796PSS | PFU | 85.5 | 10.2 | 4.7 | 7.1 |
| | CFU | 95.2 | 11.6 | 5.3 | 7.1 |
| All | PFU | 81.8 ± 3.2 | 9.8 ± 0.7 | 4.2 ± 0.8 | 6.9 ± 0.5 |
| | CFU | 78.8 ± 11.1 | 10.2 ± 1.1 | 4.4 ± 1.1 | 7.1 ± 0.6 |

[a] For each individual strain, the *D* value represents the mean for two independent experiments. *D* and *z* data for all strains in total are reported as means ± standard deviations.

**Thermal *D* and *z* values for *M. avium* subsp. *paratuberculosis*.** The *D* and *z* values calculated for the four strains of *M. avium* subsp. *paratuberculosis* heated in milk at 63, 68, and 72°C are shown in Table 1. Differences in *D* and *z* values among the four strains of *M. avium* subsp. *paratuberculosis* were not significant ($P = 0.99$ and $P = 0.63$, respectively). Mean $D_{63°C}$, $D_{68°C}$, and $D_{72°C}$ values based on the results produced using the optimized phage assay were 81.8 ± 3.2, 9.8 ± 0.7, and 4.2 ± 0.8 s, respectively, resulting in a mean *z* value of 6.9 ± 0.5°C. Comparison of corresponding *D* and *z* values calculated based on the results produced from the culture method showed that the difference was not significant ($P > 0.05$).

**Thermal inactivation curves.** Incomplete inactivation was recorded for all four strains of *M. avium* subsp. *paratuberculosis* under all three time-temperature conditions studied. The thermal inactivation curves were consistently concave and showed a progressive $\log_{10}$ reduction in viable cells followed by significant tailing (Fig. 2A, B, and C). The mean $\log_{10}$ reductions observed for each *M. avium* subsp. *paratuberculosis* strain after the longest heating times at all three temperatures are summarized in Table 2. The mean $\log_{10}$ reductions in viable *M. avium* subsp. *paratuberculosis* cells heated at 72°C for 15 and 25 s were not significantly different, at 5.6 ± 0.1 and 5.2 ± 0.3 $\log_{10}$, respectively ($P > 0.05$). A larger reduction in the number of viable cells was recorded for samples processed under the two lower time-temperature conditions; mean $\log_{10}$ reductions at 68°C for 60 s and 63°C for 9 min were 6.3 ± 0.4 and 6.6 ± 0.3 $\log_{10}$, respectively.

## DISCUSSION

Many studies have been carried out to assess the impact of pasteurization processes (both the standard holder method—63.5°C for 30 min—and the HTST method—71.7°C for 15 s) on the viability of *M. avium* subsp. *paratuberculosis* in milk (3, 10–13, 17, 22, 25, 26, 28–30). Most of these studies confirmed that *M. avium* subsp. *paratuberculosis* is more resistant to heat than other *Mycobacterium* spp. and that HTST pasteurization results in a significant reduction in the number of viable *M. avium* subsp. *paratuberculosis* cells (a >4-$\log_{10}$ reduction is generally reported), but some reported the detection of small numbers of surviving *M. avium* subsp. *paratuberculosis* cells in some pasteurized milk samples (10, 11). Results of subsequent surveys of commercially pasteurized milk in the United Kingdom, Czech Republic, and United States also tend to indicate that a small number of *M. avium* subsp. *paratuberculosis* cells may occasionally survive HTST pasteurization (1, 6, 14), although this remains a contentious subject (2).

The lack of concordance between the results of different pasteurization studies is due in part to different approaches to culturing *M. avium* subsp. *paratuberculosis* after heat treatment. In previous heat inactivation studies, culture on HEYM or in automated/semiautomated broth culture systems, such as Bactec 12B, MGIT (Becton Dickinson), or ESP (Trek Diagnostic Systems), has been used to assess the presence of sur-

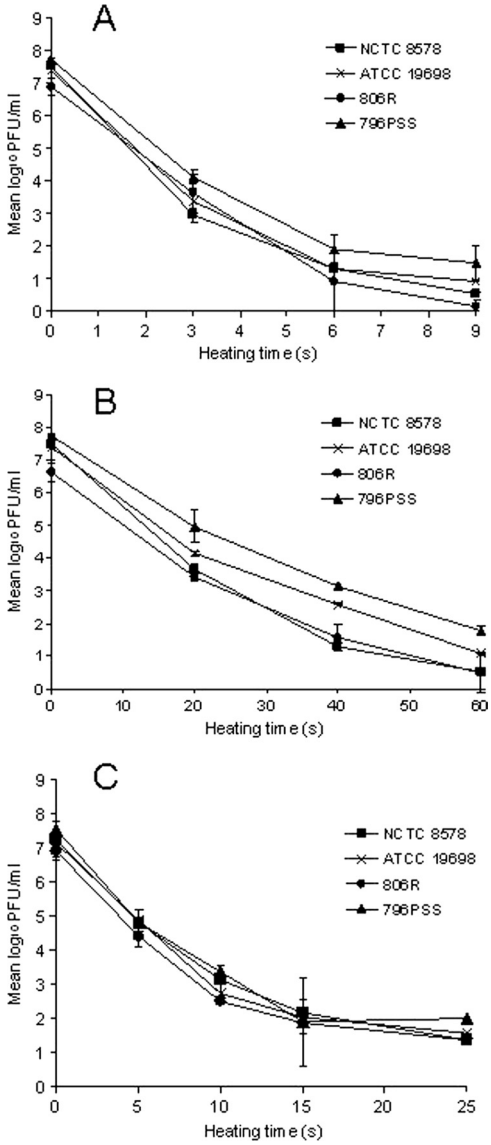


FIG. 2. Inactivation kinetics of four strains of *M. avium* subsp. *paratuberculosis* heated in milk at 63°C (A), 68°C (B), and 72°C (C), obtained using the phage assay. Each value is the mean of two independent observations ± standard error.

TABLE 2. Mean $\log_{10}$ reductions calculated for four strains of *M. avium* subsp. *paratuberculosis* heated in milk under different time-temperature conditions

| Heat treatment conditions | $\log_{10}$ reduction in *M. avium* subsp. *paratuberculosis*[a] | | | | Mean $\log_{10}$ reduction ± SD |
|---|---|---|---|---|---|
| | NCTC 8578 | ATCC 19698 | 806R | 796PSS | |
| 72°C, 15 s | 5.9 | 5.6 | 5.5 | 5.5 | 5.6 ± 0.1 |
| 72°C, 25 s | 5.1 | 5.1 | 5.0 | 5.6 | 5.2 ± 0.3 |
| 68°C, 60 s | 7.0 | 6.3 | 6.0 | 5.8 | 6.3 ± 0.5 |
| 63°C, 9 min | 7.0 | 6.5 | 6.8 | 6.3 | 6.6 ± 0.3 |

[a] Each value represents the mean of two independent observations.

viving *M. avium* subsp. *paratuberculosis* cells. However, the detection of this pathogenic bacterium based on culture takes a long time to generate results due to the slow growth of the microorganism in both solid (4 to 6 weeks minimum) and liquid (up to 40 days) growth media. In the present study, we evaluated the potential utility of a recently optimized phage amplification assay (7) for the rapid detection (within 48 h) of viable cells surviving pasteurization carried out under laboratory conditions. The method has been applied successfully to detect viable *M. avium* subsp. *paratuberculosis* cells in broth or milk spiked with cells cultivated up to stationary phase. However, Grant et al. (10) reported that heated *M. avium* subsp. *paratuberculosis* cells require a longer incubation time than unheated cells for growth on solid medium, which would be indicative of the presence of sublethally injured cells in heat-treated milk samples. These cells should have the ability, under appropriate conditions, to repair injury and become competent cells again. To demonstrate that the optimized phage assay could be employed in place of culture, its validity when applied to samples containing *M. avium* subsp. *paratuberculosis* cells that are not in optimal metabolic condition would first need to be tested. For this reason, this study evaluated the correlation between PFU and CFU counts obtained for *M. avium* subsp. *paratuberculosis* cells heated in milk at pasteurization temperatures.

UHT milk samples spiked with *M. avium* subsp. *paratuberculosis* at high levels ($>10^6$ cells/ml) were heat treated in 100-μl aliquots in thin-walled 200-μl PCR tubes in order to take advantage of the efficient thermal transfer characteristics of a thermal cycler heat block and to attain pasteurization temperature as quickly as possible. This thermal cycler approach was inspired by Gumber and Whittington (16), who used a thermal cycler to simulate temperature flux variations that *M. avium* subsp. *paratuberculosis* may be exposed to in the environment. For the pasteurization experiments, the heating rate of the thermal cycler was set to 1.2°C/s to achieve a "come-up" time to 72°C of ca. 50 s (Fig. 3), similar to the time for Franklin HTST plates (developed to simulate commercial HTST pasteurizer conditions [8]), which were used in earlier pasteurization studies by Grant et al. (10, 11). While we acknowledge that a thermal cycler is not a conventional means of simulating milk pasteurization, we contend that the results we obtained validate its use because they agree very favorably with results of numerous previous pasteurization studies.

The results of this study confirm that the optimized phage assay permits rapid and accurate enumeration of viable *M. avium* subsp. *paratuberculosis* cells in milk. The main advantages of the phage assay over culture on HEYM are as follows: first, survivor cells can be enumerated within 48 h (instead of in 4 to 6 weeks); and second, due to the larger sample volume tested (1 ml), the phage assay is able to detect smaller numbers of viable cells in milk (minimum detection limit of 1 PFU/ml rather than 10 CFU/ml for culture). This was clearly demonstrated in the case of seven heated milk samples where no colonies were observed on HEYM but corresponding PFU counts indicated the presence of 1 to 10 viable *M. avium* subsp. *paratuberculosis* cells per ml. A high correlation ($r^2 = 0.9464$) between plaque and colony counts produced from both heated and unheated cells was recorded. This result confirms that heated *M. avium* subsp. *paratuberculosis* cells can be detected and accurately enumerated using the optimized phage assay. Since D29 phage would be expected to require host cells in a fully competent metabolic state in order to replicate, this finding suggests that *M. avium* subsp. *paratuberculosis* cells surviving heat treatment were in this state when subjected to the phage assay. One of the most probable explanations is that the overnight incubation in 7H9 Middlebrook broth at 37°C (see Materials and Methods) after heating, but before the phage assay commenced, aided in the resuscitation of heat-injured cells. Incomplete inactivation of $10^6$ to $10^7$ CFU of *M. avium* subsp. *paratuberculosis*/ml was consistently recorded after heating samples under three different time-temperature conditions, once more suggesting that *M. avium* subsp. *paratuberculosis* could survive pasteurization if present in raw milk in large numbers. These results are consistent with the findings of heat inactivation experiments previously carried out at Queen's University of Belfast (10–13). The mean *D* values calculated for the four *M. avium* subsp. *paratuberculosis* strains in this study are similar to those previously reported for *M. avium* subsp. *paratuberculosis* heated in milk ($D_{71°C}$ = 11.67 s, $D_{68°C}$ = 21.8 s [30], $D_{63°C}$ = 96 to 150 s [21]). The *z* value obtained in this study is similar to one reported previously (7.11°C [30]), which confirms that *M. avium* subsp. *paratuber-*

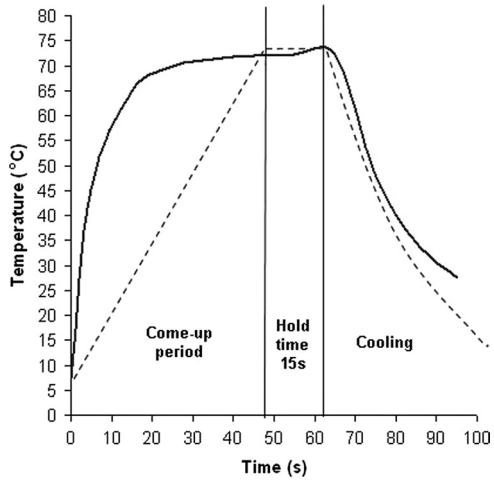


FIG. 3. Heating profiles for milk in Franklin HTST pasteurizer units (solid line), developed for lab-scale simulation of commercial pasteurization by Franklin (8) and used in previous studies by Grant et al. (10, 11), and in thin-walled PCR tubes in a Primus 96 thermal cycler (dashed line).

*culosis* is more heat resistant than *Mycobacterium bovis*, previously reported to have a *z* value in milk of 4.8 to 5.2°C (20) and to be inactivated completely after heat treatment at 70°C for 10 s (24), unlike *M. avium* subsp. *paratuberculosis* in the present study.

The factors contributing to survival of *M. avium* subsp. *paratuberculosis* during heating have not been elucidated fully but may include its thick cell wall (rich in lipids), in addition to the tendency of this bacterium to exist as large clumps of cells. Clumped cells could be less susceptible to heat treatment than individual cells (10, 12). Bacteria inside the clumps could be protected because of slow heat penetration (27), although this possibility has been refuted (2). All broth cultures used in this study were thoroughly declumped by being vortexed with glass beads before being added to the milk and processed by heat treatment. The primary purpose of this step was to reduce as much as possible the presence of large clumps, which are known to affect the correlation between PFU and CFU counts; however, it should also have ensured that single cells, not clumps, were being heat treated. The results obtained during this study suggest that single-cell or small-clump suspensions of *M. avium* subsp. *paratuberculosis* can survive pasteurization treatments and also exhibit nonlinear thermal inactivation kinetics (tailing), a phenomenon previously considered an artifact and attributed to the presence of clumped cells (15). Interestingly, it was recently reported that old (stationary-phase) cultures of both *Mycobacterium marinum* and *M. bovis* BCG contained spores, which led to tailed survivor curves when cultures were exposed to wet heat at 65°C for 15 min (9). Is *M. avium* subsp. *paratuberculosis* also able to form spores, and could the 4- to 6-week-old broth cultures used to spike milk in this study have contained spores, resulting in nonlinear thermal inactivation? This possibility merits further investigation.

It must be emphasized that the experiments reported here were not designed to determine the ability of commercial HTST milk pasteurization to inactivate *M. avium* subsp. *paratuberculosis*. For many reasons, continuous-flow HTST pasteurization as applied to commercial products cannot be simulated adequately under laboratory conditions, and direct comparison to the real situation may not be possible considering the fact that the wild strains may be less or more heat tolerant than field isolates or type strains used in the laboratory. Rather, the objective of this research was to validate the optimized phage amplification assay and its use for the rapid detection and enumeration of viable *M. avium* subsp. *paratuberculosis* cells after heat treatment. Our results clearly demonstrate that the method could be employed in place of conventional culture to speed up the acquisition of results during inactivation experiments involving spiked milk samples. The reported study was concerned with thermal inactivation. We have also monitored the effect of Sorgene disinfectant (chemical stress) on the viability of *M. avium* subsp. *paratuberculosis* by using the optimized phage assay, with similar results. The optimized phage assay may thus have applications for antibiotic susceptibility testing of this bacterium.

## ACKNOWLEDGMENT

Antonio Foddai was the recipient of a Master and Back Studentship from the Sardinian government during this study.

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