

Inactivation of *Mycobacterium paratuberculosis* by Pulsed Electric Fields

NEIL J. ROWAN,^{1*} SCOTT J. MACGREGOR,² JOHN G. ANDERSON,¹
DOUGLAS CAMERON,¹ AND OWEN FARISH²

*Department of Bioscience and Biotechnology¹ and Department of Electronic and Electrical Engineering,²
University of Strathclyde, Glasgow, Scotland*

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The influence of treatment temperature and pulsed electric fields (PEF) on the viability of *Mycobacterium paratuberculosis* cells suspended in 0.1% (wt/vol) peptone water and in sterilized cow's milk was assessed by direct viable counts and by transmission electron microscopy (TEM). PEF treatment at 50°C (2,500 pulses at 30 kV/cm) reduced the level of viable *M. paratuberculosis* cells by approximately 5.3 and 5.9 log₁₀ CFU/ml in 0.1% peptone water and in cow's milk, respectively, while PEF treatment of *M. paratuberculosis* at lower temperatures resulted in less lethality. Heating alone at 50°C for 25 min or at 72°C for 25 s (extended high-temperature, short-time pasteurization) resulted in reductions of *M. paratuberculosis* of approximately 0.01 and 2.4 log₁₀ CFU/ml, respectively. TEM studies revealed that exposure to PEF treatment resulted in substantial damage at the cellular level to *M. paratuberculosis*.

Mycobacterium paratuberculosis is a chronic enteric pathogen that causes paratuberculosis, commonly known as Johne's disease, in many different species of animals, including primates (3). It has been suggested that the etiological agent in Crohn's disease, a severe inflammatory enteritis in humans that bears extensive clinical, pathological, and systemic similarity to Johne's disease, may be mycobacterial and could be *M. paratuberculosis* (2), but this remains to be proven. Interest in the possible relationship between Crohn's disease and *M. paratuberculosis* has been recently stimulated by the detection of *M. paratuberculosis* DNA in pasteurized cow's milk samples from retail markets in England and Wales (9). Little is known about the levels of *M. paratuberculosis* that may be present in infected milk from cattle suffering from Johne's disease. However, independent research from the United States (14), Northern Ireland (5), and Australia (6) has reported that this pathogen may be capable of surviving commercial high-temperature, short-time (HTST) pasteurization and thus may be present in retail milk supplies. It is presently estimated that between 20 and 40% of U.S. dairy herds are infected with bovine Johne's disease, which may have significant health implications when it is considered that an estimated one-third of U.S. cheese is produced from unpasteurized milk (15).

The potential use of pulsed-power techniques, such as treatment with high-intensity pulsed electric fields (PEF), for food processing is currently receiving considerable attention, since inactivation of problematic microorganisms can take place under reduced-temperature conditions (7, 10). The advantages of such an electrotechnology include the potential retention of fresh-food characteristics and organoleptic qualities such as flavor, aroma, and texture (1). Previous research suggests that the application of PEF (with a magnitude usually greater than

20 kV cm⁻¹ for short durations, such as 500 ns to 4 μs) to liquids can inactivate susceptible microorganisms through irreversible electroporation of the cell membrane (7, 10, 12). The present studies were conducted to determine whether the application of PEF at different treatment temperatures (5, 20, and 50°C) effectively kills *M. paratuberculosis* cells in 0.1% (wt/vol) peptone water. *Listeria monocytogenes* cells and *Bacillus cereus* endospores were also treated under similar conditions, as these organisms also occasionally occur in milk and milk products and are of lesser and greater thermal tolerance, respectively, than *M. paratuberculosis* (4, 11). An assessment was made of PEF treatment at 50°C on *M. paratuberculosis* cells in sterilized cow's milk. Susceptibility of *M. paratuberculosis* to the lethal effects of PEF treatment and/or temperature was determined by direct viable counts and by transmission electron microscopy (TEM).

The following two strains of *M. paratuberculosis* were utilized in the PEF studies: strain ATCC 19698 (American Type Culture Collection, Manassas, Va.) and strain Linda (ATCC 43015). Strain 19698 is a laboratory strain of *M. paratuberculosis* originally isolated from ileal tissue of a cow with clinical Johne's disease. Strain Linda (ATCC 43015) was isolated from ileum tissue biopsy from a 15-year-old girl with Crohn's disease. A standard suspension of *M. paratuberculosis* cells was prepared by washing growth from slopes of Middlebrook 7H10 agar medium (containing 10% [vol/vol] Middlebrook OADC [oleic acid, dextrose, and catalase; Becton Dickinson Ltd., Oxford, United Kingdom] and 0.0002% [wt/vol] mycobactin J [Allied Monitor Inc., Fayette, Mo.] per liter) with 0.1% (wt/vol) peptone water. Washed Middlebrook 7H10 agar slopes were centrifuged at 2,500 × g for 20 min, and the pellet was resuspended in 0.1% (wt/vol) peptone water to yield a suspension containing approximately 10⁶ CFU of *M. paratuberculosis* per ml (determined spectrophotometrically at 540 nm [model UV-120-02 instrument; Shimadzu Corp., Kyoto, Japan]). Similar cell densities of *M. paratuberculosis* were also suspended in commercially pasteurized cow's milk that had been sterilized to

* Corresponding author. Mailing address: Department of Bioscience and Biotechnology, University of Strathclyde, Royal College, 204 George St., Glasgow G1 1XW, Scotland. Phone: 44 (0)141 548 2531. Fax: 44 (0)141 553 4124. E-mail: n.j.rowan@strath.ac.uk.

TABLE 1. Influence of PEF and/or temperature on the inactivation of food-borne bacterial pathogens suspended in 0.1% (wt/vol) peptone water

Bacterial strain	Reduction in viable cell or spore no. (\log_{10} CFU/ml) under the following conditions ^a :						
	5°C, 25 min	5°C + PEF ^b , 25 min	20°C, 25 min	20°C + PEF, 25 min	50°C, 25 min	50°C + PEF, 25 min	72°C, 25s
<i>M. paratuberculosis</i> ATCC 19698	0 a	1.5 ± 0.2 f	0 a	3.1 ± 0.2 h	0.01 ± 0.01 b	5.1 ± 0.3 j	2.4 ± 0.3 g
<i>M. paratuberculosis</i> Linda (ATCC 43015)	0 a	1.7 ± 0.4 f	0 a	3.3 ± 0.3 h	0.02 ± 0.005 b	5.3 ± 0.5 j	2.8 ± 0.2 g
<i>L. monocytogenes</i> 11994	0 a	1.1 ± 0.1 e	0 a	2.6 ± 0.1 g	0.16 ± 0.05 d	4.1 ± 0.3 i	7.1 ± 0.2 ^c k
<i>B. cereus</i> 11145 (endospores)	0 a	0.16 ± 0.02 d	0 a	0.14 ± 0.03 d	0 a	0.17 ± 0.04 d	0.06 ± 0.02 c

^a Values followed by the same lowercase letter do not differ at the 95% confidence interval ($P < 0.05$). Results are means and standard deviations.

^b Treatment of suspended bacterial cells with 2,500 pulses at 30 k V/cm.

^c Reduction of starting population of $7.1 \pm 0.2 \log_{10}$ CFU/ml to a nondetectable level when assessed after 15 s at 72°C.

sterility (i.e., three consecutive days of steaming for 30 min). Sterility was confirmed by the absence of microbial growth on plates of tryptone soy agar supplemented with 0.6% (wt/vol) yeast extract that were incubated for 48 h at 37°C prior to cell enumeration. Suspended bacteria were sonicated to disperse clumps prior to PEF or heat treatments, which resulted in more accurate quantification of CFU of bacteria on enumeration media.

M. paratuberculosis cells were suspended in 1.9 ml of 0.1% peptone water or cow's milk and were subjected to high-voltage PEF (~30 kV/cm) in a uniform-field static test chamber. The electrical circuit layout and experimental arrangement for PEF treatment were as described previously (7, 12). The test chamber consisted of a disk of 10 mm Perspex, with a central hole cut through it to hold a 1.9-ml volume of sample. Two separate channels were drilled from the outer edge of the Perspex to the central hole, thus allowing for syringe injection and removal of samples. Flat brass plates were fitted on both sides of the central hole, which formed the electrodes of the test chamber. Predetermined cell populations were treated with 2,500 pulses at 5, 20, and 50°C at a pulse repetition frequency that was limited to 5 pulses per s (5 Hz at 50°C) in order to ensure that there were no thermal inactivation effects associated with the energy dissipation in the test chamber. The test chamber was immersed in a circulating constant-temperature water bath (model HE30; Grant Instruments Ltd., Cambridge, United Kingdom) equipped with a thermoregulator capable of maintaining temperature to within $\pm 0.05^\circ\text{C}$ (model TE-8A; Techne Ltd., Cambridge, United Kingdom) to maintain the desired treatment temperatures at 5, 20, and 50°C. A thermocouple was also employed throughout the studies in order to verify the temperature of the treated liquid. A 100-kV high-voltage DC generator (model EH50R02; Glassmann Europe Ltd., Hampshire, United Kingdom) was used to charge a coaxial-cable Blumlein pulse generator (type TLG B-01; Samtech Ltd., Glasgow, United Kingdom) through a charging resistance of 10 M Ω . The coaxial-cable generator was constructed from 100 m of URM67 40-kV cable (Samtech). The high-voltage output pulse from the generator was 500 ns in duration, and the generator had an output impedance of 100 Ω , a switching impedance of 50 Ω , and an open circuit gain of 2. The generator was wound inductively on a 30-cm-diameter former in order to minimize secondary transmission line losses. The pulse generator was charged from one end of the cable and was fired by switching the inner conductor to ground at the

opposite end. Although both sides of the test chamber were grounded, the output from the cable generator, which was connected to one side of the test chamber, was transiently decoupled from ground during application of the voltage pulse. The Blumlein generator was fired using a triggered corona stabilized switch. The output pulse from the generator was monitored throughout the experiments using a 1,000:1 Tektronix P6015A high-voltage probe (Imex Ltd., Coatbridge, United Kingdom). Levels of microbial inactivation and cell integrity (examined with a Zeiss 902 TEM) were assessed after PEF treatment as described previously (12). Recovery of surviving populations (\log_{10} CFU of *M. paratuberculosis* per milliliter) was determined for PEF-treated suspensions and dilutions thereof by spread and spiral plating samples (model B; Spiral Systems Inc., Shipley, United Kingdom) onto Middlebrook 7H10 agar plates; these were incubated for 2 months at 37°C before enumeration.

The effect of treatment temperature on the viability of *M. paratuberculosis* cells suspended in 0.1% (wt/vol) peptone water or in tyndallized cow's milk was also determined in the static test chamber. Predetermined cell populations of *M. paratuberculosis* were transferred to the test chamber, which was immersed in a circulating constant-temperature water bath at 5, 20, or 50°C for 20 min as described above; the duration of temperature exposure was similar to that for PEF treatment. Recovery of surviving populations of *M. paratuberculosis* from the test chamber was determined as described earlier. To validate our methods, predetermined populations of *L. monocytogenes* cells (strain 11994; obtained from the National Collection of Type Cultures, Public Health Laboratory Service, Colindale, United Kingdom, and originally isolated from a patient with meningitis) and *B. cereus* endospores (diarrheagenic strain 11145, which was obtained from the National Collection of Type Cultures) were subjected to a regimen of heating and PEF treatment similar to that mentioned above. *L. monocytogenes* was grown in 100 ml of tryptone soy broth supplemented with 0.6% (wt/vol) yeast extract at 37°C for 20 h with shaking (150 rpm). *Listeria* cells were harvested by centrifugation at $3,000 \times g$ at 4°C, washed twice, and resuspended in peptone water to a cell density of approximately 10^6 CFU/ml as described above. *B. cereus* endospores were obtained by growth of the bacterium on sporulation medium (nutrient agar supplemented with 0.5 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ per liter) for 2 weeks at 37°C. The absence of *B. cereus* vegetative cells was confirmed by heating at 85°C for 15 min as described previ-

ously (13). *B. cereus* endospores were washed twice and resuspended in 0.1% (wt/vol) peptone water to a spore density of approximately 10^6 CFU/ml. After heating and PEF treatment, enumeration of surviving populations of *L. monocytogenes* cells and of *B. cereus* endospores was done after 48 h at 37°C on tryptone soy–0.6% (wt/vol) yeast extract agar and on nutrient agar, respectively.

The thermal resistance of *M. paratuberculosis* strains ATCC 19698 and Linda (ATCC 43105), *L. monocytogenes* (strain 11994), and *B. cereus* endospores (strain 11145) was also determined at 72°C for 25 s (HTST pasteurization), according to methods described previously (4, 11). To further validate the present study, *L. monocytogenes* cells were also subjected to 63°C for 30 min. *D* values (decimal reduction time; the time required to kill a 1-log-unit concentration of bacteria) were calculated from the slope of the best-fit line graphically determined by plotting the \log_{10} CFU survivors per milliliter versus time of heat exposure at 63 and 72°C. Test bacteria were suspended in 1.9 ml of preheated 0.1% peptone water to a density of approximately 10^6 CFU/ml in 3-ml crimp cap glass vials (Phase Separations Ltd., Watford, Hertfordshire, United Kingdom). The vials were sealed and kept 4 cm below the level in the water bath for the treatment period. Recovery and enumeration of surviving populations of the test bacteria were done as mentioned above. All of the experiments in this study were performed in triplicate, and results are reported as averages. Significant differences in levels of viable test bacteria recovered after PEF treatment and heating were reported at the 95% confidence interval ($P < 0.05$) using analysis of variance (balanced model) with Minitab software release 11 (Minitab Inc., State College, Pa.).

Results from PEF treatment and heating of *M. paratuberculosis* ATCC 19698 cells suspended in 0.1% peptone water revealed a greater level of cell reduction with increased treatment temperature (Table 1). *M. paratuberculosis* ATCC 19698 cells were reduced by $\sim 5.4 \log_{10}$ CFU/ml at 50°C after 2,500 pulses at 30 kV/cm. Strain Linda was similarly affected ($P < 0.05$) by PEF treatment at 50°C (Table 1). PEF treatment of *M. paratuberculosis* cells at 5°C resulted in a reduction of $\sim 1.6 \log_{10}$ CFU/ml, which was the lowest level of cell inactivation achieved using this electrotechnology. The composition and structure of the cell membrane of *M. paratuberculosis* indicate that it may be more rigid at low temperatures, and this may protect the microorganism from membrane rupture by PEF (8). TEM studies of PEF-treated *M. paratuberculosis* cells revealed substantial structural damage at the cellular level (Fig. 1b). Control TEM studies showed that the structural integrity of untreated (Fig. 1a) or heat-treated *M. paratuberculosis* cells remained intact. TEMs of untreated and heat-treated *M. paratuberculosis* cells were similar (data not shown).

Results from comparative studies with other bacteria revealed that *L. monocytogenes* 11194 cells and *B. cereus* 11145 endospores were reduced by 4.07 ± 0.3 and $0.17 \pm 0.04 \log_{10}$ CFU/ml, respectively, after PEF treatment at 50°C (Table 1). This finding suggests that *M. paratuberculosis* cells are more sensitive to the lethal action of PEF than are *L. monocytogenes* cells and *B. cereus* endospores. Thermal studies revealed that *M. paratuberculosis* strains ATCC 19689 and Linda (ATCC 43105) were similarly affected by heating (both strains were reduced by approximately $2.6 \log_{10}$ CFU/ml at 72°C), which

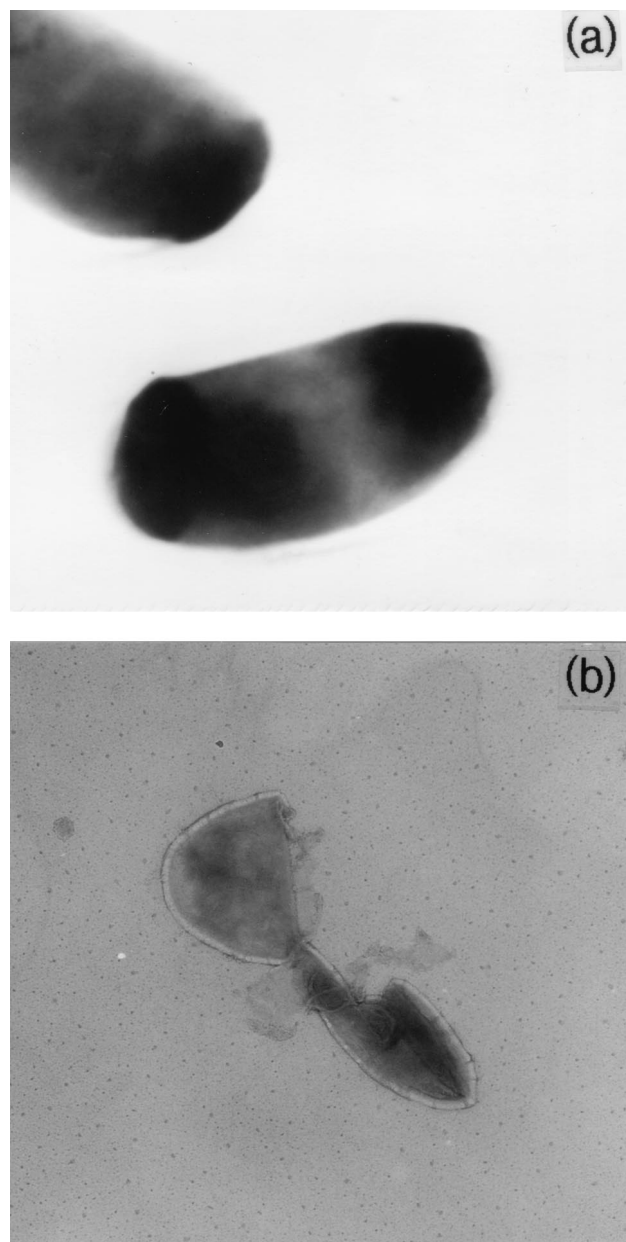


FIG. 1. TEMs of untreated (a) and PEF-treated (b) *M. paratuberculosis* ATCC 19698 cells (magnification, $\times 25,000$).

corroborated the findings of other researchers. Thermal studies showed that *L. monocytogenes* 11994 was effectively killed when examined after 15 s at 72°C (Table 1). The *D* value of *L. monocytogenes* 11994 at 63°C was also determined. The thermal death time curves were linear, and the $D_{63^\circ\text{C}}$ of 52.7 s was similar to the $D_{62.7^\circ\text{C}}$ of 54 s for *L. monocytogenes* Scott A reported by Donnelly et al. (4). Thus, it can be concluded that the method of thermal death rate or *D* value determination used in the present study is consistent with those used in other studies.

Results from *B. cereus* 11145 endospore heating experiments showed that the dormant spores of this diarrheagenic food-borne enteropathogen were far more heat tolerant than *M.*

paratuberculosis cells; these organisms were reduced by averages of 0.06 ± 0.04 and $2.6 \pm 0.3 \log_{10}$ CFU/ml, respectively. This present study demonstrates that the thermal inactivation of *M. paratuberculosis* cells and *B. cereus* endospores are not similar ($P < 0.001$). Results from PEF treatment of *M. paratuberculosis* ATCC 19698 and Linda (ATCC 43105) cells suspended in tyndallized cow's milk at 50°C revealed reductions of 5.6 ± 0.3 and $5.9 \pm 0.4 \log_{10}$ CFU/ml, respectively (data not shown). PEF treatment of *M. paratuberculosis* cells in cow's milk at 50°C produced a slightly greater level of inactivation than similar treatment in peptone water ($P < 0.05$). This improved PEF-induced killing of *M. paratuberculosis* may be due, in part, to milk having a greater conductivity.

Stimulated by the possible association between Crohn's disease and *M. paratuberculosis*, a number of independent studies from different countries have reported that this thermotolerant bacterium has the potential to survive commercial HTST pasteurization (72°C for 15 s) when suspended in cow's milk (5, 6, 14). Research by Grant et al. (5) showed that *M. paratuberculosis* may survive HTST pasteurization if present in milk at levels of 10^2 to 10^3 CFU/ml prior to heat treatment but will be completely inactivated by HTST pasteurization when low levels (10 CFU/ml) are present. As a consequence of these and other findings, and due to the detection of *M. paratuberculosis* DNA from cow's milk in retail outlets, the United Kingdom dairy industry has responded by extending the pasteurization holding period to 25 s. While the efficacy of this precautionary action has yet to be fully evaluated, it may be worth considering the inclusion of PEF as a complementary treatment to HTST pasteurization. Although the number of pulses applied during the present series of experiments was 2,500, it must be pointed out that no attempt has been made by the investigators to optimize PEF treatment of *M. paratuberculosis* cells. Based on PEF treatment of other organisms (7, 12), it can be expected that if the correct field intensity and PEF pulse frequency content are identified, the number of pulses required could be orders of magnitude lower.

Results from the present study indicate that the application of high-intensity PEF kills *M. paratuberculosis* in a test liquid and in milk and that this treatment is particularly effective

when carried out at moderately elevated temperatures. This study has also shown that PEF treatment causes substantial structural damage at the cellular level to *M. paratuberculosis* cells.

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