Detection and Fate of *Bacillus anthracis* (Sterne) Vegetative Cells and Spores Added to Bulk Tank Milk†

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ABSTRACT

A preparation of *Bacillus anthracis* (Sterne strain) spores was used to evaluate commercially available reagents and portable equipment for detecting anthrax contamination by using real-time PCR and was used to assess the fate of spores added directly to bulk tank milk. The Ruggedized Advanced Pathogen Identification Device (RAPID) was employed to detect spores in raw milk down to a concentration of 2,500 spores per ml. Commercially available primers and probes developed to detect either the protective antigen gene or the lethal factor gene both provided easily read positive signals with the RAPID following extraction from milk with a commercially available DNA extraction kit. Nucleotide sequence analysis of the *vrrA* gene with the use of DNA extracted from spiked milk provided molecular data that readily identified the spores as *B. anthracis* with a 100% BLAST match to the Sterne and Ames strains and easily distinguished them from *B. cereus*. Physical-fate and thermal-stability studies demonstrated that spores and vegetative cells have a strong affinity for the cream fraction of whole milk. A single treatment at standard pasteurization temperatures, while 100% lethal to vegetative cells, had no effect on spore viability even 14 days after the treatment. Twenty-four hours after the first treatment, a second treatment at 72°C for 15 s reduced the viability of the population by ca. 99% but still did not kill all of the spores. From these studies, we conclude that standard pasteurization techniques for milk would have little effect on the viability of *B. anthracis* spores and that raw or pasteurized milk poses no obstacles to the rapid detection of the spores by molecular techniques.

The potential for a bioterrorist attack on the U.S. food or water system appears to have increased after October 2001. The successful terrorist attack involving anthrax spores in the mail proved that there is a very real current threat of the use of biological weapons in the public environment. Furthermore, the food supply system has been used in other attacks involving microorganisms and chemicals in recent years (8). The milk supply system may be particularly vulnerable because of the increasing concentration of commercial milk production into larger and larger scale operations. A single large overland milk tanker can carry as much as 8,000 gal of milk, which could potentially be distributed to thousands of people. Following the October 2001 attack, questions arose regarding the fate of *Bacillus anthracis* (BA) spores purposely added to food products, particularly milk. We undertook a project to answer some of the questions raised. Specifically, the objectives of our research were (i) to determine whether conventional and fluorescence-based real-time PCR reagents for the detection of anthrax could be easily adapted to the detection of anthrax in milk and (ii) to measure the effects of pasteurization on the viability and detection of spores. The development of the 5′ nuclease fluorogenic PCR assay and advances in real-time fluorescence-based detection of pathogens (7, 10) is rapidly bringing this technology nearer to the marketplace, and hundreds of first responders may soon be expected to provide detection results with this new technology. Thus, it is important to provide these first responders with validated reagents.

BA can cause lethal disease in humans through two routes, respiratory and enteric, although the mortality rate for ingested BA spores is lower than that reported for inhaled spores (4). The third form of the disease, the cutaneous form, is rarely lethal. There are a variety of strains of BA, both lethal and nonlethal, and they differ with respect to the presence of the two virulence plasmids or with respect to the structure of a region known as the *vrrA* region (1, 9). The Sterne strain of BA was developed in 1937 (13). This strain (or its derivatives) has been widely used as a vaccine strain and is commonly used as a surrogate for the virulent strains of BA (4, 5, 14). It lacks the pX02 plasmid that carries the capsular protein (15) important for the virulence of the bacterium, and has a *vrrA* region indistinguishable from the virulent Ames strain region (9). The Sterne strain’s spores and spore-forming machinery are presumably indistinguishable from those of the virulent strains, and the Sterne strain carries the pX01 plasmid coding for three of the other major virulence factors, protective antigen (PA), lethal factor (LF), and edema factor (EF) (9).

† Mention of a trade name, vendor, or proprietary product or specific equipment is not a guarantee or a warranty by the U.S. Department of Agriculture and does not imply an approval to the exclusion of other products or vendors that also may be suitable.
There is a paucity of information on the fate of BA in food and, in particular, on its stability in various foods. Although rare, it has been shown that BA spores could be recovered from milk of anthrax-infected cows, and at least one study looked at the survival of vegetative and spore forms of BA in unpasteurized and pasteurized milk (3). There is no available literature, however, on the stability of BA spores during the pasteurization, storage, or processing of pasteurized milk. Consequently, the potential hazard of spores added directly to bulk milk has not been assessed. This fact, along with the increasing consumption of raw milk and products made from raw milk, prompted us to evaluate the fate of BA spores added directly to milk from the bulk tank.

**MATERIALS AND METHODS**

**Vegetative cell and spore preparation.** _B. anthracis_ Sterne and _Bacillus cereus_ were graciously provided by Dr. Catherine Fenselau, University of Maryland, College Park. Vegetative cells were obtained by scraping young colonies from blood agar plates, diluting them in phosphate-buffered saline (PBS) for immediate use, and then replating them on blood agar for enumeration. Spores from the strain were prepared by a method provided by Dr. J. Jackman, Applied Physics Laboratory, Johns Hopkins, University, Laurel, Md. New sporulation medium (NSM) contained (in distilled water [dH2O]) tryptone at 3 g/liter, yeast extract at 3 g/liter, and MgSO4·7H2O at 0.01 g/liter. NSM was autoclaved (121°C, 20 min) and poured into standard 100-mm petri dishes. Eight colonies of _B. anthracis_ Sterne from a sheep red blood cell Columbia agar plate (Remel, Lenexa, Kans.) were suspended in 2 ml of PBS, and 125 μl of stock was spread onto each of eight NSM plates. The plates were incubated at 37°C for 48 h and then at room temperature (ca. 22°C) for an additional 24 h. Biomass from each plate was suspended in 2 ml of dH2O with a plastic spreader, and each plate was washed with an additional 1 ml of dH2O. All suspensions and washes were pooled in a 50-ml centrifuge tube, and the volume was brought to 40 ml with dH2O. The suspension was incubated for 3 days at room temperature to allow the lysis of vegetative cells. Spores were recovered by centrifugation (8,000 × g, 10 min), washed with 40 ml of dH2O, pelleted by centrifugation, and suspended in 50 ml of dH2O. The spore suspension was observed by phase-contrast and Nomarski interference contrast microscopy and found to contain <0.1% vegetative cells. The highly refractive spores were counted in a hemocytometer under phase-contrast microscopy, and the suspension was found to contain 4.35 × 108 fully refractive spores per ml.

**Primer-probe synthesis.** Hybridization probe kits for the PA gene (BAPA, part no. 3481) and for the LF gene (BALF, part no. 3828) were obtained from Idaho Technology (IT), Salt Lake City, Utah, in the dried, ready-to-use form. They were resuspended and used in the IT Ruggedized Advanced Pathogen Identification Device (RAPID) essentially as described by the vendor.

**Extraction and sequencing.** A commercially available extraction kit (1-2-3 Kit, IT) was used essentially as described by the manufacturer with the exception that the volume of buffer 1 was doubled to accommodate extraction from a matrix with increased fat content. The DNA was eluted from the final column wash in 100 μl of buffer 3, which was used directly (3 to 5 μl) in the RAPID reaction mixtures resuspended from the BALF or BAPA IT detection kits. The RAPID reactions were run as specified by the manufacturer Simple 10-fold dilutions of spores were prepared in bulk milk to evaluate the sensitivity of the reactions.

**vrrA and lef gene PCR.** For PCR amplification with primers for the _vrrA_ and lethal factor (_lef_) genes, the reaction mixture contained 1 U of _Taq_ polymerase, 1.5 mM MgCl2, 5 μl of 10× PCR buffer, 50 pmol of primers GPR-4 and GPR-5 (9), and 200 μM (each) dATP, dGTP, dCTP, and dTTP (Invitrogen, Gaithersburg, Md.), with 5 μl of DNA as the template. To compare PCR amplifications for _B. cereus_ and BA, the spiking doses were ca. 76,000 spores of _B. cereus_ per ml and ca. 24,800 spores of _B. anthracis_ Sterne per ml. The “both” spike received each of these quantities of spores. Amplicons were analyzed via 1% agarose gels electrophoresed for 90 min at 50 V and ethidium bromide staining. To confirm the identity of the PCR products, an amplicon was sequenced with the use of dye-terminator chemistry on the ABI Model 3100 automated fluorescence sequencing instrument (Foster City, Calif.). The resultant sequence was analyzed with the use of NCBI BLAST and the ClustalW and Sequence Distance matrix functions of DNASTAR (DNASTAR, Inc., Madison, Wis.). Reference sequences from GenBank included BA strain B6273/93 (accession no. U63965), C93022281 (U63967), W-21 (U63964), Volum (U63968), and Ames (U63966.1) (5, 9).

**Manipulation of spore preparations in milk and culture techniques.** For each experiment, a stock of 107 spores per ml of raw milk was prepared. Various dilutions of the stock milk-spore preparation were prepared in bulk milk obtained fresh from the tank at the Beltsville Area Research Center Dairy Facility. The diluted samples were mixed by vortexing for 1 min. For thermal treatment studies, 1-ml aliquots of the samples were heated in 1.8-ml Eppendorf-style plastic tubes at pasteurization temperatures (63°C for 30 min or 72°C for 15 s) after the time to reach the desired temperature in the treatment tubes was empirically determined. Stored samples were routinely mixed to prevent the cream from separating. All platings of treated spore preparations were performed in triplicate, and the averages for the triplicates are presented in the tables and figures.

To separate and evaluate cream fractions, samples were centrifuged either at 4,000 rpm for 5 min in 10-ml tubes or at 10,000 rpm for 2 min in Eppendorf-style tubes. To quantitate bacteria in the centrifuged milk, fractions were dripped from the bottom of the sealed 10-ml tubes following centrifugation. BA samples in milk were plated in triplicate and quantitated on sheep red blood cell agar following appropriate dilutions to accommodate the initial concentration. Colonies on the triplicate plates were counted with a Q counter (Spiral Biotech, Inc., Newton, Mass.). Averages for the triplicates are presented. In control experiments, samples of _B. cereus_ spores and _Salmonella_ Typhimurium and _Escherichia coli_ vegetative cells were also spiked into 10-ml milk columns, centrifuged as above, fractionated into pellet, whey, and cream fractions, and enumerated on appropriate agar plates.

**RESULTS**

_A. anthracis_ Sterne spore preparation was shown to contain 4.35 × 109 spores per ml, with a vegetative cell contamination level of <0.1% (Fig. 1). When this spore preparation was added to raw milk and the cream fraction was separated by centrifugation, >99.9% of the spores separated into the top cream layer (Fig. 2). Conversely, 80% of vegetative cells separated with the cream, with nearly 20% of the population being in the skim fraction (Fig. 2). Heating to 63°C for 30 min apparently had little, if any, effect on the distribution of the spores in the centrifuged...
milk, with >99% separating in the cream. Heat treatment resulted in the complete death of all vegetative cells (Fig. 2). Centrifugation and fractionation of *B. cereus* spores yielded a pattern very similar to that for BA, and when *E. coli* or *Salmonella Typhimurium* cells were added to milk and centrifuged under the same conditions (4,000 rpm, 5 min), the cells were distributed throughout the milk column, with a slightly increasing cell concentration in the bottom fractions (data not shown).

When more extensive heating experiments were performed (Table 1), it was shown that both of the commonly used pasteurization temperatures had little, if any, effect on the viability of the spore preparation, while completely killing all of the vegetative cells. A second heating 24 h after the first resulted in ~2-log decreases in viable cell counts, but viable cells were still recovered after 24 h of storage. Spores that had been heated at both temperatures were stable 72 h after the heat treatment. The long-term stability of heated and unheated spores at different concentrations was then evaluated (Table 2). A preparation containing 10⁶ spores per ml of bulk milk, as counted in a slide-counting chamber, yielded 4.4 × 10⁶ CFU/ml on blood agar plates. The same preparation heated to 72°C for 15 s yielded 4.9 × 10⁶ CFU/ml. There was no significant drop in viable cell counts for these preparations after 7 and 14 days of storage after the heat treatment. When lower concentrations of spores (10⁵ and 10³) were treated, however, a ~2-log decrease in viability after 14 days of storage was observed. Such a decrease was not seen for the unheated samples we were able to quantitate (Table 2).

Raw milk appears to pose no major obstacles to extraction and analysis with the use of real-time PCR. With the slightly modified extraction protocol, we obtained DNA suitable for direct analysis in the RAPID system with the commercially available anthrax detection reagents. Spore concentrations as low as 2,500 spores per ml that were added to raw milk yielded a clear positive signal (Fig. 3). The increasing concentrations of spores in raw milk yielded in-

![Figure 1](image1.png)

**FIGURE 1.** Preparation of *B. anthracis* Sterne spores; appearance of spores and a single contaminating vegetative cell under microscopy.

![Figure 2](image2.png)

**FIGURE 2.** Distribution of spores and vegetative cells in whole milk after heating and centrifugation. Spores and vegetative cells were added to raw milk, and equal aliquots were heated to 63°C for 30 min. The samples were centrifuged at 4,000 rpm for 15 min. The tubes were then dripped from the bottom into four equal aliquots. The total concentration of *B. anthracis* in each fraction was then determined by plating in triplicate on blood agar. Data shown are averages for the triplicates.
TABLE 1. Effects of heat treatment on vegetative cells and spores of Bacillus anthracis Sterne in raw milk

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Initial count (CFU/ml)</th>
<th>Count after 24 h (CFU/ml)</th>
<th>Count after 48 h (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative cells</td>
<td>None</td>
<td>1.6 × 10^4</td>
<td>4.3 × 10^3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>63°C, 30 min</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C, 15 s</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spores</td>
<td>None</td>
<td>4.9 × 10^4</td>
<td>3.50 × 10^4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>63°C, 30 min</td>
<td>5.20 × 10^4</td>
<td>3.30 × 10^4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C, 15 s</td>
<td>2.33 × 10^3</td>
<td>2.6 × 10^2</td>
<td></td>
</tr>
<tr>
<td>Spores reheated</td>
<td>63°C: 72°C, 15 s</td>
<td>3.0 × 10^2</td>
<td>2.43 × 10^2</td>
<td></td>
</tr>
</tbody>
</table>

a Spores and vegetative cells were added to raw milk, and aliquots of the milk were immediately heated to standard pasteurization temperatures (63°C for 30 min or 72°C for 15 s). The samples were then plated on blood agar for the enumeration of viable cells. As can be seen, heating destroyed all vegetative cells but had no effect on spores. A second assay was performed after 24 h by reheating the previously pasteurized spores at 72°C for 15 s. This reheating destroyed 99% of the viable cells, indicating that most of the spores had been activated to vegetative cells by the pasteurization process and were then killed by the subsequent second heating. However, some viable cells clearly remained, indicating that pasteurization and double pasteurization would not kill all B. anthracis spores added to raw milk.

TABLE 2. Storage of spores in milk following exposure to high-temperature short-time (HTST) pasteurization conditions and following exposure to no heat

<table>
<thead>
<tr>
<th>Time post-inoculation</th>
<th>10^6 spores/ml</th>
<th>10^7 spores/ml</th>
<th>10^8 spores/ml</th>
<th>10^9 spores/ml</th>
<th>10^10 spores/ml</th>
<th>10^11 spores/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTST</td>
<td>No heat</td>
<td>HTST</td>
<td>No heat</td>
<td>HTST</td>
<td>No heat</td>
<td>HTST</td>
</tr>
<tr>
<td>1 h</td>
<td>4.9 × 10^6</td>
<td>4.4 × 10^6</td>
<td>4.0 × 10^4</td>
<td>4.6 × 10^4</td>
<td>4.1 × 10^2</td>
<td>4.5 × 10^1</td>
</tr>
<tr>
<td>7 days</td>
<td>4.8 × 10^6</td>
<td>6.5 × 10^6</td>
<td>2.1 × 10^4</td>
<td>2.3 × 10^4</td>
<td>1.7 × 10^2</td>
<td>4.5 × 10^1</td>
</tr>
<tr>
<td>14 days</td>
<td>4.2 × 10^6</td>
<td>4.0 × 10^6</td>
<td>5.0 × 10^2</td>
<td>2.2 × 10^4</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

a HTST conditions consisted of 72°C for 15 s. TNTC, too numerous to count because of overgrowth of other microorganisms; ND, not done.
guish between spores of BA and *B. cereus*, as could PCR followed by cycle sequencing (Fig. 4A). *B. cereus* and BA could also be easily distinguished with the use of a primer set specific for the *lef* gene (Fig. 4B), so contamination with *B. cereus* in milk would pose no problems in detecting BA.

There are very few published empirical data that can be used to predict the dose of consumed BA spores that would cause disease in humans. Gastrointestinal anthrax has not been reported in the United States, but reports from heavily endemic countries indicate that symptoms generally appear within 2 to 5 days after the ingestion of spore-contaminated meat from diseased animals. For purposely added...
spores, it would be assumed that spore preparations could easily reach concentrations of $10^{10}$ per ml of liquid or $10^{12}$ per g of a dried preparation. This would mean that spores could be easily delivered at concentrations of $\geq 10^6$ spores per ml in bulk milk, even in a large tanker. While mortality from outbreaks of contaminated meat has certainly been documented and can be high (16), doses of cells ingested have not been measured, so little is actually known about what to expect in the case of a purposeful introduction. There is some suggestion that a lesion in the gastrointestinal tract would be required to initiate the lethal form of the disease.

Previous studies on the stability of spores and vegetative cells have indicated that both were relatively stable when added to pasteurized and unpasteurized milk at 5°C. Incubation at higher temperatures resulted in a loss of viability of vegetative cells, but stability was measured only over a 48-h period, and the effects of pasteurization were not measured (3). The results of these studies also supported the general notion that the contamination of milk from an infected animal in a milking herd would not pose a significant health risk owing to dilution into larger quantities, but again, no quantitative data were presented. Another study (2) demonstrated that the likelihood of "natural" contamination of milk from an infected herd would be quite small. It is good that BA would not likely find its way into the milk supply system, since the results of the present studies clearly show that spores of the Sterne strain of BA are quite refractory to pasteurization and storage, particularly at higher spore concentrations. Even after double pasteurization, viable cells were not completely killed, indicating that pasteurization conditions did not activate all spores to germinate and as much as 1% of the population survived the second pasteurization. Whether these data would reflect the characteristics of the virulent strains of BA is not known for certain. Since the only difference between the Sterne strain and virulent strains is the lack of the capsule present in the vegetative cells, it seems likely that spore formation and germination, along with stability, would be the same. A further unknown factor is the effect of stabilizing additives in pasteurized milk on the stability of spores, although research on the closely related milk contaminant B. cereus indicated no significant effects on spore stability with three common stabilizers (12).

There should be concern over the potential for the use of BA as a bioweapon in food. Many foods are considered safe following pasteurization, and under circumstances in which BA vegetative cells might be introduced, cooking or pasteurization should prevent infection. Under circumstances involving the purposeful introduction of large numbers of spores, however, risks of infection may be high, and it seems prudent to develop rapid assays to evaluate levels of contamination in questionable food or dairy products. These studies demonstrate that portable real-time PCR could provide a rapid assay for the detection of reasonable levels of BA spores in raw or pasteurized milk.

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REFERENCES