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Immunomagnetic Detection of *Bacillus stearothermophilus* Spores in Food and Environmental Samples†

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There are currently no methods for the rapid and sensitive detection of bacterial spores that could be used to direct raw materials containing high spore loads away from products that pose a food safety risk. Existing methods require an overnight incubation, cannot detect spores below 10^5 CFU/ml, or are not specific to particular species. This work describes a method to specifically detect $<10^4$ CFU of bacterial spores per ml within 2 h. Polyclonal antibodies to *Bacillus stearothermophilus* spores were attached to 2.8-μm-diameter magnetic polystyrene beads by using a polythreonine cross-linker via the antibody carbohydrate moiety. A biotin-avidin-amplified sandwich enzyme-linked immunosorbent assay coupled to a fluorescent substrate was used to quantitate captured spores. The concentration of *B. stearothermophilus* spores in samples was linearly correlated to fluorescent activity ($r^2 = 0.99$) with a lower detection limit of 8×10^3 CFU/ml and an upper detection limit of 8×10^5 CFU/ml. The detection limits are not fixed and can be changed by varying the immunomagnetic bead concentration. Several food and environmental samples were tested to demonstrate the versatility of the assay.

Bacterial spores are the most heat-stable form of microorganisms, are ubiquitous in the environment, and are therefore of great concern in food products—like milk—that receive extensive heat treatments to prolong shelf life. Spore counts in milk from around the world vary between 0 and >22,000 CFU/ml depending on the climate of the region (3). *Bacillus stearothermophilus* spores are among the most heat-resistant bacterial spores and are found in large numbers in soil and water. Contaminating *B. stearothermophilus* spores survive extreme heating to germinate and grow at elevated product storage temperatures, which occur in foods transported in equatorial regions of the world.

While *B. stearothermophilus* is not commonly a problem, other bacilli often lead to foodborne illness or spoilage in a variety of foods. *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus* have all been implicated in outbreaks of foodborne illness and are commonly isolated from raw and heat-treated milk (8). *B. cereus* is responsible for a sweet curdling defect in milk as well as being pathogenic (19). A mesophilic heat-resistant bacillus similar to *Bacillus badius* has been isolated from extremely temperature-processed milk (D_{147} = 5 s) (10). *B. badius* is a mesophilic organism and grows readily at room temperature, making it a likely candidate for spoiling temperature-processed foods. There have been 52 confirmed cases of *B. badius* in UHT milk in Europe and 2 cases outside Europe (10). Lack of a rapid spore assay that can be used in milk contributes to the difficulty of predicting post-processing spoilage, thereby limiting shelf life and product safety (12). Such an assay could be used in a hazard analysis critical control point (HACCP) plan, allowing raw materials with high spore loads to be diverted to products that do not pose a food safety risk to consumers.

The standard method for quantifying spores in milk (21) involves a heat shock and an overnight plate count. This is time-consuming and yields only historical information. The food industry needs microbiological assays to yield predictive information for maximum benefit in HACCP analysis and risk assessment. An enzyme-linked immunosorbent assay (ELISA) capable of detecting $>10^6$ CFU of *B. cereus* spores per ml in foods has been reported but was unacceptable due to antibody cross-reactivity (4).

Techniques to increase the sensitivity of immunosorbent assays have focused on more efficient reporter labels, such as faster catalyzing reporter enzymes; signal amplification, such as avidin- or streptavidin-biotin enzyme complexes; and better detectors, such as luminescence and fluorescence (13, 20). Immunomagnetic antigen capture is used extensively to separate and identify *Escherichia coli* and *Salmonella* from foods (1, 6, 14–18, 22, 24). However, these methods involve either a preincubation or a subsequent incubation step—usually 18 to 24 h—to increase the cell numbers for detection. Immunomagnetic capture greatly shortens the time needed for *E. coli* and *Salmonella* testing, but long incubation times limit the use of this method to provide predictive information. Immunocapture has also been used to quantitate *Bacillus anthracis* spores in soil samples by using luminescent detection (2).

The object of this work was to develop a rapid assay for detection of *Bacillus* spores that employed the immunomagnetic capture of spores from foods, a biotin-streptavidin complex to amplify the signal, and fluorescence detection.

MATERIALS AND METHODS

Bacterial spores. Commercial preparations of spores of *B. stearothermophilus* ATCC 10149, *B. cereus* ATCC 11778, and *B. subtilis* 6633 (Fisher Scientific, Pittsburgh, Pa.) were used to produce antibodies. Viable spore numbers and germination estimates were obtained by plating these three species on plate count agar (PCA) overnight at 65, 30, and 30°C, respectively. All other spores (see Table 1) were prepared by spread plating a single colony isolate on PCA and incubating the covered plate at 30°C for approximately 2 weeks. Spores were swabbed from the surface of the agar and washed repeatedly in distilled water to remove water-soluble components. The spores were pelleted and separated from cell debris by centrifugation (1,500 × *g* for 20 min at 4°C) (7). The presence of spores was confirmed by heating to 80°C for 15 min and then plating on PCA (21). The presence of an exosporium on the spore was tested by phase-contrast microscopy with crystal violet staining (5).

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TABLE 1. Incubation temperature, source, and presence of an exosporium on the spores of *Bacillus* species used in this study

Species	Incubation temp (°C)	Source	Presence of exosporium[a]
B. stearothermophilus	65	ATCC 10149[b]	−
B. cereus	30	ATCC 11778[b]	+
B. subtilis	30	ATCC 6633[b]	+
B. circulans	30	ATCC 4513[c]	−
B. coagulans	30	ATCC 7050[c]	−
B. licheniformis	30	Raw milk isolate[d]	−
B. mascerans	30	Raw milk isolate[d]	+
B. polymyxa	30	ATCC 842[c]	+
B. pumulus	30	Raw milk isolate[d]	−

[a] Spores were observed by phase-contrast microscopy with crystal violet staining (5).
[b] From Fisher Scientific.
[c] Purchased from ATCC.
[d] Kindly donated by Floyd Bodyfelt, Oregon State University.

Polyclonal antibody production. Polyclonal anti-*B. stearothermophilus* spore antibodies (anti-BsS) were made at the Utah State University Biotechnology Center (Logan, Utah). BALB/c mice were given injections in the intraperitoneal cavity with 10^7 CFU of *B. stearothermophilus* spores per ml in sterile physiological saline (0.5 ml) three times at 3-week intervals (9). Total amounts of immunoglobulin G (IgG) in serum were purified on a protein A/G column (Pierce Chemical, Rockford, Ill.). The antibodies were desalted and concentrated to 1 mg/ml in 0.1 M sodium phosphate (pH 7.0) in a 30-kDa Centricon centrifugal concentrator (Amicon, Beverly, Mass.) at 4,500 × *g* at 4°C.

Antibody specificity. The antibody specificity was tested by measuring the cross-reactivity against the *Bacillus* spores listed in Table 1 by a standard ELISA. A suspension of each spore type (10^6 CFU/ml), suspended in 50 mM $NaCO_3$ (pH 9.5), was nonspecifically bound to wells of a microtiter plate for 12 h at 4°C. Wells containing spores were blocked with bovine serum albumin (2% in phosphate-buffered saline [PBS]) for 4 h at 25°C and washed four times with PBS containing 0.1% Tween 20 (PBST). Anti-BsS (1:10,000 serum dilution in PBS) was added to the wells, and the mixture was slowly agitated for 2 h at 25°C and washed four times with PBST. Horseradish peroxidase-labeled anti-whole mouse IgG (Sigma) was added to label anti-BsS for 2 h, and the mixture was washed four times with PBST. *o*-Phenylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.) color development was measured by using a b* color scale (blue to yellow) at 37°C for 1 h in an automated reflectance colorimeter (Omnispec 4000 bioactivity monitor; Wescor, Inc., Logan, Utah).

Antibody biotinylation. Antibodies purified from total serum were biotinylated with NHS-LC-Biotin (Pierce Chemical). The efficiency of surface biotinylation was determined by the HABA assay (Pierce); the β-mercaptoethanol step was omitted to avoid denaturing the antibodies. This modified procedure gave the number of surface biotin moieties per antibody (Sigma Technical Support).

Antibody-bead conjugation. Sodium *meta*-periodate (5 mg) was used to oxidize carbohydrate moieties on the antibodies (11). The sodium *meta*-periodate was removed after oxidation by washing five times with 0.1 M sodium phosphate (pH 7.0) in a 30-kDa Centricon centrifugal concentrator (4,500 × *g* at 4°C) and immediately cross-linked to magnetic beads.

Polythreonine (molecular weight, 12,100; Sigma) was covalently coupled to 2.8-μm, tosyl-activated polystyrene Dynabeads (Dynal, Lake Success, N.Y.) in 50 mM borate buffer (pH 9.5) via the terminal amine as described by the manufacturer. Four washes (three times for 10 min and once for 30 min) with Tris-buffered saline (pH 7.5) were used to block remaining tosyl-active sites. Adenine dihydrazine (ADH; 0.5 M in 0.1 M morpholineethanesulfonic acid [MES; pH 4.75]; Sigma) was linked to the carboxyl terminal of the bound polythreonine by an ethylenediamine carbodiimide-mediated reaction (11). Oxidized antibodies were mixed with the ADH-activated beads at room temperature for 12 h to allow cross-linking between the oxidized carbohydrate moiety of the IgG and the ADH terminal of the polythreonine linker (11). After cross-linking, the immunomagnetic beads (IMB) were stored rotating (≈50 rpm) in PBST with 0.02% sodium azide at 4°C until used.

Immunocapture. IMB (3×10^6 beads) were added to 1 ml of sample containing spores and allowed to rotate (≈50 rpm) for 30 min at 25°C. The IMB were removed from the sample for 2 min with a magnetic particle concentrator (Dynal MPC-E-1) and washed four times with PBST to reduce IMB clumping and block spore adhesion to tube walls (23). Samples containing fat were given 5 min for separation of IMB due to slower bead recovery. After each wash, IMB were transferred to a new microcentrifuge tube. The presence of bound spores on IMB was confirmed in duplicate by plate counts and examination with a phase-contrast microscope.

Determination of optimum IMB binding temperature. IMB were added to 1 ml of UHT skim milk containing 5×10^4 *B. stearothermophilus* spores and incubated at 4 to 50°C while rotating (≈50 rpm) for 30 min. The IMB were washed four times with PBST, plated on PCA, and incubated overnight at 65°C. *B. stearothermophilus* colonies were counted to quantitate bound spores.

Fluorescent detection of captured spores. Spores bound to IMB were labeled with a secondary biotinylated anti-BsS. The IMB were then washed with PBST and resuspended in biotin-streptavidin-alkaline phosphatase complex solution (Vector Laboratories, Inc., Burlingame, Calif.) for 30 min. The IMB were washed three times with PBST and resuspended in 100 μl of 0.2 M Tris buffer containing 0.1% bovine serum albumin (pH 8.5) to remove unbound enzyme complex. A 40-μl suspension of the IMB was added to 3 ml of Fluorophos substrate (Advanced Instruments, Norwood, Mass.), and fluorescence was monitored for 2 min at 38°C in a Fluorophos FLM200 fluorometer (Advanced Instruments).

Product testing. Fluid products were tested with no modification. Powdered products were dissolved to 1 g/ml. IMB (3×10^6 beads) were added to 1 ml of each product and mixed gently for 30 min at 25°C. Bound spores were quantitated by fluorescence detection.

RESULTS AND DISCUSSION

The anti-BsS were directionally oriented via the carbohydrate moiety to beads by using a polythreonine spacer. We tried several other methods of binding antibodies, but the IMB-bound anti-BsS did not capture spores from solution (Table 2). In all cases the use of horseradish peroxidase-labeled anti-IgG antibodies confirmed the presence of bound antibodies on the surface of the IMB by ELISA. While the anti-IgG spacer also acted as a spacer, only antibodies bound via the polythreonine spacer captured *B. stearothermophilus* spores. Spore capture was observed by phase-contrast microscopy, where spores could be seen against the surface of the beads after nonbound spores had been washed away. These data suggested that spacer length and flexibility may play a role in the antibody-epitope interaction.

The IMB specifically captured *B. stearothermophilus* spores from PBST containing equal numbers of *B. stearothermophilus* and *B. subtilis* spores (Fig. 1A). About 99% of nonspecifically bound organisms were removed during each wash, leaving *B. stearothermophilus* spores captured after four washes (Fig. 1B). The anti-BsS did not cross-react with any of the spore types tested (Table 1) including common aerobic spores found in raw foods. The lack of cross-reactivity may be partly due to the

TABLE 2. Method of anti-BsS IgG attachment to IMB and its influence on the number of spores captured from PBST containing 10^4 or 10^6 CFU/ml *B. stearothermophilus* spores

Method of antibody attachment to magnetic beads	Antibody modification	Antibody orientation	No. of spores bound[a] from PBST
Biotin-streptavidin	NHS-LC biotinylation[b] (3.4 biotins/antibody)	Nondirectional	0, 0
Antibody amine groups bound to tosyl-activated −OH groups on bead surface	None	Nondirectional	0, 0
Anti-Fc IgG spacer	None	Directional	0, 0
Polythreonine-ADH cross-linker	Carbohydrate oxidation	Directional	160, 3,600

[a] Determined by plate counts after four washes with PBST. The first number is for 10^4 CFU/ml, and the second is for 10^6 CFU/ml.
[b] Biotinylation did not inhibit binding as determined by ELISA.

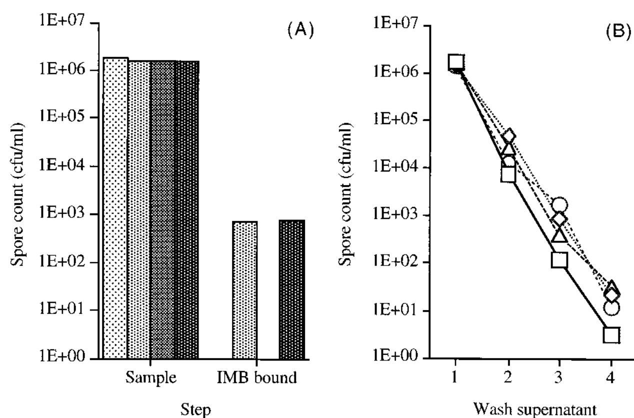

FIG. 1. Specific binding of *B. stearothermophilus* spores from a mixed population in PBST and milk. (A) Specific capture of *B. stearothermophilus* spores from a mixed population. (B) Concentration of spores in the wash. ▧, □, *B. subtilis* (PBST); ▤, ◇, *B. stearothermophilus* (PBST); ▩, ○, *B. subtilis* (milk); ▦, △, *B. stearothermophilus* (milk).

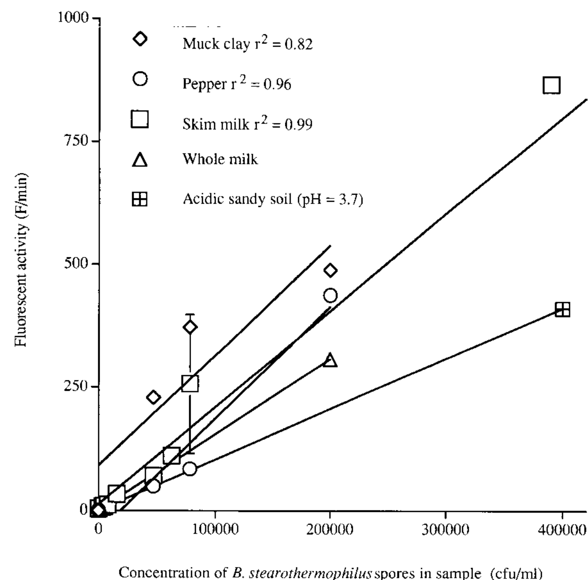

FIG. 3. Fluorescence detection of captured *B. stearothermophilus* spores in various food and environmental samples with 3×10^6 IMB. Data points represent the mean of two replications. Curves for whole milk and acidic sandy soil samples represent only two data points. Error bars represent standard error of the means.

absence of an exosporium on the *B. stearothermophilus* spores (Table 1). However, antibodies raised against *B. subtilis* and *B. cereus*, which did have exosporia, were specific only to the injected spore types, suggesting that the surface antigens of the exosporia are sufficiently different as to not cross-react (data not shown). Immunomagnetically captured *B. stearothermophilus* spores were labeled with biotinylated-anti-BsS, minimizing potential background created by nonspecifically bound organisms. The biotin moiety was used to bind an avidin-biotin-alkaline phosphatase complex for signal amplification.

Using this immunocapture sandwich ELISA, we quantified spores in UHT skim milk down to 8×10^3 CFU/ml in 2 h with no preenrichment steps and no sample preparation. Increasing the number of beads in the assay increased the fluorescence activity, suggesting that this could further increase the assay sensitivity (Fig. 2) (6, 23).

We also demonstrated the immunocapture of spores in a variety of complex backgrounds such as fluid milk, powdered milk, baby formula, spices, soil, and sand (Fig. 3). The slopes of the generated curves were similar for all samples tested, indicating that sample background did not grossly influence antigen binding. Therefore, approximate spore loads can be obtained without calibrating the assay to each product. Foods containing fat, such as raw whole milk, required a longer IMB separation time and gentle removal of the supernatant to avoid trapping the beads in the fat and removing them with the supernatant. Separation of IMB from fatty products required 5 min rather than the 2 min used for nonfatty samples. Soil samples containing a high percentage of iron fines interfered with bead recovery, although other soil types tested did not. These data support the use of this assay to test for *B. stearothermophilus* spores in food and environmental samples.

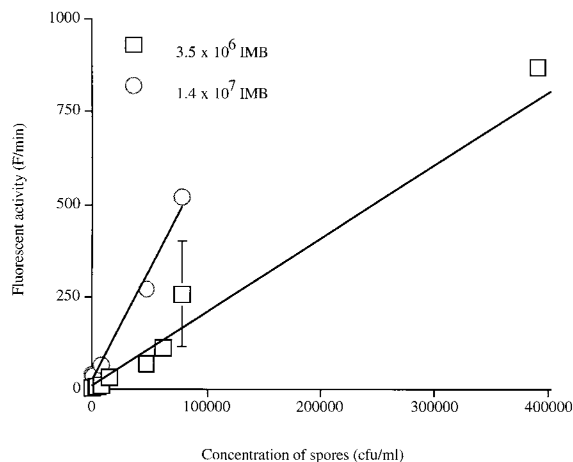

FIG. 2. Fluorescence detection of captured *B. stearothermophilus* spores in skim milk by a biotin-avidin amplified sandwich ELISA with 3×10^6 and 1.4×10^7 IMB. Data points represent the mean of two replications. Error bars represent standard error of the means.

Since the assay has been designed to be used with raw ingredients that may vary in temperature, we tested the ability of the IMB to capture *B. stearothermophilus* spores from 4 to 50°C. The number of spores captured from UHT skim milk containing 5×10^4 *B. stearothermophilus* spores did not vary significantly, thus reducing sample preparation time.

These data suggest that this approach is over 100 times more sensitive than the only other rapid spore assay (4), is about 10 times faster than any spore assay with equivalent sensitivity (21), and can be used to quantitate a single species of spore in a mixed spore population in chemically complex backgrounds.

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