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The Antigenicity of *Bacillus globigii* Spores

By:

William E. Lee, Gail Thompson and Eric Kokko*

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Suffield Memorandum No. 1500

The Antigenicity of *Bacillus globigii* Spores

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William E. Lee, H. Gail Thompson and Eric Kokko*

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ABSTRACT

Immunoassays of *Bacillus globigii* spores were carried out using a number of polyclonal antibody preparations. The limits of detection for the various antibody preparations gave similar values: all in the range of 1 ng or less. It was determined that, although spores reconstituted in distilled water could be detected in small amounts, the antibodies were directed at a soluble component in the samples, rather than the spores themselves. When the soluble component was separated from the spores by centrifugation, it was found that this component was responsible for over 90% of the immunoassay signal even though it composed less than 10% of the total weight. The nonreactivity of BG spores was also observed in transmission electron microscopy. Electron micrographs of the spores immunostained with colloidal gold did not indicate any appreciable antibody interaction.

EXECUTIVE SUMMARY

Title

The Antigenicity of *Bacillus globigii* Spores, William E. Lee, H. Gail Thompson and Eric Kokko, DRES Suffield Memorandum 1500, 1998

Introduction

Immunoassays are an important technique for detection and identification of biological materials including pathogenic and toxic agents and are a prominent assay method in the Canadian Integrated Biological Agent Detection System (CIBADS). Field trials and chamber trials of environmental monitoring equipment (such as CIBADS) are carried out regularly using specific simulants and tracers. A commonly used simulant is the spore preparation of *Bacillus subtilis* var. *niger*, often referred to as *Bacillus globigii* (BG). It is a nonpathogenic soil bacterium that can be safely released into the open air environment.

During the work leading up to the field trials, antibodies against BG spores were raised and immunoassays for BG spores developed. The purpose of the work in this report was to characterize the antigen-antibody interactions for BG and anti-BG preparations. The BG spore preparations used in this work were obtained from Dugway Proving Ground as a dry powder preparation which contained, in addition to spores, residual amounts of growth medium and debris from vegetative cells. Several preparations of polyclonal antibodies were raised against BG spores and we were interested in determining where the antibody binding was directed.

Results

Immuno-filtration assays were carried out against BG spores reconstituted in water, as well as soluble and sedimented fractions. It was found that the greater part of the antigenicity was associated with the soluble components, rather than with the spores themselves. These findings were confirmed by electron microscopy studies employing colloidal gold.

Significance of Results

This report provides an insight into the antigenicity of antibodies raised against BG spores. Although a number of vaccinations were carried out in rabbits and in goats, the antibodies produced were always directed against the soluble components of the reconstituted BG spore preparations. This was the case even when the BG spores were thoroughly washed (by centrifugation and resuspension) prior to injection in order to remove the soluble components. The soluble component seemed to persist in sufficient amount to induce antigenic response in the vaccinated animals. In total, four polyclonal preparations were assayed, all capable of detecting small amounts of the BG preparation by immunoassay, but all were found to be directed against the soluble component. The antibody preparations derived from the washed spores provided the lowest limits of detection.

A rabbit polyclonal antibody preparation, derived from washed spores, was employed in analysis of BG in chamber studies at DRES and field trials at Dugway Proving Ground. The antibodies were effective in detecting BG in liquid samples obtained from aerosol collectors. In the field trials, BG was released into the air either as a dry powder or as a wet slurry, each of which contained both the spores and the soluble antigenic material. Aerosol, when collected, contained sufficient material for detection. Thus for field trials of immunoassay identification systems (Threshold or Origen) it was not an absolute requirement that the antibodies be directed against the simulant itself (in this case BG); good results could be obtained by determining the presence of ancillary component(s) in the simulant preparation. However, in constructing protocols for field trial measurements, the antigenicity (i.e. where the immunoassay signal originates) is important knowledge for successful design and interpretation of experimental data.

Future Goals

The demonstrable importance of the soluble antigens on the immunoassays of BG spores suggests that the antigens should be characterized by extraction and synthesis to standardize the antibody stocks. Since BG spores have been characterized to have complex carbohydrates, and since the antigens do not appear to be proteins, a likely candidate for these soluble antigens is the noted sugar polymers. It would also be a worthwhile investigation to determine whether the soluble antigens are unique to different bacilli spores or a source of cross reaction between *B. subtilis* and other species such as *B. anthracis*.

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Introduction

The spores of *Bacillus subtilis* var. *niger*, often referred to as *Bacillus globigii* (BG) have been employed as tracers for sterilization, aerosol and liquid studies. As well, BG has been the subject of immunoassays and gene probe assays.

BG is a nonpathogenic soil bacteria and, as such, is suitable for use in dissemination studies in aerosol chambers and in open field settings. BG spores was one of the simulants used in the Joint Field Trials at Dugway Proving Ground, Utah, where environmental monitoring systems, such as Canadian Integrated Biological Agent Detections System (CIBADS) were tested. CIBADS is a portable multifaceted detection/identification unit housed in a 10 m trailer. It contains collection, concentration, detection and identification instruments. Identification was carried out by immunoassay on two commercial immunoassay devices: Threshold^{R} and Origen.^{R} The Origen^{R} system is based on sandwich immunoassay, magnetic bead separation and electrochemiluminescence detection. The Threshold^{R} is also based on sandwich immunoassay, but with separation by streptavidin-biotin mediated filtration onto nitrocellulose membrane and detection by enzyme-generated pH change. Threshold assays were employed in this study and will be described below.

During the work leading up to the field trials, antibodies against BG spores were raised and immunoassays for BG spores developed. The purpose of the work in this report was to characterize the antigen-antibody interactions for BG and anti-BG preparations.

Intact bacillus spores are resistant to simple lysing (enzymatic or osmotic) or solubilization and generally require mechanical degradation with glass beads for disruption. The post-breakage components can be solubilized with some difficulty. The spore coat makes up the major portion of the spore (about 50% of the volume) and consists mostly of protein, with smaller amounts of lipid and complex carbohydrate.

The BG spore preparations used in this work were obtained from Dugway Proving Ground as a dry powder preparation which contained, in addition to spores, residual amounts of growth medium and debris from vegetative cells. Several preparations of polyclonal antibodies were raised against BG spores and we were interested in determining where the antibody binding was directed.

Immuno-filtration assays were carried out against BG spores reconstituted in water, as well as soluble and sedimented fractions. It was found that the greater part of the antigenicity was associated with the soluble components, rather than with the spores themselves. These findings were confirmed by electron microscopy studies employing colloidal gold.

Materials and Methods

Reagents

Bovine serum albumin (BSA), sodium dihydrogen phosphate, Tween 20, Triton X-100, urea, phosphate buffered saline (PBS), and streptavidin-conjugated colloidal gold (20 nm particle size) were obtained from Sigma Chemical Co. (St. Louis, MO) and used without any further purification. Streptavidin was obtained from Scripps Laboratories (San Diego, CA) and was reconstituted in distilled water to yield a stock concentration of 10 mg/mL and stored at -20 ^{o}C. Antibody labelling reagents, *N*-hydroxysuccidimide esters of carboxyfluorescein and dinitrophenylbiotin were obtained from Molecular Devices Corp. (Sunnyvale, CA) and were used according to procedures described in the product literature. Nitrocellulose membrane filters (0.44 µm pore size) coated with biotinylated BSA, and anti-fluorescein urease conjugate were purchased from Molecular Devices Corp.

Antibodies

Rabbit polyclonal antibodies were produced at DRES by Elaine Fulton and Laurie Stadnyk. The antibodies were prepared by an initial vaccination and subsequent boosting of rabbits with reconstituted BG spores. Serum from a total of four rabbits was collected and pooled. A similar vaccination procedure was carried out using BG spore, that had been thoroughly washed by 6 cycles of centrifugation and resuspension in distilled water. The washing was performed to remove any soluble antigenic material present in the initial BG preparations. In effect, two pooled serum samples were obtained, one raised against the whole BG spore preparation and one against the washed spores. Additional antibodies, anti-BG (goat IgG), and anti-BG (rabbit IgG), were obtained from the Baker Laboratory, Dugway Proving Ground, Utah. All of the antibody preparations were purified by affinity

chromatography on Protein A columns using an ImmunoPure-A IgG purification kit (Pierce Chemical Co., Rockford, IL) according to procedures in the product literature.

Antibodies to BG were labelled with fluorescein or biotin using ImmunoLigand Assay kits (Molecular Devices Corporation) according to protocols provided by the manufacturer.

BG spores

Lyophilized BG spore preparations were obtained from the Baker Laboratory, (Dugway PG) and were reconstituted as a suspension in distilled water at a concentration of 1 mg/mL. Washed BG spores were prepared by taking 1 mL aliquots of the reconstituted suspension and centrifuging for 15 min at 14,000 rpm on an Eppendorf 5415C benchtop centrifuge. The supernatant was removed above the pellet; 1 mL of distilled water was added to the microfuge tube. The pellet was thoroughly resuspended and spun again in the centrifuge. The procedure was repeated for as many washings as required. The soluble antigen component was derived from the supernatant of the first centrifugation. Prior to assays, the soluble component was filtered through µStar™ 0.22 µm nitrocellulose sterilizing filters (Costar, Cambridge, MA) attached to the end of a syringe.

Apparatus

The detection apparatus was a commercially available Light Addressable Potentiometric (LAP) sensor marketed under the name Threshold Unit. It was purchased from the manufacturer, Molecular Devices Corp.

Immunoassays

For immunoassays the wash solution consisted of 150 mM NaCl, 10 mM phosphate buffer pH 6.5, plus 0.2% Tween 20 detergent. The dilution buffer was the wash solution titrated to pH 7.0, containing 0.1% bovine serum albumin (BSA) and 0.25% Triton X-100. The substrate solution was the wash solution containing 100 mM urea.

Figure 1 provides a schematic representation of the sandwich immunoligand assay. The reagent solution consisted of approximately 20 µL of biotin-labelled and fluorescein-labelled antibody preparations (typically 0.5 mg/mL) and 1 µL streptavidin stock solution

in 10 mL of dilution buffer. Aliquots of reagent solution (100 µL) were added to 100 µL portions of antigen (the test analyte), suspended in dilution buffer, mixed, incubated for the required time and filtered through the biotin-embedded nitrocellulose membrane. An aliquot (200 µL) of anti-fluorescein urease conjugate (stock solution diluted 1/10 in dilution buffer) was added to the filter wells and filtered through the membrane. The wells were then washed with 500 µL aliquots of wash solution. After the filtration-capture procedures, the membrane sticks were inserted into the reader compartment which contained the LAP sensor and substrate solution. A plunger pressed the membrane against the surface of the sensor. The instrument was designed so that the spots on the surface of the membrane aligned with the pH sensitive sites on the surface of the silicon sensor. The data was recorded and stored on the microcomputer. The rate of change of pH with respect to time at the surface of the silicon sensor was monitored by the rate of change with respect to time of the surface potential as µV/s.

Transmission Electron Microscopy

A suspension of washed BG spores (six cycles of centrifugation - resuspension) at a concentration of 1 µg/mL (dry weight) was prepared. Aliquots (10 µL) of washed BG were evaporated on the coated side of the electron microscopy grids (Formvar coated 200 mesh Ni grids, JBS Products, Dorval, QC). The grids were then floated on the BSA-containing assay buffer which functioned as a blocking agent. After a 15 min float, the blocked grids were removed and dried by wicking with filter paper. The grids were floated on 10 µL of anti-BG (6 mg/mL) for 60 min, followed by 3 floating cycles on blocking agent, 5 min blocking per cycle. The grids were subsequently floated on 10 µL of steptavidin-gold conjugate (0.5 mg/mL) for 60 min. After treatment with streptavidin-gold the grids were floated twice on 10 µL wash solution for 5 min, blotted dry with filter paper, then finally floated three times on distilled water for 5 min and blotted dry. The control grids were prepared as above but omitting the application of the anti-BG. These contained BG and streptavidin-gold only.

The specimen grids were examined and compared, using a range of magnifications, with a Hitachi H-1700 (Hitachi, Tokyo, Japan) transmission electron microscope employing an accelerating voltage of 75 kV. Conventional electron micrographs were taken using Kodak Electron Microscope Film Type 4489 (Eastman Kodak, Rochester, NY) and digital images were captured using a high-reolution Kodak MegaPlus CCD Camera, Model 1.4, interfaced with an IBM PC-based imaging workstation.

Results and Discussion

Reconstituted spore preparation

Sandwich immunoassays were carried out on the Threshold Unit using reconstituted BG spore preparations as the analyte antigen. It was possible to detect the presence of BG in solutions containing as little as 1 ng of the reconstituted preparation, based on dry weight. This value is comparable to other bacteria immunoassays carried out on the Threshold system. Standard curves of BG assayed with several antibody preparations are shown in Figures 2 - 4. All the antibody preparations showed high sensitivity to the analyte, namely, BG spores reconstituted in distilled water without any additional purification.

After running the standard curve analyses, samples of the spore preparation were washed by centrifuging the reconstituted sample, removing the supernatant and resuspending the pellet. Immunoassays of the resuspended pellet (insoluble spores) and the supernatant filtered through 0.22 µm nitrocellulose (soluble components) were run. It was found that the greater part of the immunoassay signal (the antigenicity) was associated with the soluble component, rather than the spores. The soluble components in the filtered supernatant produced over 90% of the signal (see Table I). The signal associated with the spores themselves was minor, less than 10%, even though they were, by far, the major component of the total mass. When the supernatant was dried and weighed, it was found that the soluble components accounted for 9.8% (w/w) of the initial preparation.

The antibodies used in this work were developed in either rabbit and goat. Those from the DRES collection were raised in rabbits. For one of the DRES antibody preparation, the rabbits were vaccinated with the reconstituted preparation (spores plus the soluble components). For another preparation, the rabbits were vaccinated with spores that had been washed (centrifuged and resuspended) six times to remove the soluble components. In both cases the immunoassay results were similar: the antibodies were directed against the soluble component; roughly 10% by weight produced in excess of 90% signal.

Filtration Capture Assay: Active vs Passive Capture.

Threshold assays require immobilization of analyte antigen onto nitrocellulose membrane. This can be accomplished by a biotin-streptavidin mediated active capture process as shown in Figure 1. For particulate or cellular antigens such as bacteria, the nitrocellulose membrane (pore size 0.44 μm) can provide a passive capture by physically entrapping the large antigens within the matrix structure. Thus in passive capture, only cells or particles are assayed; soluble antigens will wash through the filter. To further investigate the antigenicity, active and passive capture immunoassays were carried out on the whole reconstituted preparation and on the soluble antigen. The latter was prepared as described above by recovering the supernatant and filtering it through 0.22 μm nitrocellulose sterilizing filters. With active capture assays, the reconstituted preparation and soluble antigen produced strong signals of approximately equivalent magnitude, whereas with passive capture assays the same samples produced low signals (see Table II). For strong signals to be generated, the essential process was the biotin streptavidin-mediated capture. These results are indicative of the antigenicity residing with soluble antigen(s) that were not readily entrapped in a membrane matrix. The greater part of the antigenicity was not directed at the spores. Previous experiments have demonstrated that passive capture techniques on the Threshold are suitable for trapping and detecting bacteria cells. Thus, in this work, if the antibodies were strongly directed against the spores themselves, then the signals for reconstituted spore preparation would be of comparably large magnitudes in the active and passive capture experiments.

Washed Spores and Aged Samples

Sequential washings were carried out on a sample of reconstituted spores to determine how readily the soluble antigenic component(s) could be removed from the spore sample. The washing procedure consisted of centrifugation, filtration, and resuspension as described in the Materials and Methods section. The BG spore preparation was reconstituted at a concentration equal to 500 ng/well. Samples of the reconstituted preparation, the supernatant after centrifugation and filtrate (i.e., the supernatant after filtration through 0.22 μm nitrocellulose) were assayed on day 1. In addition, portions were set aside at 4 ^{o}C and assayed after 28 days. The data is shown in Table III. Samples from the reconstituted preparation, and the first supernatant (S1) and first filtrate (F1) produced high signals (approx. 4 - 5 x 10^4 μv/s) and about the same magnitude, in keeping the results of the above sections. After the first wash (centrifuge, filter supernatant, resuspend pellet) most of the antigen was removed. The signals in the S2 and F2 samples dropped to about 1 x 10^3 μv/s, a decrease of approximately 40-50 fold. Subsequent washings S3/F3, S4/F4 removed additional antigen, and the signals dropped further to 300 μv/s (about 3-fold). After four washes the supernatant (S5) had a signal of 290 μv/s, about 0.7% of the initial value (S1). The resuspended pellet after four washes had a signal of 1382 μv/s, 3% of the starting reconstituted sample.

When the supernatants (S1-S4) and filtrates (F1-F4) were assayed after standing for 28 days at 4 ^{o}C, the signals were slightly changed from day 1, mostly lower, ranging from 59% to 102%. However the final washed BG spore preparation (i.e., the resuspended pellet of fourth wash) increased in antigen content from 1382 to 2688 μv/s. The supernatant from the pellet (S5) produced a signal of 2025 μv/s. The background (i.e, blank: reagents, no antigen) signals for the assays in Table III were about 140 μv/s, thus 290 μv/s and 2025 μv/s (S5 on day 1 & 28) represent 150 μv/s and 1885 μv/s above background. These results indicate that with repeated washings the soluble antigenic components could be largely but not completely washed away. However, when the washed pellet was allowed to stand for 28 days, the soluble antigen component in the pellet sample replenished itself by about 12-fold (increasing from 150 μv s^{-1} to 1885 μv s^{-1}).

Limits of Detection and Measurement of Unknowns

The limits of detection (LOD) for BG spores were determined using three different polyclonal antibody preparations. For the reconstituted spores the LODs were about 1 ng or less in 0.1 mL and for washed spores it was about 17 ng. To determine the LOD of soluble antigen, the sample was dried by mild heating, weighed, resuspended in water, and assayed. The LOD was found to be 10 pg or about 100 fold less than the reconstituted preparation. These results are consistent with the above data that indicated that the soluble component which was 10% by weight gave about 90 - 95% of the immunoassay signal. Table IV summarizes the LOD data. The limits of detection of the reconstituted BG spores are comparable to other work on immunoassays of bacillus spores. Immunomagnetic-electrochemiluminescent detection of *Bacillus anthracis* spores gave LODs between 10^4-10^5 spores, with variation in the detectability of the strains assayed. Based on the size estimates from the electron micrographs (see below), the dry weight per BG spore is about 0.3 - 1 pg, thus an LOD of 1 ng of dry spore preparation represents approximately 1000 - 3000 spores (or 900-2700 spores if we consider that 10% w/w of the preparation was non-BG soluble material).

A series of BG samples were prepared as unknowns and the concentrations were determined in a blind assay by reference to a standard curve. The results are given in Figure 5 as a plot of actual values versus measured values. The average error was 6.5%

Transmission Electron Microscopy

Transmission electron micrographs were produced, documenting the BG spore preparations whereby the spores were immobilized on electron microscopy grids and treated consecutively with biotin-labelled anti-BG and streptavidin-labelled colloidal gold. The colloidal gold particles are opaque to electrons and appear as black spots on the micrographs. If the anti-BG possessed an affinity for the spores themselves, then it would be apparent from the electron micrographs. The biotinylated antibodies would attach themselves to the spores and streptavidin-colloidal gold would bind to the biotinylated antibodies. Thus, the spores treated with antibodies as above, would show a greater amount of attached gold than the control samples. In this work, the controls were the same spore samples treated with streptavidin-labelled colloidal gold, but no biotin labelled anti-BG.

The resulting electonmicrographs showed that the amounts of gold attachment in the test samples (Figure 6) and control samples (Figure 7) were not distinguishable, indicating that there was little direct interaction between the spores and the anti-BG. The micrographs gave an estimation of the size of the spores to be about 1 μm. This dimension is large enough for the spores to be physically entrapped in the nitrocellulose (see above).

Soluble Antigen

The soluble antigen was not characterized extensively. Coomassie blue protein assays carried out on solutions of 1 mg/mL of the soluble antigen showed negligible amounts of protein. Similarly SDS PAGE gel electrophoresis of the soluble components followed by Western blot staining (again with Coomassie blue) was not able to resolve any protein bands. The soluble material was tested for reactivity with polyclonal IgG anti-lectin and results were negative. No further chemical characterizations were carried out.

Conclusions

This report provides an insight into the antigenicity of antibodies raised against BG spores. Although a number of vaccinations were carried out in rabbits and in goats, the antibodies produced were always directed against the soluble components of the BG spore preparations. This was the case even when the BG spores were thoroughly washed (by centrifugation and resuspension) prior to injection in order to remove the soluble components. In total, four polyclonal preparations were assayed, all capable of detecting small amounts of the BG preparation by immunoassay, but all were found to be directed against the soluble component. The soluble antigen, although readily reduced in concentration by repeated washing, was present in residual amount sufficient to induce antibody response. The antibodies preparation derived from the washed spores provided the lowest LOD.

A rabbit polyclonal antibody preparation, derived from washed spores, was employed in analysis of BG in chamber studies at DRES and field trials at Dugway Proving Ground. The antibodies were effective in detecting BG in liquid samples obtained from aerosol collectors.[7] In the field trials, BG was released into the air as dry powder or as a

wet slurry each of which contained both the spores and the soluble antigenic material. Aerosol, when collected, contained sufficient material for detection. Thus for field trials of immunoassay identification systems (Threshold or Origen) it was not an absolute requirement that the antibodies be directed against the simulant itself (in this case BG); good results could be obtained by determining the presence of ancillary component(s) in the simulant preparation. However, in constructing protocols for field trial measurements, the antigenicity (i.e. where the immunoassay signal originates) is important knowledge for successful design and interpretation of experimental data.

The demonstrable importance of the soluble antigens on the immunoassays of BG spores suggests that the antigens should be characterized by extraction and synthesis to standardize the antibody stocks. Since the antigens do not appear to be proteins and that BG spores have been characterized to have complex carbohydrates, a likely candidate for these soluble antigens is the noted sugar polymers. It would also be a worthwhile investigation to determine whether the soluble antigens are unique to different bacilli spores or a source of cross reaction between *B. subtilis* and other species, such as *B. anthracis*.

References

1. Klapes, N. A. and Vesley, D. "Vapor-Phase Hydrogen Peroxide as a Surface Decontaminant and Sterilant," Appl. Environ. Microbiol. 56, 503-506, 1990.

2. Gammon, R. A. "The Effects of Temperature and Concentration of Ethylene Oxide on *Bacillus subtilis* var. *niger* Strain *globigii* Spores." Developments in Industrial Microbiology, Vol 16 pp 313-17, 1975.

3. Cross, G. L. C. and Lach V. H. "The Effects of Controlled Exposure to Formaldehyde Vapour on Spores of *Bacillus globigii* NCTC 10073," J. Appl. Bacteriol., 68, 461-469, 1990.

4. Anderson, J. D. "Biochemical Studies of Lethal Processes in Aerosols of Escherichia coli," J. Gen. Microbiol. 45, 303-313, 1966.

5. Horan, N. J., Parr, J., Naylor, P. J. "Evaluation of Tracers for the Determination of the Mixing Characteristics of Activated Sludge Reactors," Environ. Technol. 12, 606-608, 1991.

6. Houston, J., Learner, M. A. and Dancer, B. N. "Selection of an Antibiotic-Resistant Strain of *Bacillus subtilis* var. *niger* (*B. globigii*) for use as a Tracer in Microbially Rich Water," Wat. Res. 16, 387-388, 1989.

7. Boulet, C.A., Ho, J., Stadnyk, L., Thompson, H.G., Spence, M. R., Luoma, G. A., Fulton, R. E., Lee, W. E., Suffield Report 662, Report on the Canadian Integrated Biological Detection System Results of the Combined Joint Field Trials II, for Biological Detection (U), Defence Research Establishment Suffield, 1996 UNCLASSIFIED.

8. DRES Contract Report No W7702-0-R210, "Detection of Bacteria, Viruses and Toxins," Microtek Research and Development, 1994.

9. Bader, D. E., Fisher, G. R. and Lee, W. E., "Gene Probe Assay of Viral Nucleic Acid using a Silicon Biosensor (U)," Suffield Memorandum 1471, Defence Research Establishment Suffield, 1995, UNCLASSIFIED.

10. Hiragi, Y, Physical, "Chemical and Morphological Studies of Spore Coat of *Bacillus subtilis*," J. Gen. Microbiol. 72, 87-99 (1972).

11. Goldman, R. C. and Tipper, D. J., "*Bacillus subtilis* Spore Coats: Complexity and purification of A Unique Polypeptide Component," J. Bacteriol. 135, 1091-1106, 1978.

12. Pandey, N. K. and Aronson, A. I., "Properties of the *Bacillus subtilis* Spore Coat," J. Bacteriol. 137, 1208-1218, 1979.

13. Russell, A. D. "The Destruction of Bacterial Spores," Academic Press, London, 1982, pp 2-8.

14. Lee, W. E. and Thompson, H. G., unpublished data.

15. Lee, W. E. and Thompson, H. G., "Rapid Immunofiltration Assay of *Francisella tularensis* (U)", Suffield Memorandum 1376, Defence Research Establishment Suffield, 1992 UNCLASSIFIED.

16. Bruno, J. G., and Yu, H., "Immunomagnetic-Electrochemiluminescence Detection of *Bacillus anthracis* Spores in Soil Matrices," Appl. Environ. Microbiolog. 62, 3474-3476, 1996.

17. This value is based on an estimated diameter of 5×10^{-5} cm and density of 1.45 g cm^{-3}.

18. Nagata, L. P., personal communication.

Table I. Immunoassay Signal from Reconstituted Spore Preparations and Filtrates of *Bacillus globigii*

Antibody Source	BG in sample (ng/well)	Signal Ratio (filtrate/reconstituted)	
[1]rabbit vs washed BG	25	96.5 (2.5)*	
[1]rabbit vs washed BG	12.5	99.0 (4.1)*	
[2]rabbit vs reconst. BG	200	90.0 (3.2)**	

*Mean (SD) of 4 determinations

**Mean (SD) of 3 determinations

[1]DRES polyclonal (rabbit) IgG raised against washed *Bacillus globigii* spores

[2]DRES polyclonal (rabbit) IgG raised against reconstituted *Bacillus globigii* spores

Table II. Active - Passive Capture Immunoassay of *Bacillus globigii*

Capture	**Sample**			
	Reconst (µv/s)	**filtrate (µv/s)**	**blank (µv/s)**	**ratio (%) filtr/reconst**
active	1448 (39)	1368 (5)	193 (12)	94.5[a]
active	1811 (66)	1708	219 (3.6)	94.3[a]
passive	153 (22)	66 (4)	64 (16)	n.a.
passive	156 (12)	66 (6)	74 (5)	n.a.

[a] similar measurement to that in Table I, however different data and different antibodies. The antibodies were produced by Dugway Proving Ground and were raised in goats vaccinated with the whole reconstituted preparation.

Data are average of three determinations, standard deviations are given in brackets.

Each sample contained the equivalent of 200 ng/well determined from the dry weight of the spores. The reconstituted samples were prepared by dilution with distilled water.

Table III. Washing and Aging of *Bacillus globigii* spores

Sample	Signal (µv/s)	
	Day 1	Day 28
reconst.	51,400	52,100
S1	44,733	37,933
F1	41,067	42,033
1st wash		
S2	1044	889
F2	915	713
2nd wash		
S3	506	342
F3	362	290
3rd wash		
S4	395	235
F4	298	214
4th wash		
resus. pellet	1382	2688
S5	290	2025

Samples were assayed on day 1 and day 28. Each sample contained the equivalent of 500 ng/well

reconst. = reconstituted spore preparation, S1 = supernatant of reconst. after centrifugation, F1 = -1 filtered through 0.22 µm nitrocellulose sterilizing filters.

S2 = supernatant after pellet from first centrifugation was resuspended in buffer centrifuged, F2 = S2 filtered through 0.22 µm nitrocellulose sterilizing filters.

S3, F3, S4, F4,S5 are subsequent supernatants and filtrates following from S2,F2.

Resus. pellet = the pellet from the fourth centrifugation was resuspended in buffer.

The antibodies used were produced at DRES and were raised in rabbits vaccinated with washed BG spores.

Table IV. Limits of Detection of *Bacillus Globigii*

BG sample	*antibody source	**LOD (ng)
recon. spores	[1]rab., recon	0.60
recon. spores	[3]goat recon	0.46
recon. spores	[4]rab., wash	0.39
recon spores	[2]rab., recon	0.32
soluble antigen	[4]rab., wash	0.010
washed spores	[4]rab., wash	17

Assays were 60 min incubations

*antibody source: see Materials and Methods section for details.

**taken as the background (blank) of the assay plus two standard deviations

1 - polyclonal IgG raised in rabbit against reconstituted spores at DRES

2 - polyclonal IgG raised in rabbit against reconstituted spores at Dugway Proving Ground

3 - polyclonal IgG raised in goat against reconstituted spores at Dugway Proving Ground

4 - polyclonal IgG raised in rabbit against washed spores at DRES

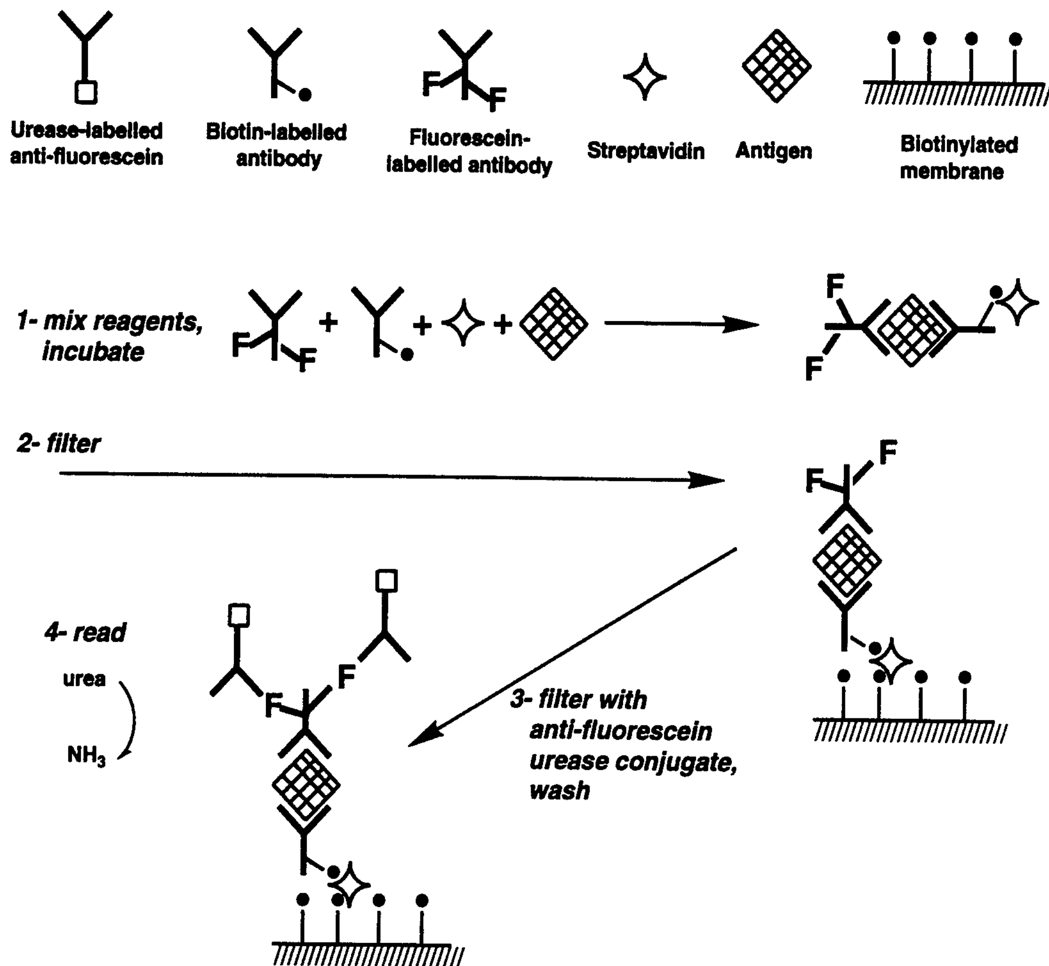

Figure 1. Reaction Assay scheme for Threshold assays of *Bacillus globigii*.

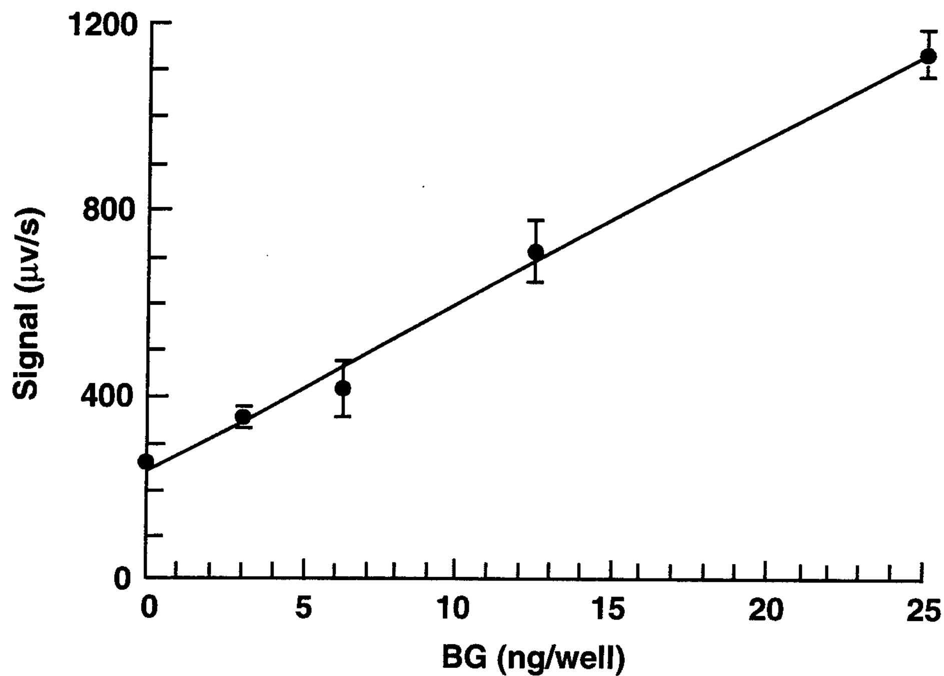

Figure 2. Standard curve of *Bacillus globigii* spores reconstituted in distilled water. The antibodies used were produced at DRES and were raised in rabbits vaccinated with the whole reconstituted preparation.

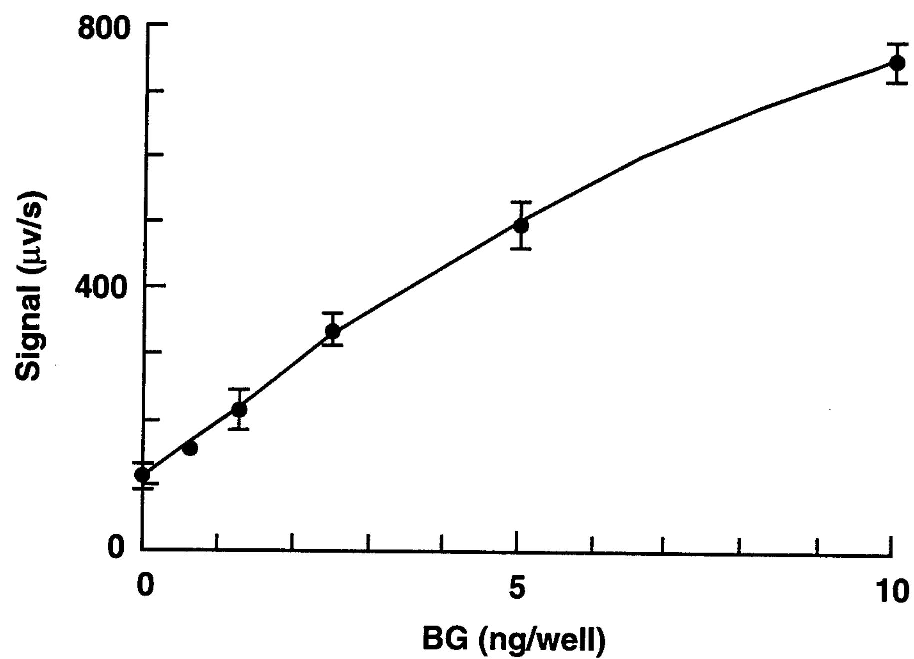

Figure 3. Standard curve of *Bacillus globigii* spores reconstituted in distilled water. The antibodies used were produced at DRES and were raised in rabbits vaccinated with washed BG spores.

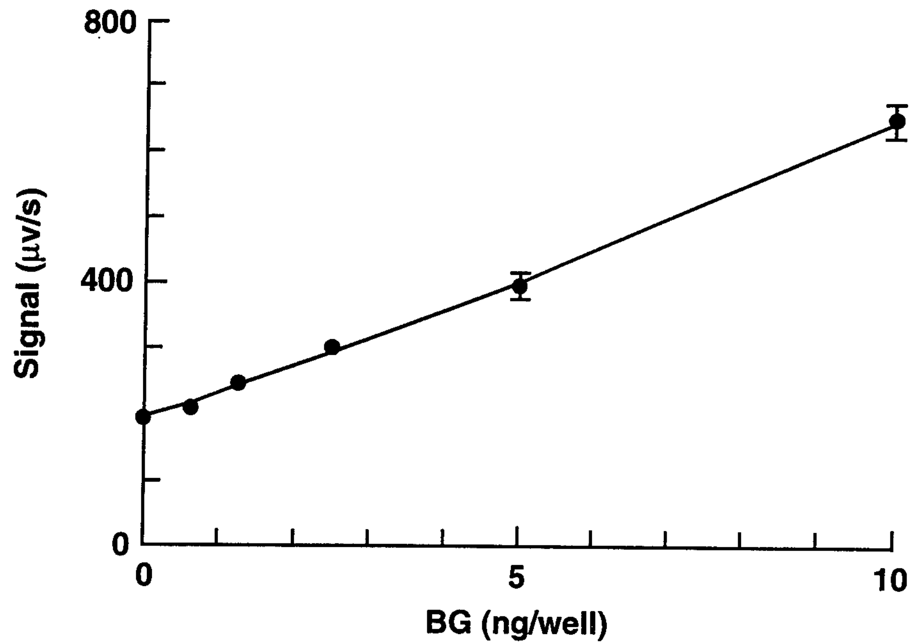

Figure 4. Standard curve of *Bacillus globigii* spores reconstituted in distilled water. The antibodies used were produced at Dugway Proving Ground and were raised in rabbits vaccinated with the whole reconstituted preparation.

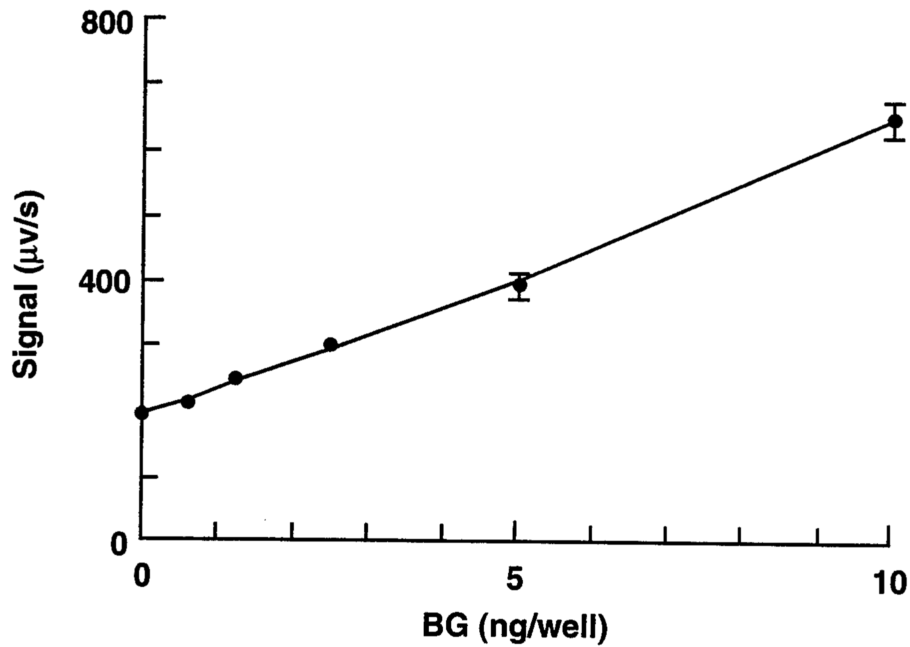

Figure 5. Quantitative determination of blind samples of BG spores reconstituted in distilled water. The antibodies used were produced at Dugway Proving Ground and were raised in rabbits vaccinated with the whole reconstituted preparation. The curve fit is a least squares regression: $y = 1.09x + 0.95$ ($r^2 = 0.991$).

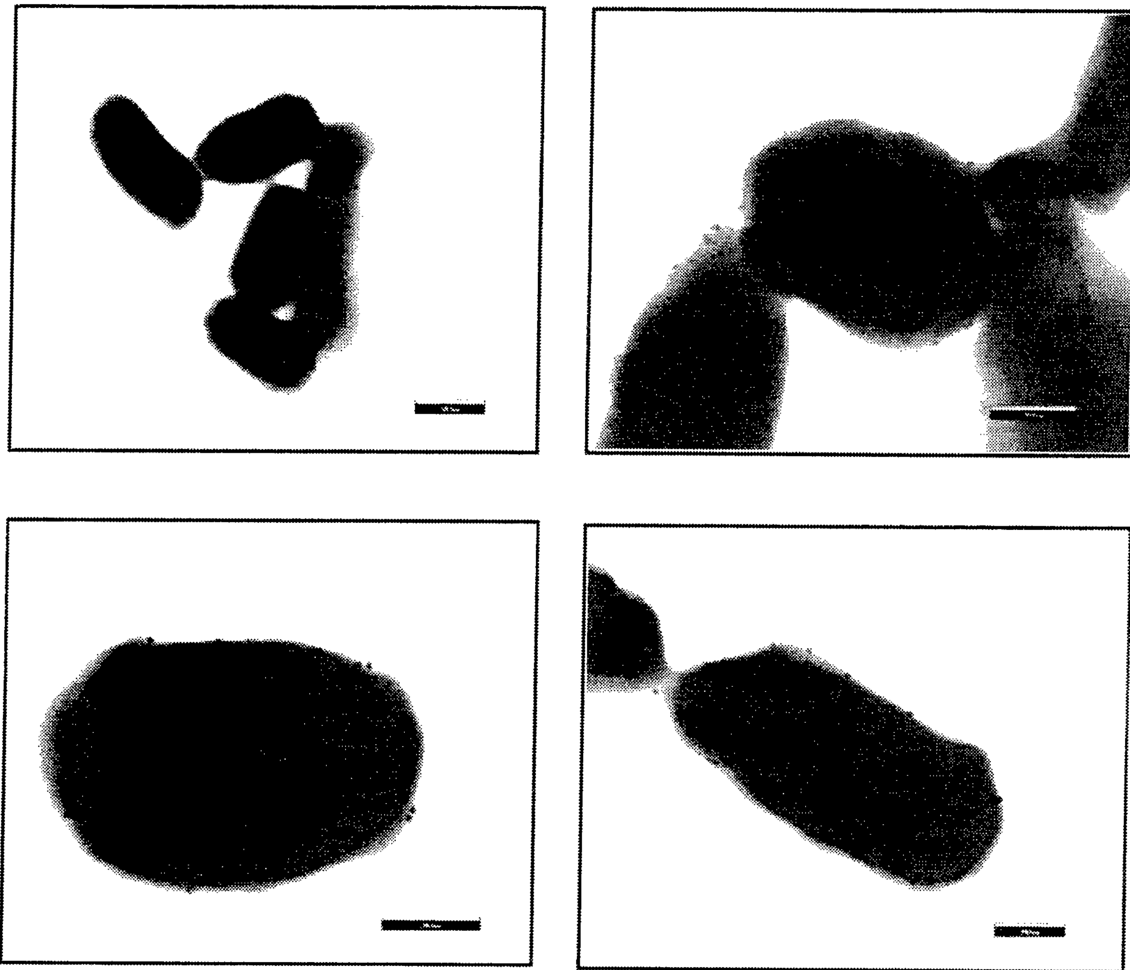

Figure 6. Electron micrograph of *Bacillus globigii* spores treated with biotin-labelled anti-BG and streptavidin colloidal gold.

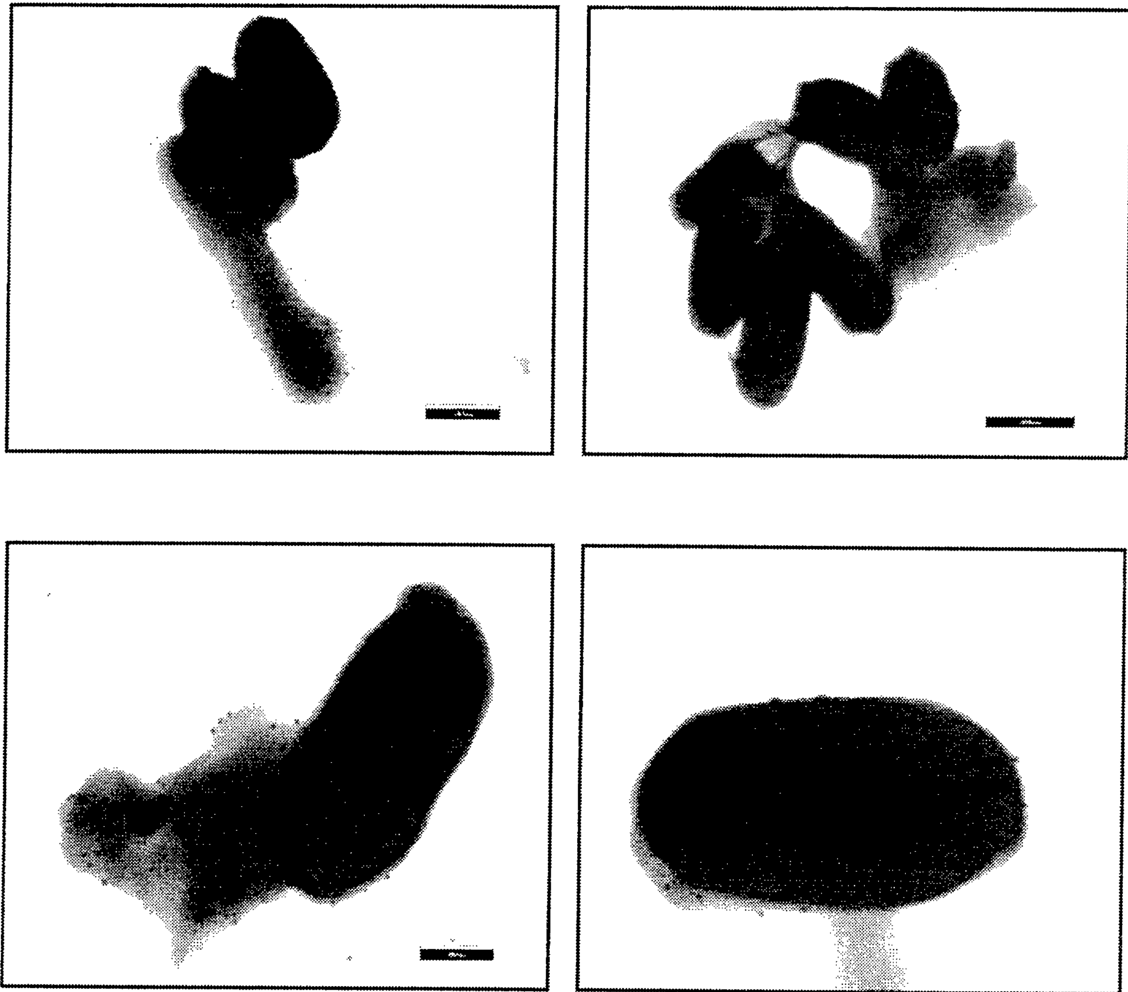

Figure 7. Electron micrograph of *Bacillus globigii* spores, controls samples for Figure 6. The spores treated with colloidal gold, no antibody. The antibodies used in Figs 6 & 7 were produced at DRES and were raised in rabbits vaccinated with washed BG spores.

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Immunoassay of Bacillus globigii spores were carried out using a number of polyclonal antibody preparations. The limits of detection for the various antibody preparations gave similar values: all in the range of 1 ng or less. It was determined that, although spores reconstituted in distilled water could be detected in small amounts, the antibodies were directed at a soluble component in the samples, rather than the spores themselves. When the soluble component was separated from the spores by centrifugation, it was found that this component was responsible for over 90% of the immunoassay signal even though it composed less than 10% of the total weight. The nonreactivity of BG spores was also observed in transmission electron microscopy. Electron micrographs of the spores immunostained with colloidal gold did not indicate any appreciable antibody interaction.

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Immunoassay

Threshold

Antibody

Bacillus globigii

Spores

Limit of detection