

A Label-Free Method for Detection and Differentiation of *Bacillus* spp. Endospores

Shannon B. Murphy and Stephen M. Wright
Middle Tennessee State University, Department of Biology

Abstract

The ease of manufacture, distribution, and resiliency of *Bacillus anthracis* endospores make this bacterium an attractive bioweapon to terrorists. Rapid identification of *B. anthracis* endospores is crucial for implementing a response strategy in the event of an attack. Because current detection methods are based on time-consuming culture and fluorescence techniques, an optical biosensor is being developed to identify an unknown endospore in real-time without fluorescent labels. This study was conducted to enumerate, detect, and differentiate endospores of *B. atrophaeus*, *B. pumilus*, and *B. thuringiensis* as simulants for *B. anthracis*, and to perform preliminary testing of the biosensor. Fluorescence analysis showed polyclonal *B. atrophaeus* antibodies bound all three organisms, monoclonal *B. atrophaeus* antibodies cross-reacted with *B. pumilus*, and monoclonal *B. thuringiensis* antibodies bound only *B. thuringiensis*. The limit of fluorescence-based detection was approximately 10 viable endospores. The biosensor showed comparable sensitivity, with results complete within 30 minutes. The results of this study demonstrate that the biosensor is able to detect endospore-antibody binding without fluorescent labels in timely fashion, suggesting that this system holds promise for rapid identification of *B. anthracis* endospores.

Introduction

Rapid detection and differentiation of *B. anthracis* endospores is crucial for implementing a response strategy during a suspected terrorist attack. Currently used methods of detection require culture or the use of fluorescent labels and are not conducive to field testing (1). An optical biosensor based on surface electromagnetic wave resonance (SEW) in photonic band gap (PBG) materials is being developed at MTSU that potentially can determine the identity of an unknown endospore in real time without fluorescent labels (Fig. 1) (2).

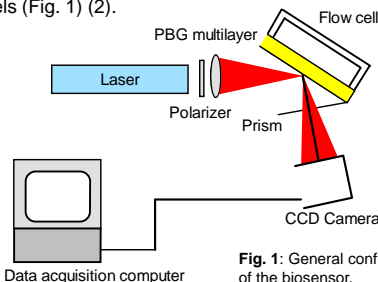


Fig. 1: General configuration of the biosensor.

This study was conducted to:

- 1) Enumerate, detect and differentiate endospores of nonvirulent simulant organisms (*B. atrophaeus*, *B. pumilus*, and *B. thuringiensis*) using fluorescence.
- 2) Perform preliminary testing of endospore detection with the biosensor.

Materials and Methods

Endospore Enumeration

- *Bacillus* spp. were grown on sporulation medium and harvested according to sporulation percentages.
- Washed samples were heated to kill vegetative cells.
- Dilutions were grown on tryptic soy agar plates to determine endospore counts.

Specificity of Antibody Binding

- *Bacillus* spp. were applied as microarrays to slides and antibody was added (polyclonal anti-*B. atrophaeus*, monoclonal anti-*B. atrophaeus*, and monoclonal anti-*B. thuringiensis*).
- A fluorescently-labeled antibody was added for visualization (Fig. 2).
- A confocal laser scanner was used to analyze the specificity of the endospore-antibody binding.

Limit of Detection

- Endospore dilutions of each *Bacillus* sp. were applied as a microarray to slides.
- Monoclonal *B. atrophaeus*, polyclonal *B. atrophaeus* and monoclonal *B. thuringiensis* antibodies were added to the appropriate slide (*B. atrophaeus*, *B. pumilus*, and *B. thuringiensis* respectively).
- Secondary fluorescently-labeled antibody was added for visualization with a laser scanner (Fig. 2).

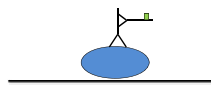


Fig. 2: Fluorescence detection of endospores. Endospore (blue oval) bound by primary antibody which is bound by a secondary fluorescently-labeled antibody

Antibody "Trapping" of Endospores

- Polyclonal anti-*B. atrophaeus* antibodies were applied to a slide and *B. atrophaeus* endospores were added.
- Monoclonal anti-*B. atrophaeus* antibodies were added, followed by a secondary fluorescently-labeled antibody for visualization.

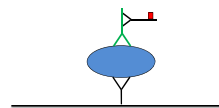


Fig. 3: Fluorescence detection of antibody trapping an endospore.

Biosensor Detection

- Polyclonal anti-*B. atrophaeus* antibodies were hand-applied to a PBG slide and spread over an area of 10 mm x 10 mm.
- The biosensor flow cell was assembled, slide inserted and primed using de-ionized H₂O.
- The first scan was performed and initial position of the surface mode was recorded.
- *Bacillus atrophaeus* endospores were injected and the change in surface mode was recorded.

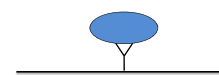


Fig. 4: Biosensor detection of endospores. Antibody is applied to the slide and traps the endospore.

Results

Endospore Enumeration

- Endospores were harvested on day 8 for *B. atrophaeus* and *B. pumilus*, and day 18 for *B. thuringiensis* based on optimal sporulation. Numbers of spores/mL at optimal sporulation are listed in the Table below.

<i>Bacillus</i> spp.	<i>Bacillus atrophaeus</i>	<i>Bacillus pumilus</i>	<i>Bacillus thuringiensis</i>
Average # spores/mL	$1.48 \times 10^{10} \pm 4.2 \times 10^9$	$9.50 \times 10^9 \pm 5.0 \times 10^7$	$9.35 \times 10^7 \pm 5.64 \times 10^7$
Approximate % spores	90	95	90

Specificity of Antibody Binding

- Fluorescence analysis showed that polyclonal anti-*B. atrophaeus* bound to all three simulants (Fig 5). Monoclonal anti-*B. atrophaeus* antibody bound to *B. atrophaeus* and showed cross reactivity with *B. pumilus*. The monoclonal anti-*B. thuringiensis* antibody bound only to *B. thuringiensis*. None of the antibodies bound with the negative control, *Streptococcus pyogenes*.

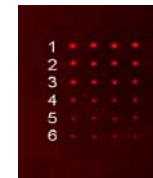


Row 1: *Bacillus atrophaeus*
Row 2: *Bacillus pumilus*
Row 3: *Bacillus thuringiensis*
Row 4: *Streptococcus pyogenes*
Row 5: Goat IgG (red label, + control)

Fig. 5: *Bacillus* spp. were spotted in triplicate. Green spots indicate endospore-antibody binding.

Limit of Detection

- Approximately 10 viable endospores of each *Bacillus* sp. were detected with fluorescence (Fig. 6).



Row 1: 320 endospores
Row 2: 160 endospores
Row 3: 80 endospores
Row 4: 40 endospores
Row 5: 20 endospores
Row 6: 10 endospores

Fig. 6: *Bacillus atrophaeus* endospores were spotted in quadruplicate.

Antibody "Trapping" of Endospores

- Fluorescence analysis showed that *B. atrophaeus* endospores specifically bound to immobilized polyclonal anti-*B. atrophaeus* antibodies when applied as a microarray or when hand-applied.

Biosensor Detection

- The initial baseline surface mode was measured at 310 pixels prior to endospore addition.
- After addition of endospores, a shift in surface mode was measured to 330 pixels (Fig. 7).

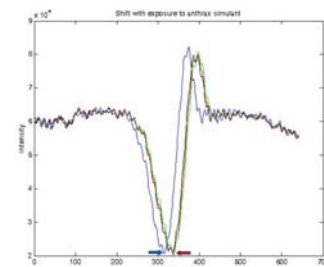


Fig. 7: Shift in surface mode upon addition of endospores to the biosensor. The blue arrow indicates initial surface mode and the red arrow is after endospore addition.

Discussion

There is well-founded concern over the use of *B. anthracis* as a bioweapon. In the event of a suspected anthrax event, it is essential to have a reliable, rapid means of detection. Current antibody-based detection requires a fluorescent or chemiluminescent label. The time necessary to identify a suspect organism with this method or by culture may have detrimental consequences to public health. Advantages to using the novel optical biosensor include:

- No fluorescent labels were required,
- Detection was complete within 30 minutes of endospore addition, and
- Comparable sensitivity was shown with SEW-based detection.

Based on preliminary testing with *B. anthracis* simulant organisms, this system may hold promise for rapid identification of *B. anthracis* endospores.

Literature Cited

1. Popovic T and Glass M. 2003. Laboratory aspects of bioterrorism-related anthrax—from identification to molecular subtyping to microbial forensics. *Croat Med J.* 44:336-341.
2. Robertson WM, Friedli AC, Wright SM. 2008. Biosensors based on surface optical wave resonance in photonic band gap multilayers. National Science Foundation. Proposal 0854423.

Acknowledgements

This study was funded by the Department of Homeland Security through the Southeast Region Research Initiative.

