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Differential detection of a surrogate biological threat agent (*Bacillus globigii*) with a portable surface plasmon resonance biosensor

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ABSTRACT

New methods and technology are needed to quickly and accurately detect potential biological warfare agents, such as *Bacillus anthracis,* causal agent of anthrax in humans and animals. Here, we report the detection of a simulant of *B. anthracis* (*B. globigii*) alone and in a mixture with a different species of *Bacillus* to test non-specific interference using a portable surface plasmon resonance (SPR) biosensor (SPIRIT 4.0, Seattle Sensor Systems). Both direct capture and antibody amplification were used to determine the limit of detection for spores of *B. globigii*, and to detect spores of *B. globigii* in a mixed sample containing another *Bacillus* spp. Spores of *B. globigii* were detected by anti-*B. globigii* (anti-Bg) coated sensors by direct capture at a concentration of 10^7 spores/mL, and with a secondary antibody amplification at a concentration of 10^5 spores/mL. Spores of *B. globigii* were differentially detected in a 1:1 mixture with *B. pumilus* spores from equal concentrations (10^7 spores/mL) with a secondary antibody amplification. To our knowledge, this is the first report of the differential detection of *B. globigii* with SPR in a mixed sample containing at least one additional *Bacillus* spp., highlighting the potential for SPR to detect any target bacterium in a mixed sample of closely related species. With the availability of portable instrumentation to accurately detect biological warfare agents such as *B. anthracis*, emergency responders can implement protocols in a timely fashion, limiting the amount of exposed individuals.

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1. Introduction

Early and specific detection of biological warfare agents are critical aspects of defense in the event of a bioterrorist attack (Eubanks et al., 2007; Lane et al., 2001; Pohanka et al., 2007). Detection systems need to be rapid, sensitive, portable, and specific to a biological threat (Byrne et al., 2009). Optical detection methods offer the advantage of a label-free and real-time detection of a target pathogen, and one optical method known as surface plasmon resonance (SPR) has recently been integrated into a portable biosensor (Chinowsky et al., 2007). Portable SPR biosensors may be used in manned (Naimushin et al., 2005) or unmanned (Palframan et al., 2014) biodetection operations in a variety of environments (Usachev et al., 2014) (Fig. 1).

Bacillus anthracis is a gram positive, rod-shaped, spore-forming bacterium that can cause the disease anthrax in humans and animals (Blackburn, 2010; Kracalik et al., 2011; Read et al., 2003; Spencer, 2003). This bacterium has been used as a biological warfare agent, and is still considered to be a threat to biosecurity (Webb, 2003). Consequently, new methods and technology are needed to accurately detect *B. anthracis* in advance of an attack (Rao et al., 2010).

A number of simulants (surrogates or mimics) for *B. anthracis* have been used to develop and test new biosensors. *B. globigii*, a non-pathogen with distinct colony morphology on agar plates, has been used as a simulant in military operations for over 60 years (Gibbons et al., 2011; Stratis-Cullum et al., 2003). Other studies have used *B. thuringiensis*, a close relative of *B. anthracis*, as a simulant (Tufts et al., 2014). Different factors must be considered when selecting appropriate surrogates for pathogenic microorganisms, such as phylogenetic relationships, morphology, and ease of access (Sinclair et al., 2012).

Antibodies are commonly used as recognition elements for SPR (Byrne et al., 2009; Shankaran et al. 2007). Though antibodies have been used in SPR to detect a variety of different agents including bacteria, virus-like particles, and toxins (Chinowsky et al., 2007; Koubova et al., 2001), they have not yet been used in SPR to differentiate among *Bacillus* spp. in a mixed sample containing more than one *Bacillus* spp. To address this knowledge gap, we used portable SPR detection methods to rapidly and accurately detect a simulant of *B. anthracis* (*B. globigii*) in a mixed sample. *B. globigii* was selected as a simulant in our studies because it is a non-pathogen of humans and domestic animals and it does not have any

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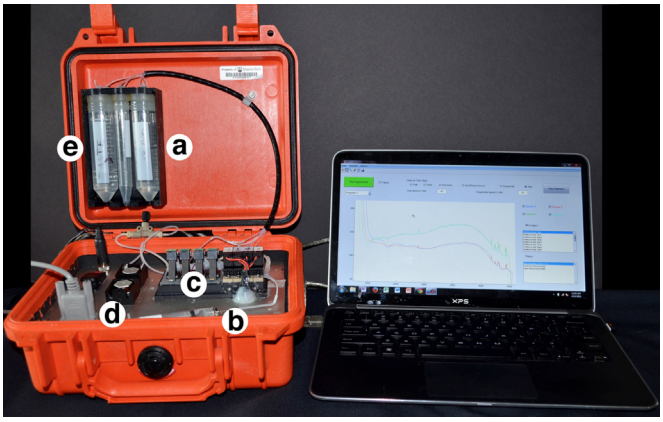

Fig. 1. The portable SPR biosensor (Seattle Sensor Systems SPIRIT). The biosensor has a tube for buffer (A), a syringe for injection of a sample (B), 4 sensors (C), a peristaltic pump (D), and a waste container (E). The pressurized buffer (A) is metered by a peristaltic pump (D) across the sensor surfaces and into a waste container (E). A syringe (B) is used to inject samples into an injection loop before the sample enters the flow cell. The flow cell is where the sample interacts with the sensor surfaces. Results are viewed on a laptop computer in real-time.

special handling restrictions (Gibbons et al., 2011). The specific objectives of our work were to: (1) screen antibodies for their specificity against spores of *B. globigii* and other related *Bacillus* spp., (2) determine the limit of detection (LOD) for spores of *B. globigii* with direct capture and antibody amplification methods, and (3) differentially detect spores of *B. globigii* in a mixed sample containing at least one other *Bacillus* spp. With the availability of portable instrumentation to accurately detect biological warfare agents such as *B. anthracis*, emergency responders can implement emergency protocols in a timely fashion, limiting the amount of people and/or domestic animals exposed.

2. Materials and methods

2.1. Generation and purification of spores of Bacillus spp.

Cultures of *B. globigii, B. circulans, B. polymyxa, B. thuringiensis* (ATCC13367), *Lysinibacillus sphaericus* (ATCC33722), and *B. albolactis,* were obtained from Katherine Rodgers of the Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA. Cultures were used to generate spores following the nutrient depletion and spore separation protocol developed by Harrold et al. (2011). Plates of 3.0% trypticase soy broth (TSB) (Cat. No. 211768, Becton Dickinson, Sparks, MD) and 0.5% yeast extract (Cat. No. Y1625-250G, Sigma Aldrich, St. Louis, MO) were inoculated and incubated overnight at 37 °C. Resulting single colonies were transferred to 3.5 mL of 3.0% TSB and 0.5% yeast extract liquid media and incubated at 37 °C overnight. The resulting cultures were transferred to 500 mL of 0.3% TSB on a shaking incubator (Excella E24, New Brunswick Scientific, Enfield, CT) at 37 °C for 6 days.

Spores were isolated from the liquid cultures through a series of washing steps, based in part on the hydrophobic tendencies of spores (Harrold et al., 2011). First, 1 L of the spore suspension was centrifuged at 6000 g for 15 min. The supernatant was discarded, and the pellet at the bottom of the centrifuge tube was washed 3 times by vortexing with 30 mL of 0.1 M NaCl solution. To reform the pellet again, the solution was centrifuged at 10,000 g for 5 min and the supernatant was discarded. After the washing steps, the pellet containing the spores and cell debris was resuspended in 5 mL of diH_2O water. To isolate the spores from the cell debris, a solution containing 11 mL 50% PEG-4000 (Cat. No. H273-61, Mallinckrodt Chemicals, Phillipsburg, NJ), 17 mL 3 M potassium phosphate consisting of 1.76 M K_2HPO_4 (Cat. No. P-3786, Sigma, St. Louis, MO) and 1.24 M KH_2PO_4 (Cat. No. 3246, JT Baker, Center Valley, PA), and 22 mL diH_2O water was added to the pellets and vigorously shaken by hand for 15 min. The resulting suspensions were centrifuged for 2 min at 100 g, and 12–15 mL of the top layer containing the purified spores was collected.

Aliquots of 10 µL containing purified spore suspension were transferred to microscope slides, flooded with 5.0% malachite green (Cat. No. 211079, MP Biomedicals, Solon, OH), and heated over a beaker containing boiling water for 5 min. After 5 min, the malachite green was washed off with diH_2O for 30 s. A counter stain of 0.6% safranin (Cat. No. ICN15204025, Fisher Scientific, Hanover Park, IL) was added to visualize vegetative cells. Slides were flooded with this stain for 30 s and then washed with diH_2O. Microscope slides were then visualized under a light microscope, and images of spores were captured with a digital camera.

Spores previously isolated (from the top layer) were diluted with diH_2O to a final volume of 50 mL and centrifuged at 6000 g for 30 min. The resulting pellet was washed four times with 5 mL of 0.1 M NaCl, resuspended by shaking, and centrifuged at 10,000 g for 5 min. After the fourth wash, the pellet was vortexed once more and resuspended in 5 mL of Dulbecco's Phosphate-Buffered Saline (DPBS) (Cat. No. 14190-136, Life Technologies, Grand Island, NY) for SPR sample preparation. Viability of the resulting suspension was assessed by distributing aliquots of 20 µL of the final spore suspension on plates of TSB, and colonies were observed the next day. Spore suspensions were quantified using a Neubauer haemocytometer (Cat. No. 1492, Hausser Scientific, Buffalo, NY). Ten-fold dilutions of lab-generated spores and commercial spore suspensions of *B. globigii* were prepared at 10^7, 10^6, 10^5, 10^4, 10^3 spores/mL in DPBS.

2.2. Dot blots

Dot blots were performed to observe the antibody specificity for spores of different *Bacillus* species with commercial anti-*B. globigii* antibodies (anti-Bg). A 0.45 µm nitrocellulose membrane (Cat. No. 162-0115, Bio Rad, Hercules, CA) was used to blot the spore suspensions.

Aliquots of 20 µL of suspensions (10^7 spores/mL) of lab-generated *B. globigii*, *B. pumilus* (ATCC27142), and *Lysinibacillus sphaericus* (ATCC33722) spores in PBS, and 20 µL of ovalbumin (10 µg/mL) in PBS (Cat. No. 825051, Fisher Scientific, Hanover Park, IL) and PBS alone (negative controls), were pipetted onto the nitrocellulose membrane and dried for 20–30 min. The membrane was then blocked using TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20) with 3.5% milk for 1 h at room temperature. After blocking, the membrane was incubated with the 1° antibody solution (rabbit anti-Bg at a concentration of 3.7 mg/mL was diluted 1:5000 in TBST with 3.5% milk) for 1 h. After incubation, the membrane was washed 3 times for 15 minutes using TBST with 3.5% milk. Binding of anti-Bg antibody to spores was observed through the addition of a secondary anti-rabbit Alkaline phosphatase at a concentration of 0.6 mg/mL was diluted 1:10,000 (Cat. No. 31345, Thermo Scientific, Rockford, IL) for chemiluminescent detection. After the second incubation, the membrane was washed with TBST 3 times for 15 min, followed by a final wash with TBS (10 mM Tris, pH 8, 150 mM NaCl) for 15 min. After the final washing step, the chemiluminescent signal was observed upon application of substrate Lumi-Phos WB (Cat. No. 34150, Thermo Scientific, Rockford, IL) with a minimum of 0.125 mL per cm^2 of membrane and incubated for 3 min followed by exposure using a ChemiDoc XRS+ (Bio Rad, Hercules, CA) gel doc system.

2.3. SPR biosensor and sensor configuration

A Seattle Sensors SPIRIT portable SPR biosensor was used for all of the experiments (Seattle, WA). The running buffer used in all experiments was DPBST: DPBS + 0.1% Tween-20 (BP337-100, Fisher Scientific, Hanover Park, IL). Spore suspensions were manually injected into a 25 °C temperature compensated injection loop and then flowed at a rate of 30 µl/min through the cast-silicone flow cell where the sample contacts the four prepared SPREETA sensor chips. The surface area of the flow cell was $1.9 \times 5.3 \times 0.1$ mm (S. Soelberg, Seattle Sensor Systems, Seattle, WA). The sensing channels were 100 µm by 3.8 mm, and the area that was analyzed was 0.5 mm × 0.1 mm (S. Soelberg, Seattle Sensor Systems, Seattle, WA). Results were viewed in real-time with custom graphical user interface (GUI) program provided with the instrument (Seattle Sensor Systems, Seattle, WA).

For detection of *B. globigii* spores, four SPR SPREETA sensors (Cat. No. SS01A, SensiQ, Oklahoma City, OK) were used in the SPR biosensor. Three sensors were coated with a commercially available rabbit anti-Bg (Cat. No. TC-7008-001, Tetracore, Rockville, MD). The fourth sensor was coated with a commercial rabbit anti-ovalbumin antibody (anti-OVA) (Cat. No. TC-7003-001, Tetracore, Rockville, MD) or commercial mouse anti-DON antibody (anti-DON) (Cat. No. ABIN1022126, Antibodies-Online.com, Atlanta, GA) to serve as a reference sensor and an indicator of any potential non-specific binding. Antibodies were physisorbed to clean gold sensor surfaces with 20 µL of the antibody solution (3.75 mg/mL in PBS) and incubated overnight at room temperature in a humidity chamber. After incubation, excess antibody solution was removed by pipetting. Detection of the specific agent (*B. globigii* spores) was determined at the end of the direct capture step and at the end of the amplification step as a difference in the refractive index units (RIU) between the detection and reference sensors, consistent with the methods of Chinowksy et al. (2007).

2.4. SPR experiment 1: direct capture method

Injections of 1 mL samples of *B. globigii* were done sequentially from 10^7 to 10^3 spores/mL, with each injection followed by regeneration step using a 50 mM NaOH (Cat. No. S3201, Fisher Scientific, Fairlawn, NJ) solution to remove bound spores from the immobilized antibodies on the sensor surface.

2.5. SPR experiment 2: antibody injection method

New sensors were coated as described previously, but with the addition of a blocking step. First, clean sensors were coated with 20 µL of anti-Bg antibodies and incubated overnight at room temperature in a humidity box. Second, antibodies were recovered for later use and the sensors were rinsed with PBST in the SPR biosensor with a flow rate of 20 µL/min for 10 min. Third, sensors were removed from the SPR biosensor and blocked with 20 µL of PBSTB (PBS with 0.05% tween − 20, 2% BSA) and incubated for 1 hour at room temperature within the humidity box. PBSTB was then recovered, disposed of, and the sensor was rinsed once more with PBST by insertion into the SPR biosensor, flowing at a rate of 20 µL/min. Antibody injection samples were prepared at a concentration of 10 µg/mL and rotovaped using the CentriVap Concentrator (Labconco, Kansas City, MO) to evaporate trace reagents that may interfere with the SPR signal. Antibody samples were injected after the entire spore sample passed completely through the flow channel. This point was chosen to maximize the amount of binding of spores to antibodies, and can be observed graphically in real time as the point when the RIU signal begins to drop. Antibody samples of 1 mL were injected in the same fashion to that of the spore sample and allowed to run completely through the flow channel at a rate of 30 µL/min. After the antibody solution passed through the flow channel, as evident by another dip at the end of the curve, 1 mL of regeneration buffer at 50 mM was injected to release both antibodies and captured spores. After 2–3 min of flowing regeneration buffer, 1 mL of PBST was injected to return the system to the original running buffer.

2.6. SPR experiment 3: detection of target in mixed spore samples

Samples of both *B. pumilus* and *B. globigii* were rotovaped at room temperature using a CentriVap Concentrator from the 1 mL original volume to 0.5 mL. The 0.5 mL suspensions were then combined to make a total volume of 1 mL with 10^7 spores/mL of both *B. globigii* and *B. pumilus* within a single sample. Samples were vortexed and directly injected into the SPR biosensor with a sterile needle and flowed across the sensors surfaces at a rate of 30 µL/min for both the direct capture and antibody injection methods. Once the spore sample had completely passed through the flow channel to maximize interaction with all sensor surfaces, an antibody solution of anti-Bg at a concentration of 10 µg/mL was injected and flowed across the sensor surface at a rate of 30 µL/min. The regeneration process of the sensors was the same for both direct capture and antibody injection methods. Once all injections had been completed, the regeneration buffer (50 mM NaOH) was injected and flowed at 30 µL/min across the sensor surface to remove bound antibodies and spores for future injections. After 3–5 min of regeneration, PBST was injected to return the system to its running buffer state.

2.7. SPR experiment 4: LOD with antibody injection

Samples with a volume of 1 mL were prepared and injected in sequential order from 10^7 to 10^4 spores/mL in PBS. The differential between detection and reference sensors was recorded for each sensor. The mean response of the detection sensors was generated and subtracted from the reference sensor for each sample injection.

2.8. Statistical analyses

Analysis of Variance (ANOVA) was used to test for significant differences between sensors for sequential injection of *B. globigii* spores using the direct capture method. JMP (SAS Institute, Cary, NC) was used to assess any statistical significance between sensor treatment (factor) and RIU levels (response).

3. Results

3.1. Generation and purification of spores of Bacillus spp.

Spores were generated and quantified for *B. globigii*, *L. sphaericus*, *B. albolactis*, *B. thuringiensis*, *B. circulans*, and *B. polymyxa* (data not shown).

3.2. Dot blots

Results from the dot blots showed that lab-generated spores of *B. globigii* interacted with anti-Bg antibodies at concentrations of 10^7 and 10^5 spores/mL (Fig. 2). Spores of *L. sphaericus* and *B. pumilus* did not interact with anti-Bg antibodies at these same concentrations (Fig. 2). Ovalbumin and PBS were used as negative controls to indicate non-specific binding.

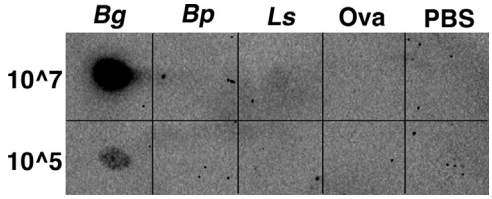

Fig. 2. A dot blot showing the potential interaction among commercial antibodies of *B. globigii* and spores of *B. globigii* (Bg), spores of *B. pumilus* (Bp), spores of *L. sphaericus* (Ls), Ovalbumin (Ova), and PBS buffer. The membrane was dotted from left to right with lab-generated *B. globigii*, commercial *B. pumilus*, lab-generated *L. sphaericus*, Ovalbumin, and PBS. Ovalbumin and PBS were used as negative controls to indicate non-specific binding and residual background interference. The top row was dotted at a concentration of 10^7 spores/mL and the bottom row was dotted at a concentration of 10^5 spores/mL. Anti-Bg antibodies demonstrated a strong interaction with lab-generated *B. globigii* at both 10^7 and 10^5 spores/mL. Spores of *B. pumilus* and *L. sphaericus* did not interact with anti-Bg.

3.3. SPR experiment 1: direct capture method

Lab-generated spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) at 10^7 spores/mL with direct capture using the SPR biosensor (Fig. 3). Spores were not detected with this SPR method at concentrations lower than 10^7 spores/mL (data not shown). This might be explained by sensor attrition of the signal over multiple injections (data not shown). Fig. 3 illustrates an injection of 10^7 spores/mL of *B. globigii* on a new sensor with freshly coated antibodies. After 21 min, there was a mean response of 123 RIUs for sensors coated with anti-Bg, compared to the reference sensor response of 108 RIUs (Fig. 3). The time of 1476 s was selected because it corresponds to the time at which the maximum amount of spores was expected to interact with the antibodies on the sensors surface and was consistent with the end of detection observed in Chinowsky et al. (2007). There was not a statistically significant difference between detection and reference sensors based on

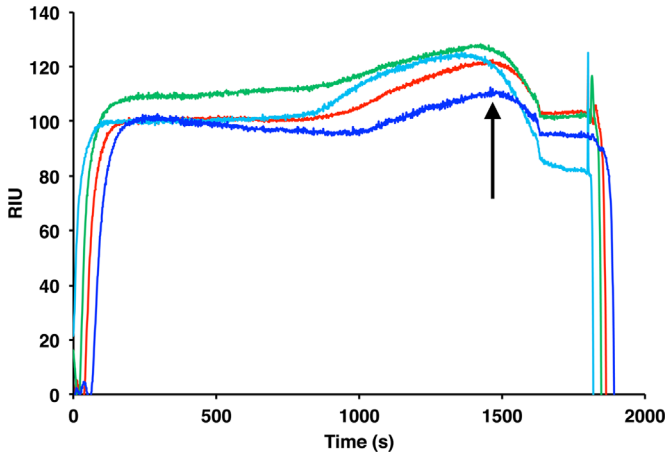

Fig. 3. A sensorgram showing the reaction of sensors coated with physisorbed antibodies to Bg and Bg spores at a concentration of 10^7 spores/mL. Sensors coated with anti-Bg are represented by (4) light blue (middle top), (3) green (top), and (2) red (middle bottom) lines shown above. Anti-Ovalbumin (anti-OVA), the reference sensor, is represented as the (1) dark blue line. The initial upshift indicates the injection point of the sample, followed by a leveling off where the rate of binding equilibrates. The sharp drop in signal is the point where regeneration buffer (50 mM NaOH) is injected to remove bound spores from the immobilized antibody to allow for multiple injections. The signal analysis point was chosen to be when the highest signal is reached (time = 1476 s) and is indicated by the black arrow. Sensor data reflected at this point (black arrow) were 125 ± 3 for sensor 4 (light blue), 125 ± 7 for sensor 3 (green), 120 ± 12 for sensor 2 (red) and 108 ± 14 for sensor 1 (dark blue). Spores of *B. globigii* were detected using this method, since all detection sensors are >10 RIUs above the reference sensor (4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

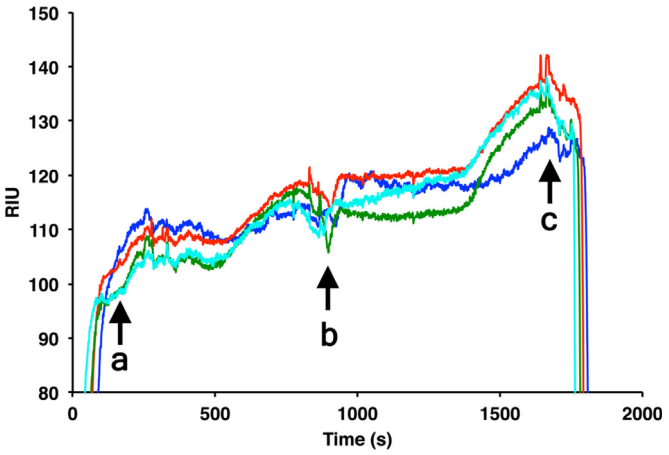

Fig. 4. A sensorgram showing an injection of spores of *B. globigii* at 10^7 spores/mL, followed by an injection of *B. globigii* antibodies (anti-Bg) at a concentration of 10 μg/mL. Arrow (a) indicates the point of spore injection, arrow (b) indicates the injection of anti-Bg, and arrow (c) indicates the point of detection. Sensor 4 (light blue) was coated with anti-Bg, sensor 3 (green) was coated with anti-Bg, sensor 2 (red) was coated with anti-Bg and sensor 1 (dark blue) was coated with anti-DON. Sensor 4, 3, and 2 have signal responses of 137 ± 3, 135 ± 12, and 141 ± 1 respectively, while sensor 1 had a signal response of 127 ± 6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4 sequential, replicate injections ($P=0.51$).

3.4. SPR experiment 2: antibody injection method

An injection of anti-Bg into the SPR biosensor further increased the RIUs above the reference sensor (Fig. 4). Fig. 4 shows an injection of *B. globigii* spores with an injection of anti-Bg. Fig. 5 illustrates an injection of *B. pumilus* spores followed by an injection of anti-Bg. The time from injection to assessment of detection with spores of *B. globigii* and *B. pumilus* was estimated to be 1440 s following the methods of Chinowsky et al. (2007). The mean response of the anti-Bg sensors with an injection of

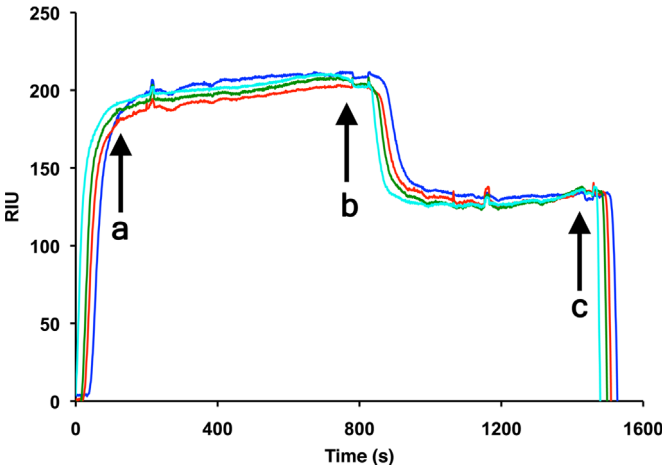

Fig. 5. A sensorgram showing an injection of spores of *B. pumilus* 10^7 spores/mL, followed by an injection of *B. globigii* antibodies (anti-Bg) at a concentration of 10 μg/mL. Arrow (a) indicates the point of spore injection, arrow (b) indicates the injection of anti-Bg, and arrow (c) indicates the point of detection. Sensor 4 (light blue) is coated with anti-Bg, sensor 3 (green) was coated with anti-Bg, sensor 2 (red) was coated with anti-Bg and sensor 1 (dark blue) was coated with anti-DON. Sensors 4, 3 and 2 had signal responses of 138 ± 7, 136 ± 18, and 135 ± 8 respectively, while sensor 1 had a signal response of 133 ± 5. These results show that the injection of an antibody post injection of spores of *B. pumilus* did not increase the difference between detection and reference sensors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

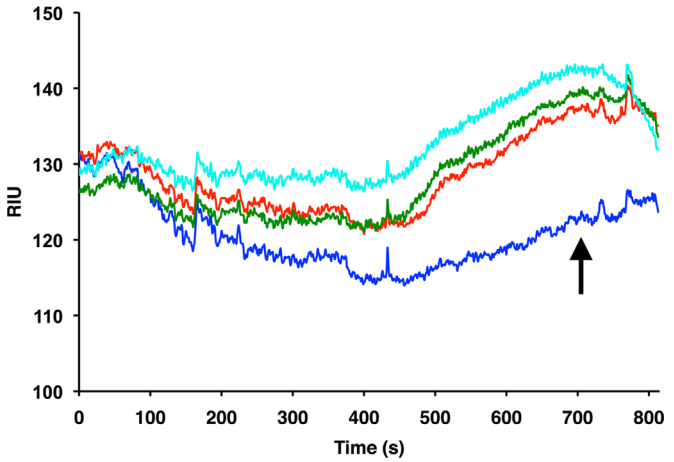

Fig. 6. Injection of mixed spore suspension *B. globigii* and *B. pumilus* at equal concentrations (10^7 spores/mL), followed by injection of anti-Bg at a concentration of 10 μg/mL. The sensorgram shows the portion of the run following the injection of anti-Bg. Sensor 4 (light blue), sensor 3 (green) and sensor 2 (red) were coated with anti-Bg. Sensor 1 (dark blue) was coated with anti-DON. The arrow indicates the detection point at 730 s. Sensors 4, 3, and 2 showed signal responses of 143 ± 3, 140 ± 13, and 140 ± 13. Sensor 1 showed a signal response of 123 ± 9. A divergence of detection from reference sensors was observed, indicating differential detection of *B. globigii* with in a mixed sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

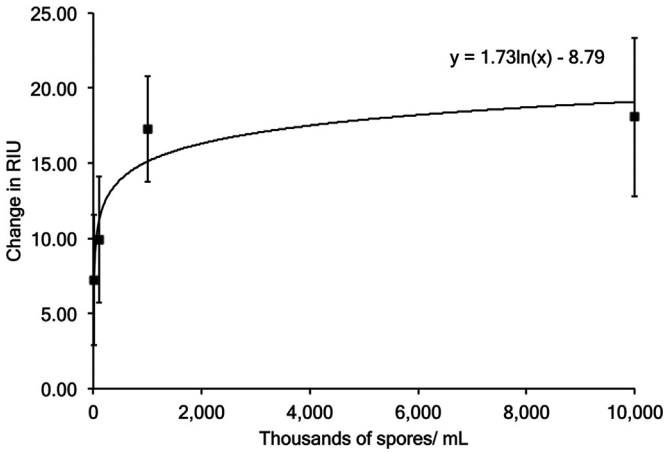

Fig. 7. Limit of detection (LOD) of *B. globigii* spores using antibody injection. This figure shows that as the concentration of spores decreases, the difference in response between detection and reference sensors also decreases. The injection of an antibody solution post-sample injection amplified the difference between detection and reference sensors at the original limit of detection (LOD) of 10^7 spores/mL. Error bars represent the standard error of the mean. Using the trendline equation listed in the figure, we can extrapolate two different LOD with RIU differences of 10 (52,118 spores/mL) and 5 (2,896 spores/mL).

10^7 spores/mL of *B. globigii* was 138 RIUs, compared to 127 RIUs for the reference sensor; the anti-Bg sensors were significantly greater than anti-OVA sensors over 2 replicates ($P=0.03$). For a single injection of spores of *B. pumilus*, sensors 4, 3, and 2 had signal responses of 138 ± 7, 136 ± 18, and 135 ± 8 respectively, while sensor 1 had a signal response of 133 ± 5 at a time 1347 s (Fig. 5). These results demonstrated that the injection of an antibody post injection of spores of *B. pumilus* did not increase the difference between detection and reference sensors (Fig. 5). Though these results were based on a single injection, the non-detection of spores of *B. pumilus* was also supported by the dot blot results that showed spores of *B. pumilus* did not bind to anti-Bg (Fig. 2).

3.5. SPR experiment 3: detection of target in mixed samples

Spores of *B. globigii* were not detected when mixed with *B. pumilus* at 10^7 spores/mL using direct capture (data not shown), but were detected in a mixed sample at equal concentrations using antibody injection (Fig. 6). When anti-Bg was injected, there was a sharp drop in RIU signal, rather than an increased or amplified signal observed in pure *B. globigii* injections (Fig. 4). However, the response leveled off and detection sensors diverged from the reference sensor (showing detection) at about 200 s. In Fig. 4, detection sensors produced an averaged signal response of 138 RIUs while the reference sensor response was 127 RIUs at the 730 s time mark. Results showed that anti-Bg sensors were significantly greater than anti-DON sensors over 2 replicates ($P < 0.01$). The overall time it took to achieve detection beginning with the spore sample injection was 1620 s. These responses were similar to that of the injected sample of pure *B. globigii* at 10^7 spores/mL followed by antibody amplification (Fig. 4).

3.6. SPR experiment 4: LOD with antibody injection

The injection of anti-Bg after sample injection lowered the LOD from 10^7 to 10^5 spores/mL (Fig. 7). Fig. 7 shows the dose response of spores of *B. globigii* from 10^7 to 10^4 spores/mL using antibody injection. There was a maximum difference between detection and reference sensors, as evidenced by the trendline (Fig. 7).

4. Discussion

New methods and technology are needed to quickly and accurately detect potential biological warfare agents, such as *B. anthracis*, causal agent of the potentially lethal disease anthrax in humans and animals. Here, we show that a portable SPR biosensor can be used to detect spores of *B. globigii*, a surrogate for *B. anthracis*, using commercial antibodies at a concentration of 10^5 spores/mL. Chinowsky et al. (2007) identified their LOD to be 9.0×10^4 CFU/mL. Their LOD, however, was based on CFUs which represents viable (culturable) spores in their suspension. It is likely that some of the spores in their suspension were not viable, and since their SPR method could not distinguish between dead and living spores, their reported LOD likely underestimates the true number of spores in suspension. This work could extend to the rapid, onsite detection of other biological threat agents in a variety of emergency response scenarios.

Commercially produced antibodies were able to differentially detect *B. globigii* from *B. pumilus*. Dot blots were inoculated with *B. globigii* and other *Bacillus* spp. and showed that anti-Bg specifically binds to *B. globigii* and not to *B. pumilus*. The dot blot results were extended to SPR; sensors coated with anti-Bg showed strong responses to spores of *B. globigii* and not *B. pumilus* using direct capture and antibody injection. This differential response is noteworthy, since many *Bacillus* spp. have similar proteins on the surface of their spores (Giorno et al., 2007; Lai et al. 2003) which could present a challenge in the accurate detection of the target. Previous work has shown that monoclonal antibodies can detect *B. anthracis* from several other *Bacillus* spp. with separate injections using SPR and subtractive inhibition assays (Wang et al., 2009). Some studies have attempted to detect targets in complex samples/matrices from environmental samples using SPR (Hang et al., 2008; Sípová et al., 2010).

Spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) with direct capture at a concentration of 10^7 spores/mL. It is important to note that previously exposed sensors were not as sensitive as freshly coated sensors in detecting spores of *B. globigii* (data not shown), thus this observed LOD might vary if the sensors had been previously exposed to spores. The LOD of 10^7 spores/mL is high compared to other biodetection methods (Byrne et al., 2009). However, these results are

in agreement with previous literature, indicating that larger molecules (in our case higher concentrations) are required for direct detection to be successful (Shankaran et al., 2007). SPR using direct capture was unable to differentiate a mixed sample of spores of *B. globigii* and *B. pumilus* at a concentration of 10^7 spores/mL. Thus, additional methods (such as antibody injection, discussed next) were warranted to further decrease the LOD and detect the target in a mixed sample.

When antibodies were injected at the phase in which spores had achieved maximum contact with immobilized antibodies, spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) at a concentration of 10^5 spores/mL. We identified a logarithmic trend between sample concentration and the difference in RIUs between the detection and reference sensors. This might be explained by a saturating effect with how many spores can be captured as well as how many injected antibodies can bind to captured spores on the sensors. Using the trendline equation, we can extrapolate two different LOD with RIU differences of 10 (52,118 spores/mL) and 5 (2,896 spores/mL).

The logic behind injecting an antibody stems from the generation of what is known as a 'sandwich assay' with the constituents of immobilized antibody, spore, and injected antibody (Chinowsky et al., 2007; Hang et al., 2008). The addition of the injected antibody, and the resulting formation of the 'sandwich,' increases the molecular weight of the binding complex, further increasing the RIU signal (Shankaran et al., 2007). The use of the sandwich assay reduced the LOD by 100 fold. The antibody injection method is a technique that has been used to strengthen the detection signal and provide more specific signal responses for many different biodetection methods (Byrne et al., 2009; Chinowsky et al., 2007; Hang et al., 2008).

The work described here provides evidence that SPR might be suitable for the detection of *B. anthracis* spores in a proposed biological attack. In a real world scenario, the infectious dose for *B. anthracis* in a building is dependent on factors such as room size, amount of air circulation, and pulmonary ventilation (Fennelly et al., 2004). The mathematical model developed by Fennelly et al. (2004) suggests that if 10,000 people are exposed to between 77,500–275,000 *B. anthracis* spores for 1 h in a moderate-size room (61 m^3), 2–5% of exposed individuals will contract inhalation anthrax, depending on the pulmonary ventilation rate. This model suggests that if all spores within this hypothetical room could be collected and suspended into a 1 mL sample, detection of *B. anthracis* spores would be achieved for the upper 89% of this spore range (100,000–275,000). However, it should be noted that *B. anthracis* infections may occur from about 100 spores (Glassman, 1966). This is an obvious limitation of the technology described here, and likely an unrealistic expectation with antibody methods in the current SPR configuration that we used.

There is the possibility of pushing the technology to detect an even lower concentration of spores. Additional experiments using a higher concentration of injected antibodies may be able to lower the LOD into the lethal dose range for *B. anthracis*, but this was not tested in our experiments. With a higher concentration of injected antibodies, saturation of the captured spores will be more likely to occur, which may further increase the RIU signal of detection sensors (Chinowsky et al., 2007). Nevertheless, there would be some tradeoffs between how effective high concentrations of antibody injection would be at decreasing the LOD and the overall cost of purchased antibodies. Eventually, captured spores would be saturated with antibodies, leading to an excess or waste of injected antibodies at higher concentrations. Previous work has also used direct capture and antibody injection to detect concentrations of *B. globigii* spores at 9.0×10^4 CFU/mL and detailed the use of subtractive inhibition assays in SPR to obtain a LOD of 10^4 CFU/mL (Chinowsky et al., 2007; Wang et al., 2009). A food spiking experiment using a method known as phage-mediated bioluminescence produced much lower LOD (< 100 CFU/mL after enrichment) (Sharp et al., 2015). We identified our LOD in spores/mL, which is a true number of the target in suspension. However, this also has limitations because both viable and non-viable spores are detected; some of the spores detected may not be able to cause disease. Additional methods to concentrate samples within the range of the LOD should also be considered (e.g., Humrighouse et al., 2015).

Spores of *B. globigii* were also detected when mixed in a sample with *B. pumilus* at equal concentrations (10^7 spores/mL) using antibody injection. The injection of the antibody post-mixed sample injection produced a sharp drop in RIUs, eventually leading to divergence between detection and reference sensor signals (Fig. 6). One possible explanation for this drop might also be a slight difference in RIUs between the spore solution and the antibody solution. Though SPR has been used to identify targets in environmental samples (Hang et al., 2008), additional work is needed to determine the limitations of this method in complex matrices containing other potential contaminants that might interfere with the antibodies. Though polyclonal antibodies have been used to detect different serovars of *Salmonella* within a mixture (Barlen et al., 2007), our work is the first to differentially detect *B. globigii* with SPR in a mixed sample containing at least one additional *Bacillus* spp.

Though not an objective of this work, future SPR work could extend our laboratory measurements to field observations of *B. globigii* and/or *B. anthracis* in natural and industrial environments. Such applications could leverage assays using nucleotides and immunomagnetic beads (Soelberg et al., 2009), which could ultimately enhance specificity and lower limits of detection. SPR methods might also incorporate carbohydrates for the detection of metabolically active bacteria (Bulard et al., 2015). One promising approach might be the use of small RNAs (sRNAs) or micro RNAs (miRNAs) that exist in high numbers in individual cells (Iyer et al., 2012; Sipova and Homola, 2013). Immobilized oligonucleotides have the potential to be more advantageous than immobilized antibodies because binding would be sequence specific and there are multiple copies of sRNA within a single spore (Sipova and Homola, 2013). sRNA detection has the potential for more oligonucleotide receptors to be bound with a lower spore concentration as compared to antibody receptors. This approach, however, may create some additional challenges, such as optimization of spore lysis and subsequent harvesting of sRNA prior to degradation by enzymes such as RNases. Oligonucleotide-based SPR assays could also help target the exotoxin that is responsible for pathogenicity of *B. anthracis* (Smith and Keppie, 1954).

5. Conclusions

Methods using a new, portable SPR biosensor were extended from the work of Chinowsky et al. (2007) to detect a simulant of *B. anthracis* (*B. globigii*) in a mixed spore sample. Two methods (direct capture and antibody injection) were used to determine the limit of detection (LOD) for spores of *B. globigii* and to detect spores of *B. globigii* in a mixed sample containing at least one other *Bacillus* spp. Spores of *B. globigii* were detected on freshly coated sensors (not previously exposed to spores) with direct capture at a concentration of 10^7 spores/mL, and with antibody injection at a concentration of 10^5 spores/mL and are consistent with previous literature. Spores of *B. globigii* were differentiated from *B. pumilus* in a mixed sample at equal concentrations (10^7 spores/mL) using antibody injection. With the availability of portable instrumentation to accurately detect biological warfare agents such as *B. anthracis*, emergency responders can implement emergency

protocols in a timely fashion, limiting the amount of people and/or domestic animals exposed. Future work could integrate nucleotide-based assays (such as sRNAs) to improve specificity and decrease the LOD.

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