

A rapid screening method for the detection of viable spores in powder using bioluminescence

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ABSTRACT: A rapid diagnosis of a biological threat in a powder sample is important for first responders who have to make decisions on-site. The present culture-based method does not provide timely results, which is a critical barrier for a quick response when a suspicious powder sample is found. The ATP bioluminescence method, combined with a heat shock, was investigated to determine the presence of spores in powder. The results show that only spore-containing powder samples provided a dramatic increase in the bioluminescence signal after the heat shock, which induces germination of the spores. Various conditions were tested to find the most effective and rapid germination procedure. Elevated temperatures (37°C and 50°C) were more effective in germination than room temperature. At 50°C, a double-strength germinant was more effective in germination than the regular strength. The 37°C/15 min procedure induced the germination of spores most effectively, while a 50°C/2 min procedure provided reasonably high signals, so it could make the entire procedure even faster (< 5 min). The detection limit of the bioluminescence method is < 100 spores. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: spore detection; powder; ATP bioluminescence; rapid method

INTRODUCTION

Shortly after the anthrax scare of 2001 in the mail, the number of powder samples received by government and public health laboratories was tremendous (1). These samples have to be treated as though they are biologically active during processing until confirmed to be harmless. Currently used analyses (e.g. colony counting or PCR) take one to several days to provide results, which delay decision making and impose a heavy burden on public health laboratories. If an analysis of the sample in question could be made on-site very rapidly (e.g. within minutes), the workload of the laboratories and the local disruptions could be minimized. A timely decision about further processing could be made if the sample contained viable spores and an appropriate decontamination procedure implemented.

Bacterial spores are inert and show no biological sign until they are given a favourable environment (e.g. nutrients, temperature, moisture) and turn into vegetative cells, which is called germination. In our study we have developed a rapid test method to allow first responders to test the biological activity of a powder on-site in less than 5 min. Our test procedure is to induce the spores in the powder to germinate, lyse the emerging vegetative cells, and then determine their ATP using a

bioluminescence method which allows a quantification of viable bacterial cells within minutes (2). The procedure does not identify *Bacillus anthracis* (anthrax) spores specifically. It can be used as a fast screening tool for detecting viable spores in powder samples. Thus, if spores are detected, further identification steps can be implemented.

MATERIALS AND METHODS

Powder samples

The powder of *Bacillus thuringiensis* (Bt) was used as a surrogate for powder containing *Bacillus anthracis* because Bt is not pathogenic to humans. The Bt powder used in this experiment was Dipel 150 Dust (Bonide Products, Yorkville, New York). For the detection limit of the bioluminescence technique in determining spores, a liquid type of Bt concentrate (Bonide Products, Yorkville, New York) was used because it can be easily diluted serially so as to provide more accurate data than the Bt powder. A group of white powders that have been used in hoaxes includes flour, baking soda, baby powder, foot powder, dried milk and powdered sugar. These were tested for the presence of biological activity due to the presence of spores in powder, using ATP bioluminescence before and after a germination step during the preliminary study. None of them showed an increased signal above the background after the germination treatment.

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Germination of Spores

To 1 mg powder, 1 mL tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) was added in a small tube. TSB was used as a nutrient to induce germination of the spores (3). Various conditions were tested, which included the length of germination period (2, 15, 30 min), germination temperature (room temperature, 37°C, 55°C) and concentrations of the nutrient (regular strength, double-strength) in order to find the fastest test procedure while having a significantly high signal.

ATP Bioluminescence

After the germination, 50 µL spore-containing liquid was transferred into a Filtravette™ [New Horizons Diagnostics (NHD), Columbia, MD, USA], which is a combination of a filter with a pore size of 0.45 µm and a cuvette. Gentle air pressure was used to filter out the liquid portion. At this stage, the emerging vegetative cells remain on the surface of the Filtravette™ and then 50 µL bacterial cell releasing agent was added. After holding for 1 min, the bioluminescence reaction was initiated by the addition of 50 µL luciferin/luciferase and the light emission was measured in relative light units (RLUs) using a microluminometer (Model 3550, NHD, Columbia, MD, USA). The RLU values were converted to RLU/mg powder. The activity of luciferin and luciferase was checked with a standard ATP solution (NHD, Columbia, MD, USA) according to the manufacturer's instructions. To compare ATP bioluminescence with the agar plate method in counting bacterial cells, germinated cells were serially diluted using a phosphate buffered solution (PBS) and measured by the two methods. The colonies were counted after incubation at 30°C for 24 h using tryptic soy agar medium (Becton Dickinson, Sparks, MD, USA). All the tests were performed in triplicate.

RESULTS AND DISCUSSION

Effect of Heat Shock

This experiment was carried out to verify that only a spore-containing powder sample shows increased light emission after germination. Foot powder and Bt powder were used for non-spore-containing powder and spore-containing powder, respectively. Throughout all conditions, the foot powder showed no biological activity. However, Bt powder showed a significantly increased signal once treated with the germination treatments (Fig. 1). Various germination conditions were used: (a) addition of TSB and determining RLU immediately; (b) addition of TSB and germinating at room temperature for 30 min; (c) addition of TSB and germinating at 37°C for 15 min. The Bt powder did not show a positive

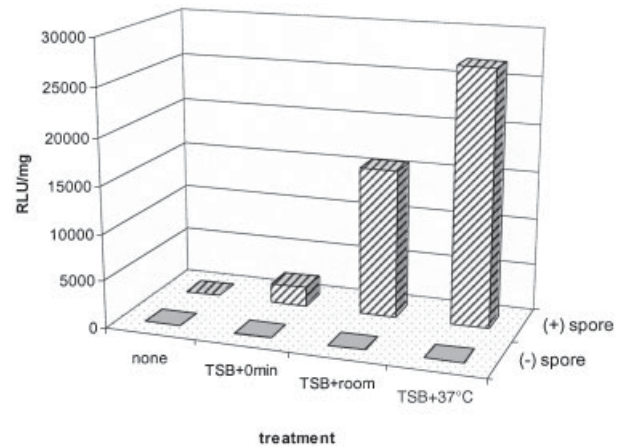


Figure 1. Comparison of the RLU values between the spore-containing powder and the non-spore-containing powder at various germination treatments.

signal if no nutrient was added. The 37°C/15 min germination treatment showed a more dramatic increase in the bioluminescence signal than the room temperature/30 min treatment. These phenomena indicate that the addition of nutrients is critical to initiate germination and an elevated temperature accelerates it.

Fastest germination procedure

This experiment was performed in order to seek a faster germination procedure. For this, combinations of higher temperature (50°C) and double-strength germinant were tested (Fig. 2). Longer germination time (15 min) at 37°C certainly generated the highest signal. At 50°C, using a higher-strength nutrient was more effective in inducing germination than the regular-strength nutrient. Although the 50°C/double-strength treatment provided a lower signal than the 37°C/15 min treatment, the signal from the 50°C/2 min/2 × procedure was sufficiently significant. This finding helps decision making in choosing an optimal test procedure, e.g. when getting the highest signal is the priority, such as when only a very limited amount of powder sample is available, the 37°C/15 min

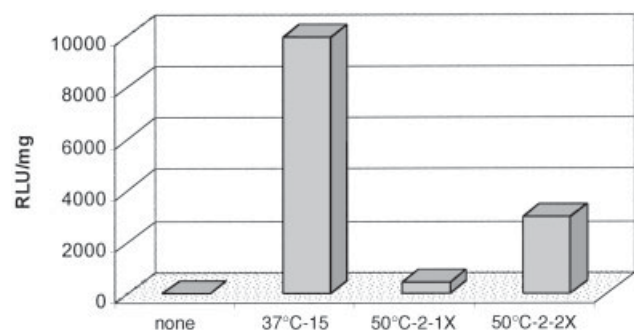


Figure 2. Comparison of various germination treatments to seek a faster procedure.

Table 1. The sensitivity of the bioluminescence technique in detecting spores. The CFU and RLU values are geometric means of triplicate measurements

Target spore no. (CFU/mL)	Measured spore no. (CFU/mL)	(-) Germination (RLU/mL)	(+) Germination (RLU/mL)	Change in signal above background (RLU/mL)
2000	3960 ± 1480.1 ^a	88	1600	1512 ± 273.1 ^a
1000	1670 ± 481.1	35	836	801 ± 178.8
500	700 ± 198.6	35	355	320 ± 34.6
250	360 ± 93.6	33	209	176 ± 24.9
125	140 ± 27.8	30	117	87 ± 34.6
60	60 ± 12.0	26	65	39 ± 16.0
30	30 ± 11.2	25	66	41 ± 21.5
15	7 ± 2.6	24	51	27 ± 11.9

^aStandard deviation.

procedure would be the choice, or an even longer germination time may be necessary; when the speed of the test is the priority, then the 50°C/2 min procedure using double-strength nutrient would be the choice. The procedure can be slightly modified depending upon the purpose of the tests.

Detection limit

The detection of bioluminescence technique in detecting spores is shown in Table 1. Based upon the preliminary experiment, the target spore numbers were estimated. Bt concentrate was serially diluted with buffer until it reached the target concentrations (2000 to as few as 15 spores). The data shown in Table 1 are the summary of three experiments. The spores were measured by bioluminescence (RLU) at each dilution after germination treatment (37°C/15 min). The spore numbers were also measured by plate counts on tryptic soy agar (CFU). The results show that our method can clearly detect as few as 60 spores, which is a desirable detection limit suggested by first responders (4). This detection limit is about 1000 times lower than that of the commercially available assay kits for *B. anthracis* spores, which is 10⁵ spores (5). A PCR-based method has a detection limit of 100 spores but it does not provide information on the viability of the spores (6). The result of the study also shows that the light emission (RLUs) correlated very well with the CFUs ($R^2 = 0.99$). This therefore indicates that the bioluminescence method can replace the time-consuming plate count method. Another advantage is that using the bioluminescence technique as a screening tool for the detection of spores is very economical. The cost of commercial assay kits for *B. anthracis* is in the range \$20–52/assay (5); the consumables for the bioluminescence test cost about \$3/assay.

CONCLUSIONS

The presence of viable spores in a powder sample can be determined by ATP bioluminescence after heat shock, which is a procedure to induce germination of spores with a warm nutrient. The longer germination produced a higher signal, but even the 2 min treatment was sufficiently effective to give a significant signal when using a higher concentration of nutrient. The entire procedure, the heat shock and the bioluminescence, takes less than 5 min and the detection limit is < 100 spores. The results of spore measurements using the bioluminescence technique agreed very well with the results from the traditional plate count method. The method can be also used as a verification tool to assess the viability of spores in a powder sample together with standard protocols for first responder testing, which do not provide information on viability. By doing this, sensitivity, specificity and viability can be combined.

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