Detection of *Bacillus anthracis* spores from non-porous surfaces using 'bioluminescent' reporter bacteriophage

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Abstract

Bacillus anthracis is a pathogenic spore-former and etiological agent of anthrax. Spores are naturally found in the environment where they can persist and remain infectious for more than 200 years. A contaminated area has potential to cause extensive disruption as it is uninhabitable until successful remediation. To ensure public health and preparedness for such an event, an efficient and rapid environmental detection system for spores is essential. To address this need, we previously generated a 'light-tagged' *B. anthracis*-specific reporter phage (Wβ::*luxAB*) which can rapidly and sensitively detect pure cultures from germinating spores by conferring a bioluminescent response.

The efficacy of Wβ::*luxAB* to detect *B. anthracis* ΔSterne spores from 3 non-porous contaminated surfaces was assessed. 2x2 inch coupons of stainless steel, glass and polycarbonate were used to represent the various surfaces. Coupons were inoculated with spores (10^1 to 10^4 CFU/coupon) suspended in 95% ethanol (EtOH), then left overnight for EtOH to evaporate, leaving 'dried' spores on the coupon surfaces. To sample, macrofoam swabs moistened with phosphate-buffered saline with 0.02% Tween 80 (PBST) were used to methodically wipe the coupon surface to 'collect' spores, which had an estimated processing time of 1 min per coupon. Extraction efficiency was assessed by plating samples and controls for CFU onto brain heart infusion (BHI) agar plates. Swabs were submerged in media containing reporter phage (10^9 PFU/mL), vortexed vigorously for 2 min, incubated at 35°C with continuous shaking (250rpm) to allow for germination and phage infection, and then analyzed for bioluminescence after 4-8h. To emulate 'real life' environmental samples, swabs were also deliberately 'dirtied' by moistening in PBST harboring either Arizona test dust (10mg/mL), *Bacillus thuringiensis* spores (10^4 CFU/mL), *Staphyloccocus epidermidis* (10^4 CFU/mL) or all three contaminants combined before sampling.

Swab sampling extraction efficiency was similar from all 3 surfaces, consistently yielding 50-70% recovery of spores from coupons. *B. anthracis* was detectable from 'clean' coupons deliberately inoculated with spores, yielding a limit of detection of 10^1 CFU/coupon within 6 h or 8 h for polycarbonate, stainless steel and glass surfaces, respectively. Wβ::*luxAB* was able to detect 10^1 CFU within 8h from 'dirty' stainless steel, glass and polycarbonate coupons. As the methodology is simple with minimal hands-on time, the technology displays potential for rapid detection of viable spores from various non-porous surfaces under fieldable or laboratory conditions.

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Problem

Table 1. Persistence of biowarfare bacteria

Species	Matrix/host	Persistence (time)
B. anthracis	Soil	40 years
	Human remains	Over 200 years
	Pondwater	18 years
Y. pestis	River water	>28 days
	Soil	24 days
	Surfaces	Up to 3 days
B. mallei	Not specified	Weeks
B. pseudomallei	Soil	Years
	Water	Months
F. tularensis	Water	>30 days

Spores are problematic if released in the environment

- Infectious agent
- Resistant to treatments
- Easily weaponized and disseminated
- Stable for 200+ years

Table 2. National planning scenario for aerosol anthrax

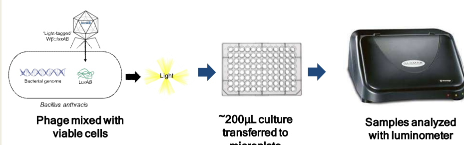
Item	Anthrax attack
Attack method	Wide dispersion of *B. anthracis* spores in an urban environment
Cases/fatalities	13,342/13,208
# Exposures	328, 000
# Clinical tests	300,000 in 14 days
# Clinical tests/day	60,000
# Environmental tests	1,000,000 over 2 months
Peak # environmental tests/day	50, 000

- Spores viable for decades
- Massive # of tests
- Diagnostic requirements:
  - High throughput
  - Cost effective
  - Viability
  - Low complexity

(Adapted from the Department of Homeland Security and 2005 National Planning Scenarios)

Detection System:

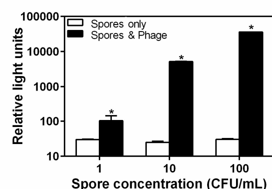
Fig. 1. *B. anthracis* ΔSterne Wβ::*luxAB* phage assay



Phage mixed with viable cells
~200µL culture transferred to micropiate
Samples analyzed with luminometer

LuxAB genes encoding luciferase enzymes were integrated into *B. anthracis*-specific Wβ reporter phage via homologous recombination. Engineered Wβ::*luxAB* infects the cell and uses the host's metabolic machinery to produce luciferase. The phage alone cannot express *luxAB* reporter genes. Bioluminescent signal can only be produced in the presence of viable cells, which is then detectable by a luminometer.

Fig. 2. Bioluminescent signal response of ΔSterne spores

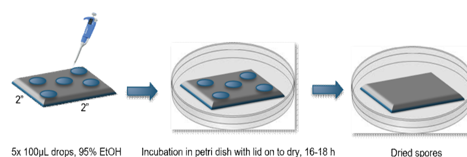


- Detection of 10^1 CFU/mL in 8h
- Dose-dependent signal

*significant ($p<0.05$) signal increase compared to phage only controls

Methods/Results:

Fig. 3. Coupon spore inoculation



5x 100µL drops, 95% EtOH
Incubation in petri dish with lid on to dry, 16-18 h
Dried spores

Fig. 4. Spore extraction and detection

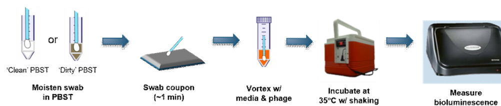
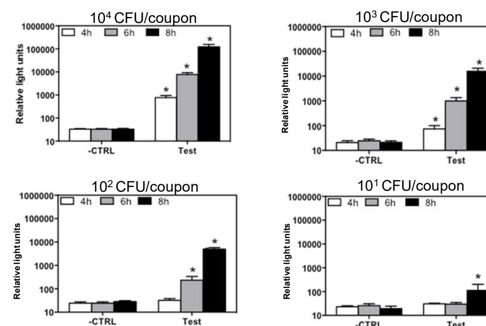


Table 3. Steel coupon extraction efficiency

	Control (no spores, n=5)					Test (n=5)				
Reps	1	2	3	4	5	1	2	3	4	5
Colony count (neat)	0	0	0	0	0	160	147	128	128	172
CFU/mL	0	0	0	0	0	$1.60x10^3$	$1.47x10^3$	$1.28x10^3$	$1.28x10^3$	$1.72x10^3$
Total CFU (5 mL)	0	0	0	0	0	$8.00x10^3$	$7.35x10^3$	$6.40x10^3$	$6.40x10^3$	$8.60x10^3$
% Recovery	0	0	0	0	0	**69.6**	**63.9**	**55.7**	**55.7**	**74.8**

- Nationally-validated recovery range: **10-30%**[1]
- Reported extraction efficiency: **50-70%**
- Fairly consistent within sample set (low variation)

Fig. 5. Spore detection from 'clean' steel coupons

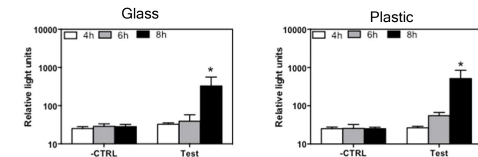


Dose-dependent detection:

- Detection of 1.0 x 10^4 CFU/coupon within 4h
- Detection of 1.0 x 10^3 CFU/coupon within 4h
- Detection of 1.0 x 10^2 CFU/coupon within 6h
- Detection of 1.0 x 10^1 CFU/coupon within 8h
- **Limit of detection: 10^1 CFU/coupon**

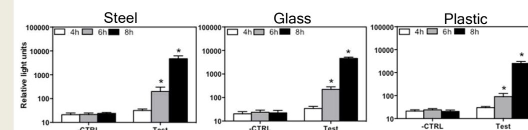
Numbers for all graphs are mean (n=3)± S.D. $p<0.05$ students t-test, one-way ANOVA, or two-way ANOVA

Fig. 6. Detection from 'clean' glass & plastic coupons



- Detection of 1.0 x 10^1 CFU/coupon within 8h from both glass & plastic

Fig. 7. Detection of 10^1 CFU from 'dirty' coupons



- Swabs moistened in PBST deliberately contaminated with 10mg/mL Arizona Test Dust, 10^4 CFU/mL Bt spores and 10^4 CFU/mL *S. epidermidis*.
- **Limit of detection** for 'dirty' steel, glass and polycarbonate coupons:
  - **1.0 x 10^1 CFU within 8h**

*significant ($p<0.05$) signal increase compared to phage only controls

Conclusions

- **Established spore extraction protocol:**
  - Adapted protocol from a nationally-validated study[1] using sterile macrofoam swabs
  - 50-70% extraction efficiency from steel
  - Low variation within sample set
- **Detection of spores from 'clean' coupons with minimal processing:**
  - **Steel:** 10^1 CFU/coupon in 8h
  - **Glass:** 10^1 CFU/coupon in 8h
  - **Plastic:** 10^1 CFU/coupon in 8h
- **Spores detectable in presence of commensal bacteria and other contaminants (ATD, Bt spores, *S. epidermidis*):**
  - **Steel:** 10^1 CFU/coupon in 8h
  - **Glass:** 10^1 CFU/coupon in 8h
  - **Plastic:** 10^1 CFU/coupon in 8h

References

[1]Hodges, L.R., Rose, L.J., O'Connell, H., Arduino, M.J. 2010 National validation study of a swab protocol for the recovery of *Bacillus anthracis* spores from surfaces. Journal of Microbiological Methods. 81, 141-146.

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