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Novel Sample Preparation Method for Safe and Rapid Detection of *Bacillus anthracis* Spores in Environmental Powders and Nasal Swabs

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***Bacillus anthracis* spores have been used as a biological weapon in the United States. We wanted to develop a safe, rapid method of sample preparation that provided safe DNA for the detection of spores in environmental and clinical specimens. Our method reproducibly detects *B. anthracis* in samples containing <10 spores.**

Bacillus anthracis spores, a recent threat, can remain dormant for years while retaining full virulence (5, 10, 13, 20, 21, 22; S. Endicott, E. Hagerman, and M. Furmanski, Letter, JAMA **284:**561–562, 2000). Powders and environmental samples, the most common nonclinical specimens, and nasopharyngeal swabs are submitted to designated laboratories in the national Laboratory Response Network (LRN), which has facilities to work safely on the specimens (6, 19, 23). High numbers of specimen can overwhelm a laboratory's capability and capacity to perform the tests in a timely fashion. From October to December 2001, the Florida Department of Health's three LRN laboratories each received hundreds of samples per day, which revealed the need for a method to render the samples harmless so the DNA could be safely extracted under biological safety level 2 conditions, to alleviate the bottleneck, and to decrease the turnaround time to the final result. Specimens may contain <10 spores, and the isolation and identification of *B. anthracis* may take days (5, 15). The purpose of this work was to develop a method of sample preparation that would provide safe DNA for the detection of ≤10 *B. anthracis* spores.

Previous studies used sonication probes in open tubes and cartridges to hold samples, a method that necessitated the use of a biological safety level 3 environment and sterilization between each sample preparation (2, 3, 7). Dang et al. demonstrated that DNA from autoclaved spores was usable for PCR assays (9). No one explored the sensitivity of these methods or if combinations of methods could be used.

(A preliminary report of this work has been presented previously [V. A. Luna, M. S. Robeson, M. Ewert, P. Amuso, A. Cannons, C. Davis, L. Heller, D. King, K. K. Peak, A. Rycerz, D. Wingfield, J. Cattani, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol., abstr. C-256, 2002].)

Spore suspensions of *B. anthracis* Pasteur (BC 3132) in phosphate-buffered saline (PBS; pH 7.4) from the Centers for Disease Control and Prevention, Atlanta, Ga., were vortexed for 5 min, centrifuged at 16,000 × *g,* and washed with PBS (pH 7.4) (Fisher, Pittsburgh, Pa.) containing 0.01% Triton X-100 (Sigma-Aldrich Chemical, St. Louis, Mo.) (1). This process was repeated three times. The final centrifugation at 300 × *g* for 1 min produced a supernatant of single, nonaggregated spores. Light microscopy confirmed aggregate dissolution of the spores. Tenfold dilutions were made, and concentrations were confirmed by multiple cultures on blood agar (Remel, Lenexa, Kans.) incubated at 30°C for up to 48 h. The bacteria isolated from powders were identified by standard biochemical tests (12, 15) and the API/CH50 and API 20E tests (bioMérieux, St. Louis, Mo.) according to the manufacturer's directions.

Dilutions (10^6 to 10^0 CFU) in 300 μl of Trypticase soy broth (TSB) (BD Biosciences, Sparks, Md.) were processed in screw-top, autoclavable 1.5-ml tubes by (i) autoclaving (121°C, 20 min), (ii) sonication (0, 10, 30, or 60 min) in a model 1500 sonicator (Branson Ultrasonics Corp., Danbury, Conn.), (iii) autoclaving and sonication (0, 10, 30, or 60 min), or (iv) sonication, autoclaving, and heat shock (60 or 80°C) for 2 and 10 min followed by 1 h of incubation at 30°C (11, 15). Each protocol was tested with 20 to 30 sets of a series of seven 10-fold dilutions (10^0 to 10^6 CFU/sample); after the dilutions were processed, the DNA was concentrated and tested in triplicate. Aliquots (50 μl) plated unto blood agar plates before and after processing were incubated for up to 4 days. The negative controls contained only PBS or TSB.

B. anthracis genomic DNA was extracted either with a MasterPure DNA purification kit (Epicenter, Madison, Wis.) or manually (4). Sample DNA was extracted with MagNaPure (Roche, Indianapolis, Ind.) and concentrated tenfold by using Microcon PCR centrifugal filters (Millipore Corp., Bedford, Mass.).

For the PCR, we used as a target the chromosomal Ba813 (EMBL accession no. U46157) that is present in all *B. anthracis* isolates and that is used by the LRN (8, 16, 17, 18, 23). We used ABI PRISM 7700 Gene Express (Applied Biosystems, Foster City, Calif.) to design the following primers and probe: BaFW1 (5′AAT-TTG-AAG-CAT-TAA-CGA-GTT 3′), BaREV2 (5′

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TABLE 1. Comparison of sample preparation methods for DNA extraction and PCR[a]

Method	Sonication time (min)	No. of spores (range) detected by PCR
Autoclaving only (121°C, 20 min)	None	10^3–10^6
Sonication only	0	10^4–10^5
	10	10^4–10^5
	30	10^0–10^4
	60	10^2–10^4
Sonication followed by autoclaving	0	10^2–10^6
	10	10^0–10^4
	30	10^1–10^5
	60	10^0–10^3
Autoclaving followed by sonication	0	10^3–10^5
	10	10^1–10^5
	30	10^3–10^6
	60	10^2–10^6
Germination at 80°C for 2 min, followed by sonication and autoclaving	0	10^0–10^2
	30	10^0–10^1
	60	10^0–10^2
Germination at 80°C for 2 min, followed by autoclaving and sonication	0	10^0–10^2
	30	10^0–10^3
	60	10^0–10^3
Germination at 80°C for 10 min, followed by sonication and autoclaving	0	10^0–10^4
	30	10^0–10^3
	60	10^0–10^3
Germination at 80°C for 10 min, followed by autoclaving and sonication	0	10^0–10^4
	30	10^0–10^3
	60	10^0–10^6
Germination at 60°C for 10 min, followed by sonication and autoclaving	0	10^0–10^5
	30	10^0–10^5
	60	10^0–10^5
Germination at 60°C for 10 min, followed by autoclaving and sonication	0	10^0–10^4
	30	10^0–10^4
	60	10^0–10^5

[a] Each protocol was tested with 20 to 30 sets of a series of dilutions, and the DNA in each dilution tube was tested in triplicate.

TABLE 2. PCR detection of lowest number of spores in powders and nasal specimens

Powder[a]	Wt (g)[b]	Lowest no. of spores with positive PCR results	Positive CT[c] (range)
Wheat flour	0.0050	<20	31.62–36.48
	0.0025	<10	38.62–39.72
Baking soda	0.0050	<20	27.36–30.92
	0.0025	<10	38.18–38.39
Talcum powder	0.0025	<10	38.36–39.07
Cornstarch	0.0025	<10	33.06–40.56
Baking powder	0.0025	<10	39.06–39.46
DOH powder 465	0.0025	<10	26.17–35.71
DOH powder 689	0.0025	<10	27.39–39.04
DOH powder 750	0.0025	<10	25.42–38.76
DOH powder 761	0.0025	<10	32.55–39.26
Nasal set A	NA	<10	29.60–39.54
Nasal set B	NA	<100	34.54–41.4
Nasal set C	NA	<10	34.99–40.93
Nasal set D	NA	<10	28.66–44.09
Nasal set E	NA	<25	31.96–33.42
Nasal set F	NA	<50	28.04–28.83
Nasal set G	NA	<10	29.60–30.01
Nasal set H	NA	<10	28.49–34.73

[a] DOH, Florida Department of Health.
[b] NA, not applicable.
[c] CT, cycle number in which the sample is considered positive by the instrument. The higher CT values in the ranges were observed with the Roche LightCycler. A background CT of 50 (50 cycles) was found in tests of all negative controls.

TTC-TTT-CTG-ACT-TGG-AAT-AGC 3′), and C1 (5′ GCC-AGG-TTC-TAT-ACC-GTA-TCA-GCA-A 3′). C1 was labeled with a FAM (6-carboxy-fluorescein) reporter and a TAMRA (6-carboxy-tetramethyl-rhodamine) quencher for direct PCR product detection during the reaction. For tests with the ABI PRISM 7700 sequence detector, each reaction mixture contained 10 μl of template; 0.30 pmol of primers; 0.10 pmol of probe; 25 μl of a master mixture (Applied Biosystems) containing *Taq* DNA polymerase, Tris-HCl, KCl, $MgCl_2$, and nucleotides; and enough sterile deionized water to achieve a total volume of 50 μl. Amplification conditions were 95°C (10 min) and 50 cycles of 95°C (15 s), 50°C (1 min), and 60°C (1 min). Each LightCycler (Roche) reaction mixture contained 2 μl of template, 0.6 pmol of primers, 0.05 pmol of probe, 2 μl of a master mixture, and enough H_2O to obtain a total volume of 20 μl. Amplification conditions were 95°C (4 min); 50 cycles of 95°C (10 s), 50°C (15 s) and 60°C (15 s); and an extension at 40°C (1 min). Prior tests with *Bacillus cereus* and *Bacillus thuringiensis* American Type Culture Collection strains were negative. Positive or negative controls were *B. anthracis* and water or *B. cereus* ATCC 11778, respectively. Samples were tested in triplicate. To determine the reproducibility of the optimal preparation method, 22 sets of a series of five dilutions (10^4 to 10^0 CFU/sample) were made and processed. Eight to 10 μl of DNA from each dilution was used as a template and tested ten times.

Dry, sterile, synthetic-tipped swabs (Copan Diagnostics Inc., Corona, Calif.) were used by four adult volunteers on different days to self-collect 171 nasopharyngeal secretion specimens. The nasal secretion-soaked swabs received either 10 μl of PBS or 5.5 to 10 μl of suspension to yield ≤10 to 15,000 spores/sample and then were placed into 300 μl of TSB and processed by the optimal method. Aliquots (50 μl) were cultured and held for 3 days.

Sterile synthetic swabs were dipped into one of three solutions: PBS, PBS with 0.01% Tween 20 (Sigma-Aldrich), or PBS with 0.01% Tween 20 and 0.03% lecithin (Sigma-Aldrich). The swabs, coated with 0.0025 to 0.005 g of powder (baking powder, baking soda, cornstarch, flour, or talcum) and 5.5 to 10 μl of spore suspension (≤10 to 15,000 spores), were placed into 300 μl of TSB (with a minimum of 200 samples per powder), processed, and tested in triplicate. Chi-square statistical tests analyzed the different solutions. Powders ($n = 33$) received by the Florida Department of Health Tampa Laboratory were

TABLE 3. Detection of spores by PCR and culture in unknown environmental powders submitted to Florida Department of Health analysis

Powder no.	Culture results[a]	PCR results
8	NG	Neg
36	NG	Neg
64	NG	Neg
207	NG	Neg
260	NG	Neg
377	NG	Neg
456	NG	Neg
465	NG	Neg
499	NG	Neg
501	NG	Neg
535	NG	Neg
547	NG	Neg
665	NG	Neg
689	NG	Neg
750	NG	Neg
761	NG	Neg
1038	NG	Neg
34	Infrequent mixed *Bacillus* spp.	Neg
100	Infrequent mixed *Bacillus* spp.	Neg
114	Infrequent mixed *Bacillus mycoides*	Neg
115	Infrequent mixed *Bacillus* spp.	Neg
200	Moderate mixed *Bacillus* spp.	Neg
246	Infrequent mixed *Bacillus* spp.	Neg
371	Infrequent mixed *Bacillus* spp.	Neg
402	Few mixed *Bacillus* spp.	Neg
453	Few mixed *Bacillus* spp.	Neg
558	Moderate *B. cereus*	Neg
718	Many mixed *Bacillus* spp.	Neg
835	Many *Bacillus thuringiensis*	Neg
845	Many *Bacillus thuringiensis*	Neg
225	Many mixed *Bacillus* spp. and moderate *B. cereus*	Pos
595	Moderate mixed *Bacillus* spp. and few *B. cereus*	Pos
658	Few mixed *Bacillus* spp. and *B. cereus*	Pos

[a] None of the bacteria isolated were *B. anthracis.* NG, no growth. Infrequent, ≤5 colonies; few, >5 and ≤10 colonies; moderate, >10 and ≤100 colonies; many, >100 colonies.

cultured and processed in duplicate. For four of the culture-negative powders, a third set of swabs was spiked with spore suspensions.

Our results show that DNA that was only autoclaved did not produce reliably sensitive results. Samples that received only sonication allowed PCR detection of 10 to 100 spores but contained viable spores (Table 1). When autoclaving and sonication were combined, irrespective of order, there was a slight improvement in the overall results (Table 1). After heat shock (2 min, 80°C) was administered prior to sonication and autoclaving, the PCR assay detected target DNA at ≤10 spores/sample (Table 1).

The ABI PRISM 7700 and LightCycler assays detected the target sequence in 6.45 and 2.58 pg of whole-cell DNA, respectively. After the DNA was concentrated to one-half to one-third of the total volume, the PCR assay consistently identified samples containing ≤10 spores regardless of powder type or presence (Table 2). No interference was seen. The sensitivities and specificities of the solutions were as follows: PBS only, 99.3 and 99%, respectively; PBS-Tween, 82.9 and 99%; and PBS-Tween-lecithin, 84.3 and 97%. Chi-square analysis ($P < 0.05$) gave P values of 0.0247 for PBS versus PBS-Tween, 0.0020 for PBS versus PBS-Tween-lecithin, and 0.1546 for PBS-Tween versus PBS-Tween-lecithin. All nasopharyngeal samples with ≥10 spores were identified. Due to poor sampling distribution, we could not accurately determine the true sensitivity and specificity of samples with <10 spores.

Of the 33 powders, results for 17 (51.5%) were negative by PCR and culture (Table 3). Thirteen (39.3%) PCR-negative powders grew infrequent to many numbers of colonies of *Bacillus* species. The remaining 3 (9.1%) PCR-positive powders grew a variety of *Bacillus* spp. (no *B. anthracis*) upon multiple cultures. When individually tested, one isolate (*B. cereus*) from each powder was PCR positive. The presence of Ba813 in *B. cereus* has previously been reported and demonstrates the importance of testing for the virulence plasmids in current LRN protocols (17, 18, 19, 23).

A combination of heat shock, sonication, and autoclaving, followed by sample concentration, allows the detection of ≤10 *B. anthracis* spores and infrequent Ba813-positive isolates in nasal specimens and powders in a reproducible, sensitive, simple, and safe method. Automated DNA extraction allows high throughput without cross-contamination. Real-time PCR shortens turnaround time to ≤6 h. Because current LRN protocols split specimens for culture and molecular tests, sampling distribution errors are possible in specimens with infrequent (1 to 2) spores. All positive culture or PCR results would be sent to the Centers for Disease Control and Prevention for further examination.

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