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Forensic Application of Microbiological Culture Analysis To Identify Mail Intentionally Contaminated with *Bacillus anthracis* Spores‡

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ABSTRACT

The discovery of a letter intentionally filled with dried *Bacillus anthracis* spores in the office of a United States senator prompted the collection and quarantine of all mail in congressional buildings. This mail was subsequently searched for additional intentionally contaminated letters. A microbiological sampling strategy was used to locate heavy contamination within the 642 separate plastic bags containing the mail. Swab sampling identified 20 bags for manual and visual examination. Air sampling within the 20 bags indicated that one bag was orders of magnitude more contaminated than all the others. This bag contained a letter addressed to Senator Patrick Leahy that had been loaded with dried *B. anthracis* spores. Microbiological sampling of compartmentalized batches of mail proved to be efficient and relatively safe. Efficiency was increased by inoculating culture media in the hot zone rather than transferring swab samples to a laboratory for inoculation. All mail sampling was complete within 4 days with minimal contamination of the sampling environment or personnel. However, physically handling the intentionally contaminated letter proved to be exceptionally hazardous, as did sorting of cross-contaminated mail, which resulted in generation of hazardous aerosol and extensive contamination of protective clothing. Nearly 8×10^6 CFU was removed from the most highly cross-contaminated piece of mail found. Tracking data indicated that this and other heavily contaminated envelopes had been processed through the same mail sorting equipment as, and within 1 s of, two intentionally contaminated letters.

On 15 October 2001 an envelope addressed to United States Senator Tom Daschle was discovered to contain a threatening letter and dried *Bacillus anthracis* spores. Subsequently, all of the mail in congressional buildings and at the postal facility that serves the U.S. Congress was collected, placed into large plastic bags, packed in drums, and quarantined. Subsequently, the Washington Field Office of the Federal Bureau of Investigation (FBI) arranged to search for additional intentionally contaminated letters that might be in the sequestered mail.

Analysts from the FBI Behavioral Analysis Unit indicated that any additional spore-filled mail would most likely closely resemble the three spore-filled letters already discovered, which engendered a plan for each piece of congressional mail to be examined for similarities in appearance to those letters.

However, this approach would have several disadvantages. In particular, handling each item of mail would maximize the hazard to personnel by maximizing their exposure to contamination. A manual search would also be very time-consuming and tedious and may have failed to identify a spore-filled letter if it had a different appearance than the other letters. Therefore, a search strategy aimed at assessing spore levels inside the bags containing mail was adopted. The rationale for this strategy was simple. Unopened spore-filled letters had shed enough spores to cause respiratory infections and extensive contamination at large postal facilities; therefore, the number of spores inside a plastic bag containing such a letter should be enormous, and the spores should be easily identifiable by culture analysis. This approach would also take advantage of the prior bundling of the mail into manageable batches.

Work within hazardous areas is often protracted and stressful because of the physical demands imposed by working in personal protective equipment (PPE). Previous FBI microbiological sampling endeavors included extensive delays because of laboratory bottlenecks, which endanger hazardous materials (hazmat) sampling personnel by prolonging their mission. Bottlenecks occur because large field teams employed for these activities quickly generate numerous samples, each of which must be documented and processed for culture or other analysis by the relatively few personnel typically employed in laboratories. The more complex the sample preparation process in the laboratory is, the more prone it is to backlogs.

Generally, hazmat teams collect environmental samples on swabs or gauze pads (wipes), which are then transported to the laboratory. For the undertaking described here, the sampling and analysis process was streamlined by having hazmat personnel inoculate culture media directly in the hazardous work area, leaving the laboratory simply to receive and document the inoculated cultures, incubate them overnight, and interpret them the following morning.

Culture analysis was chosen because the relative ease of culture inoculation would facilitate higher throughput compared to immunochemical or PCR methods, which involve multiple sample preparation and assay steps, which require extensively trained technicians. This approach led directly to the discovery of a spore-filled letter addressed to Senator Patrick Leahy within several days, rather than the weeks that would have been required for visual examination alone.

MATERIALS AND METHODS

Containment facility. A temporary facility that had appropriate containment features and was large enough to permit processing of 230 barrels was built within a large warehouse. The dimensions and layout of the facility are shown in Fig. 1. The area of the workroom was approximately 547 m^2 (excluding the 3-m^2 loading dock) and the height was 5 m, which resulted in an air volume of approximately 2,735 m^3. All internal walls were coated with two layers of 6-mil polyurethane, and the doors between rooms were covered by overlapping hanging plastic sheets. A net negative pressure and directional airflow were maintained for the duration of the operation using four portable air handlers, each of which drew 59.5 m^3 of air/min and was equipped with high-efficiency particulate air (HEPA) filters. The workroom contained tables comprising two workstations and a station for packaging evidence and for monitoring contamination on personnel. Two class II type A biological safety cabinets equipped with ultra-HEPA (0.2-μm) filters (Air Science Technologies, Ft. Myers, FL) were used for activities requiring physical manipulation of mail pieces. Four slit-to-agar air samplers (item no. M1252-0002; New Brunswick Scientific, Edison, NJ) were placed in the workroom, one was placed adjacent to the decontamination lines, and one was placed in the perimeter at the entryway from the cold zone.

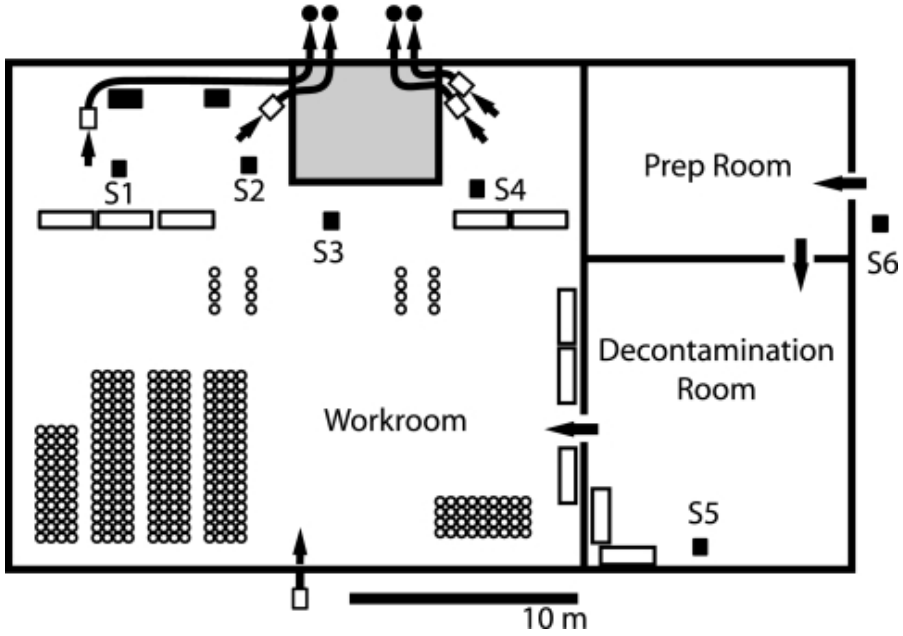

FIG. 1.

Diagram of the negative pressure isolation facility. The workroom dimensions were 25 by 22 by 5 m. Solid rectangles, class II type A biological safety cabinets; solid circles, monitoring of exhaust air using SBA plates during handling of exposed mail (see Materials and Methods for details); open circles, barrels; large open rectangles, work tables; small open rectangles, air handlers with HEPA filters; lines from air handlers, air exhaust or supply hoses (diameter, 30.5 cm) (arrows indicate the direction of airflow); solid squares labeled S1 to S6, slit-to-agar air samplers; large shaded rectangle, loading bay separated by walls from the workroom. The drawing is to scale, and the locations of items (except barrels, which changed positions) were determined by triangulation.

Prior to transfer of the barrels into the workroom, swab samples were obtained from around the lip of each barrel lid to ensure that spore leakage had not occurred during transit. Negative results permitted transfer and handling of the previously decontaminated barrels by personnel without respiratory PPE. After the barrels were positioned and before they were opened, the workroom was sealed and entry was restricted to appropriately trained and equipped hazmat personnel.

Swab sampling. Workdays in the hot zone were limited to about 8 h so that sampling personnel could be given adequate recovery time between each of two 2-h entries and so that samples could be delivered to the laboratory by 5:00 p.m. All sampling was performed by teams consisting of two persons who were trained to properly sample bags and inoculate agar culture media in order to obtain maximal coverage and swab-to-agar contact. All sampling personnel practiced the techniques and demonstrated proficiency prior to entering the workroom. One member manipulated the bags and performed the sampling, changing outer gloves between samples. An assistant handed unused items to the sampling member and attended to the required paperwork. This is a common evidence collection technique, often referred to as clean man/dirty man, that is intended to prevent cross contamination between samples.

The sealed plastic bags containing the mail were agitated to distribute spores. After agitation, a piece of precut duct tape with a folded tab (to avoid contact of adhesive with safety gloves) was placed on the outside of the bag for reinforcement, and a hole that was approximately 1 cm in diameter was cut

through the tape and the plastic with safety scissors. A rayon swab moistened with sterile water was inserted through the hole and wiped thoroughly on the surrounding interior plastic. The swab was removed and immediately used to inoculate a culture plate. The hole was resealed with a second piece of duct tape (also precut and folded).

Culture media and inoculation. Standard 85-mm-diameter sheep blood agar (SBA) plates (Becton Dickinson) were used for analysis of swabs after they were removed from the plastic bags. Contact petri dishes containing sheep blood agar (Becton Dickinson) were used to monitor the extent of spore contamination on personnel. Each person entering the workroom pressed contact petri dishes onto the surface of his or her protective coveralls immediately prior to exiting the sampling room and prior to entering the decontamination line. Personnel inside the workroom during swab and air sampling of bags and during physical examination of most mail pressed one dish one time onto each sleeve. Personnel involved in the examination of the contents of the single most contaminated bag (as determined by air sampling) pressed five separate plates onto different locations on their PPE. The smooth-surface protective coveralls were composed of Tychem SL (Lakeland, Ronkonkoma, NY), a laminate of Tyvek (Dupont, Wilmington, DE) and an outer layer of Saranex 23-P film (Dow Chemical Company, Midland, MI). Plates were transferred to the laboratory for interpretation and confirmatory analysis.

Environmental air monitoring. Slit-to-agar air samplers were run continuously while sampling and decontamination were in progress. Air was drawn at a rate of 30 liters/min through the sampler slit onto a rotating 150-mm petri dish containing SBA. The times of day at which airborne spores were deposited on the plates were estimated using a segmented template to correlate colony location with time, based on the start time and rotation rate (e.g., one revolution per 100 min). Air exhausted from the sampling facility was monitored whenever mail was removed from bags and manipulated, which was considered a hazardous activity. Monitoring of exhaust air was performed by positioning a standard SBA plate in the center of each exhaust hose at a 90° angle to the airflow. Plates were changed every 30 min to limit dehydration of the culture medium.

Air sampling inside plastic bags. An all-glass liquid impinger with a 12.5-liter/min critical orifice was fixed in place with a ring stand and clamps atop a piece of Styrofoam to avoid breakage (impingers were provided by Louise Pitt, United States Army Medical Research Institute of Infectious Diseases). The impinger, containing 10 ml of sterile water, was connected to the slit barrel of slit-to-agar air sampler S3 or S4 (Fig. 1) by Tygon Formula R-3603 tubing (inside diameter, 6.35 mm; Fischer Scientific, Pittsburgh, PA) attached to a rubber stopper. The inlet tube of the impinger was fitted with a length of Tygon tubing, the end of which was connected to a rigid sampling tube. The rigid sampling tube was fashioned from a plastic 10-ml pipette with each end removed. A vacuum was drawn on the liquid impinger using the slit-to-agar sampler containing a 150-mm SBA plate rotating at one revolution per 2 min to catch flowthrough spore particles. For sampling, the bag was agitated, the duct tape sealing the previously cut hole was removed, and the sampling probe was inserted into the bag. The probe was moved around in the bag for 2 min and removed. Each of 20 bags was sampled with a separate impinger-probe apparatus. Each impinger was disassembled, and the sample liquid was transferred to a 50-ml sterile conical plastic centrifuge tube for transport to the laboratory. The liquid samples were subjected to standard plate count analysis.

Visual examination of mail from contaminated bags. Mail examiners compared envelopes with photographs of the spore-loaded letters addressed to television news anchor Tom Brokaw, the New York Post, and U.S. Senator Tom Daschle. All mail was examined inside one of two class II type A biological safety cabinets. Nineteen bags were examined in order of increasing contamination as determined by the swab sampling results. These bags were examined in shifts by multiple two-member teams, and several teams occupied the workroom simultaneously. The 20th bag appeared, from air

sampling results, to be orders of magnitude more contaminated than the rest of the bags and to pose a significantly greater safety hazard. This bag was the last bag to be examined by a single three-member team composed of two examiners and one observer. No other personnel were present in the workroom.

Quantification of contamination on envelopes. A specific set of 20 letters, all postmarked 9 October 2001, Trenton, NJ, were segregated and photographed for analysis of routing information by the United States Postal Inspection Service. After photography, each letter was thoroughly swabbed on one side over a 100-cm^2 area with a moist rayon-tipped swab. The swabs were submitted to the laboratory for viable plate count analysis. High counts from these swabs suggested that further analysis would be useful, and the letters were transferred in separate plastic bags to the same laboratory for additional plate count analysis.

Laboratory culture analysis. All microbiological culture analysis was performed by the Naval Medical Research Center Biological Defense Research Directorate (Silver Spring, MD) under the direction of Al Mateczun, Robert Bull, and Joan Gebhardt. Colonies were counted on overnight direct plate and contact plate cultures inoculated at the mail containment facility the preceding day. When necessary, cultures were confirmed to be *B. anthracis* by gamma phage assay. One set of swab samples from 20 envelopes obtained at the mail containment facility was subjected to standard viable plate count analysis. Following transfer of the letters to the laboratory, viable plate count data were obtained for each letter by thoroughly wiping one side using a moist gauze wipe (rayon/polyester), which was then added to a tube containing 5 ml of sterile phosphate-buffered saline and vigorously mixed with a vortex mixer to extract spores. The extracts were subjected to standard viable plate count analysis. Five separate viable plate counts were obtained for each side of every envelope. Ten counts per side were obtained for 5 of the 20 envelopes.

RESULTS

Swab sampling of bags. Despite limitations imposed by stringent hazardous materials safety protocols, all 642 bags in 230 drums were sampled within 3 days (16.4 working hours). A significant amount of that time was devoted to maneuvering barrels and attending to the meticulous record keeping required by law enforcement evidence collection protocols. Preliminary results were received between 9:00 and 11:00 a.m. each morning after sampling, and a maximum of 353 results were received on the third day of sampling. Nearly all growth that occurred on the SBA plates under the sampling conditions used was *B. anthracis*. Minimal interference from other bacterial species occurred; therefore, presumptive results based on colony phenotype were sufficient to guide subsequent actions.

Of the 642 bags sampled, 62 yielded 1 CFU or more. Of these 62 bags, 26 yielded 1 CFU, 22 yielded 2 to 19 CFU, 8 yielded 20 to 99 CFU, and 5 yielded 100 to 300 CFU; the plate from one bag had growth that was too numerous to count.

Air sampling within bags. The ability to differentiate between contamination levels with the direct plating method described above is limited. The uptake and release of spores with swabs are inherently variable (9), and the spores in the bags were probably distributed unevenly despite mixing. In addition, the maximum number of colonies that can be counted before they become too crowded to distinguish from one another is roughly 300, which places a very low quantitative ceiling on the method. Because of these limitations, air sampling within bags was conducted in an attempt to better estimate the relative contamination of bags. The 20 bags that yielded 10 CFU or more when swabbing was used were chosen for sampling. *B. anthracis* was isolated from only three air samples. Two bags produced 300 CFU or fewer, while the third produced roughly 19,000 to 23,000 CFU or 760 to 920 CFU per liter of air sampled. During the 2-min sampling time, a 150-mm-diameter SBA plate on the slit-to-agar air sampler was used to monitor flowthrough from the impingers. These plates contained 0, 1, and 87 CFU. Colonies on the latter plate were distributed relatively evenly on the entire plate, indicating that

significant numbers of spores passed through the impinger liquid. The apparatus used for sampling air within bags was improvised from available materials when the need arose and was not tested for collection efficiency. Significant quantities of spores probably adhered to the plastic inlet tubing, decreasing the sensitivity of the method.

Environmental air monitoring. Airborne *B. anthracis* was not detected in the decontamination area, in the indoor cold zone, or in the exhaust air at any time during the operation. It was detected inside the hot zone only twice during the 3 days during which mailbag interiors were sampled. One of these events corresponded to the swab sampling of the bag that was later found to contain a letter that had been intentionally loaded with spores. During the air sampling of the 20 selected bags described above, slit-to-agar samplers S1 and S2 (Fig. 1) remained in place for air monitoring and detected significant contamination; 10 and 30 CFU were collected on plates in samplers S1 and S2, respectively, during 70 min of operation.

Figure 2 shows the detection of airborne *B. anthracis* throughout one workday, 16 November 2001. The detection of airborne *B. anthracis* beginning just prior to 5:00 p.m. corresponded with the identification and handling of an envelope that was addressed to Senator Patrick Leahy and was intentionally loaded with dried *B. anthracis* spores. The absence of data after 5:30 p.m. marks the cessation of air monitoring for the day. *B. anthracis* was detected at other times throughout the day prior to 5:00 p.m. (Fig. 2), but no additional envelopes containing spore powder were found.

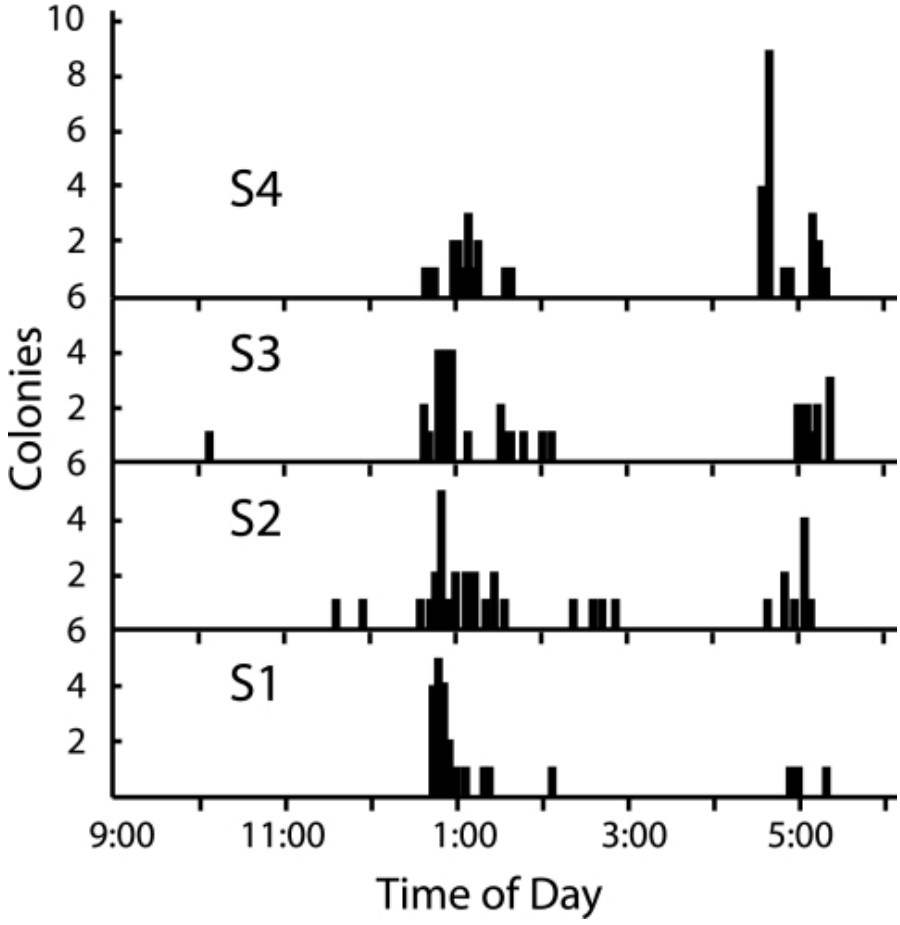

FIG. 2.

Detection of airborne *B. anthracis* spores during handling of mail. The bars indicate the numbers of colonies discernible on sections of slit-to-agar sample plates representing 100 liters of sampled air (3.3-min exposure) at the time of day indicated. Graphs S1 to S4 show the results for air samplers S1 to S4 (Fig. 1). The *x* axis begins at 9:00 a.m. and ends at 6:00 p.m. Air monitoring ceased at 5:30 p.m.

Contamination of personal protective garments. Growth occurred on only 1 of 104 personal monitoring plates used to sample personal protective garments during the 3 days of swab sampling of the mailbags. Sampling logs indicated that the bag containing the spore-laden letter addressed to Senator Leahy was sampled by the single contaminated person.

Physically handling mail presented a greater potential for contamination of personnel. Table 1 shows the contamination of the three people who examined mail from the bag containing the spore-laden letter addressed to Senator Leahy. As might be expected, all of these people were significantly contaminated. However, the PPE of numerous people who examined only cross-contaminated mail earlier in the day was contaminated. Of 24 personal contact plates (each representing a separate hot zone entry), only 5 were free of *B. anthracis* growth. The growth on the other 19 plates ranged from 2 colonies to too numerous to count (one plate), and five plates contained more than 50 colonies.

TABLE 1.

Contamination of personnel handling the Leahy letter

Location	No. of colonies on personal SBA contact plate[a]		
	Examiner 1	Examiner 2	Observer
Right arm	TNTC	0	5
Left arm	75	14	29
Chest	130	78	39
Right thigh	165	170	32
Left thigh	5	82	22

[a]A sheep blood agar contact plate was pressed one time onto the protective garment at the location indicated immediately before the individual left the hot zone. Each plate represents a 125-cm^2 sample area for the approximately 9,600 cm^2 of material comprising the front of each protective suit from knee to shoulder. TNTC, too numerous to count.

Identification of a letter intentionally loaded with *B. anthracis* spores.

All of the mail from the 20 most highly contaminated bags (described above) was examined manually and visually in order to identify envelopes that might have been intentionally loaded with powdered *B. anthracis* spores. Suspicious mail was not found in the first 19 bags examined. The 20th bag, which was the most highly contaminated bag, yielded a letter addressed to “Senator Leahy” whose appearance was nearly identical to the appearance of the spore-loaded letters addressed to the New York Post, Tom Brokaw, and Senator Daschle. Subsequent examination of the envelope contents revealed a quantity of powdered *B. anthracis* spores. The time at which this letter was identified and handled corresponded with the detection of airborne spores (Fig. 2).

Identification and analysis of cross-contaminated mail.

As described above, the PPE of numerous people was contaminated while they examined mail from the 19 bags in which no suspicious mail was found. In addition, Fig. 2 shows the detection of airborne *B. anthracis* between about 12:30 and 2:00 p.m. The degree of contamination appeared to be comparable to that produced by the handling of the Leahy letter (between 4:45 and 5:30 p.m.). These results prompted reexamination of the mail in all of the bags that had been opened during the period in which airborne spores were detected in order to determine whether a spore-loaded letter had been overlooked during the first search. During the reexamination, all letters were closely examined, opened, and inspected for the presence of powder within the envelope. Again, no suspicious mail was identified.

As the mail was visually inspected, 20 letters postmarked 9 October 2001, Trenton, NJ, were segregated and photographed for analysis by the United States Postal Inspection Service. Each letter was then swabbed on one side over a 100-cm^2 area. Viable plate counts from these swabs revealed that every letter was contaminated, and the counts ranged from 1×10^1 to 4×10^5 CFU per 100 cm^2. These letters were transferred to the laboratory for additional plate count analysis. Table 2 shows viable plate count data for each letter and compares the colony counts to the United States Postal Inspection Service tracking data for these letters and the Daschle and Leahy letters.

TABLE 2.

Cumulative plate counts and sorting histories relative to the Daschle and Leahy letters for 20 letters with postmarks of 9 October 2001, Trenton, NJ[a]

New Jersey P&DC[b]		Washington P&DC[c]		
AFCS	DBCS	DBCS	Time in DBCS Z (min:s)[d]	CFU[e]
B	M	X	NA	200
C	O	X	NA	750
B	O	Z	7:55	165,700
C	O	Z	7:56	1,200
B	O	Z	7:56	4,200
D	O	Z	8:09	8,200
B	O	Z	8:11	300
D	O	Z	8:14	6,100
C	O	Z	8:14	8,300
C	P, Q	Z	8:23	2,300
B	P, Q	Z	8:24	6,300
D	Q	Z	8:28	1,400
A	O	Z	8:30	29,300
B	N	Z	10:35	117,000
C	N	Z	10:40	382,300
B	N	Z	10:41	328,100[f]
B	N	Z	10:41	1,731,000[g]
B	N	Z	10:41	7,026,300[h]
B	N	Z	10:41	Daschle
B	N	Z	10:41	Leahy
B	N	Z	10:54	197,100[i]
C	N	Z	11:15	115,800[j]

Open in a separate window

[a]Each row shows the sorting history of an individual letter.
[b]The initial mail sorting in the New Jersey processing and distribution center (P&DC) with separate Advanced Facer Canceller System (AFCS) machines labeled A, B, C, and D was followed by sorting with separate Delivery Bar Code Sorters (DBCS) labeled M, N, O, P, and Q.
[c]Mail sorting in Washington, DC, involved individual DBCS machines labeled X and Z.
[d]Relative time at which letters passed through DBCS Z. NA, not applicable.
[e]Additive data for six successive colony counts rounded to the nearest 100 CFU. “Daschle” and “Leahy” indicate the times at which intentionally contaminated mail to United States senators was processed in DBCS Z (see Materials and Methods for details).
[f]Plate counts were obtained from five additional wipes from this letter. The final cumulative count was 3×10^5 CFU.

[g]Plate counts were obtained from five additional wipes from this letter. The final cumulative count was 1.8×10^6 CFU.
[h]Plate counts were obtained from five additional wipes from this letter. The final cumulative count was 7.9×10^6 CFU.
[i]Plate counts were obtained from five additional wipes from this letter. The final cumulative count was 4.4×10^5 CFU.
[j]Plate counts were obtained from five additional wipes from this letter. The final cumulative count was 1.8×10^5 CFU.

The quantity of CFU recovered decreased only incrementally after each successive wipe, and large numbers of spores were still removed after one swabbing and five wipes. Five letters were wiped an additional five times (Table 2). One letter produced a total of 7.9×10^6 CFU, and more than 10^5 CFU was recovered from the 10th and final wipe.

Although the precise pathway of each letter in Table 2 relative to the pathways of the Daschle and Leahy letters cannot be determined, there is a general correlation between the contamination levels and the histories of the envelopes in sorting machines. Seven of the eight letters that produced more than 10^5 CFU passed through the same Delivery Bar Code Sorters in the Trenton and Washington, DC, postal processing and distribution centers. The four letters exhibiting the highest contamination levels all passed through the Delivery Bar Code Sorters in Washington within 1 to 2 s of the Daschle and Leahy letters. One letter yielded $>10^5$ CFU but did not follow the path of the other letters. It was, however, the first of the letters in Table 2 to follow the Daschle and Leahy letters through the Advanced Facer Canceller System in Trenton.

DISCUSSION

All of the mail from Capitol Hill was packed in large plastic bags to expedite its removal from government buildings, which fortuitously bundled the mail into a manageable number of batches for the subsequent microbiological analysis described here. The appearance of the letter addressed to Senator Leahy found in this mail was nearly identical to the appearance of the other letters sent during the anthrax attacks of 2001, just as behavioral analysts had anticipated. In retrospect, the initial plan to locate spore-laden mail by visually inspecting each piece would probably have located this letter; however, efficiency and safety made the sampling approach far superior.

Physical manipulation of contaminated mail proved to be exceptionally hazardous, whereas the release of airborne spores was effectively limited by swab sampling through small holes in the bags containing the mail. The efficiency of the method enhanced safety by reducing the number of manually searched bags, as well as the overall processing time. It took only 5 days to sample and search all of the mail, compared to an estimated minimum of 30 working days for visual inspection alone. The time saving minimized hazard exposure, heat stress, fatigue, and the anxiety commonly experienced by individuals working in hazardous environments. Quantifying spores also provided an estimate of the relative hazard of each bag so that it could be handled with appropriate precautions.

All of the culture plates generated during this search were analyzed by only four laboratory workers, who provided 642 results for presumptive *B. anthracis* in 3 days. It has been necessary to process large numbers of samples for semiquantitative analysis in several investigative sampling efforts, which places a premium on high throughput. Ironically, traditional culture methods, often viewed as too slow, proved to be more efficient than rapid molecular methods would have been. The simplicity of direct plating permitted inoculation of culture media in the hot zone by nonspecialists, eliminating laboratory bottlenecks experienced previously. However, one can envision situations in which simple culture

methods do not exist for a microorganism or are precluded by circumstances. The availability of other dependable, high-throughput, quantitative or semiquantitative assay platforms would greatly enhance investigative capabilities in such cases.

Although confirmatory results for *B. anthracis* are generally not available for 72 h, the growth of non-*B. anthracis* microorganisms in the sampling environment used was relatively rare, and overnight presumptive results were sufficient to guide subsequent actions. This highlights the importance of using appropriate culture media (or other analytical tools) in a given sampling environment. Implicit in this requirement is the need for familiarity with the background microbiota and potential competitors or inhibitors that might be encountered in that environment. However, most sampling environments have not been extensively characterized with available sampling and analysis methods.

Contamination of the air and of personnel occurred while mail was being handled inside class II biological safety cabinets. Cabinet performance may have been adversely affected by the airflow in the workroom, as well as by the unconventional activity for which the cabinets were used (5, 7). Although class II cabinets under controlled laboratory conditions may provide protection better than that seen here, they are not designed for total containment, and escape of spore aerosols is concentration dependent (5). Therefore, considering the extremely high spore concentration that may characterize some powders and the remarkable ease with which particles disseminate, laboratories should carefully consider safety and contamination issues before handling such materials.

The contamination seen on PPE highlights the hazards of handling contaminated mail, and it raises other issues as well. The clean man/dirty man sample collection protocol used here, which is meant to prevent cross contamination between samples, appears to have limited value in a heavily contaminated environment. Also, the protective garments used here were adapted from a standard hazmat repertoire generally used for chemical splash protection. While there is little doubt that that the material protects the skin of the wearer from exposure, the consequences of surface contamination by dry spores are unknown. Decontamination and doffing of contaminated protective garments might create an aerosol hazard, the risk of which may be a function of the affinity of spores for the material. A strong affinity might maximize contamination on the material but minimize the hazardous release of spores. A material that repels spores might prevent contamination completely. Unfortunately, few data are available on this subject.

Most sampling related to the 2001 attacks was used for remediation of contaminated buildings, which relies heavily on the sensitivity of the sampling methods used to reliably declare a space inhabitable. In contrast, effective use of sampling methods for the purpose described here relies less on sensitivity than on consistency, dynamic range, and pragmatic interpretation of sample-to-sample differences. Contact petri dishes, used here for personal monitoring, probably provide the greatest consistency between samples because of their ease of use and uniform contact area. The direct plating method used to interrogate mailbags is inherently variable due to the inconsistency of swab sampling (9). However, differences between sample results were great enough to confidently parse bags for subsequent analysis. Finally, air sampling with liquid impingers, the least sensitive and possibly most variable method but the one with the largest dynamic range, produced the single most informative sample.

The large number of spores shed from the Leahy letter during the activities described here might have been expected considering the extent of contamination and the attendant consequences that the unopened spore-filled letters caused in postal facilities. However, the level of shedding from cross-contaminated mail was notable and vividly illustrates its potential hazard (Fig. 2). The capacity for envelopes to accumulate and retain dried spores was also remarkable, and the colony counts in Table 2 probably underestimate the actual contamination because of the inherent inefficiency of surface sampling (9, 10).

Although some cross-contaminated mail contained high numbers of spores, the contamination associated with the Leahy letter was orders of magnitude greater than the contamination associated with any other mail. This leads to the expectation that the immediate proximity of a source of a deliberately released biological agent will have an extremely heavy specific microbial load that will decrease sharply with distance. Informed interpretation of contamination levels is necessary to avoid being misled by cross contamination, particularly when workers are trying to identify evidence or drawing inferences from sampling data. Indeed, detection of trace quantities of *B. anthracis* on mail would be highly significant in many circumstances but was essentially meaningless here.

It has become common practice to open mail in a containment device for protection from a biological attack. Given the observations presented here, it appears that it is virtually impossible to intentionally place dried spores inside a standard envelope without heavily contaminating its outside surfaces. Even if it were possible to perfectly seal a spore-laden envelope so that no spore could escape from the inside, the outer surface would be so heavily contaminated that spores shed from the surface would present a hazard. Significant contamination of a facility would likely occur simply by carrying such a letter to the containment device in which it is to be opened. This mechanism of spore dissemination is illustrated by the shedding of spores from cross-contaminated mail seen here.

Individuals familiar with the compositions of the powders in the letters have indicated that they were comprised simply of spores purified to different extents (6). However, a widely circulated misconception is that the spores were produced using additives and sophisticated engineering supposedly akin to military weapon production. This idea is usually the basis for implying that the powders were inordinately dangerous compared to spores alone (3, 6, 12; J. Kelly, Washington Times, 21 October 2003; G. Gugliotta and G. Matsumoto, The Washington Post, 28 October 2002). The persistent credence given to this impression fosters erroneous preconceptions, which may misguide research and preparedness efforts and generally detract from the magnitude of hazards posed by simple spore preparations.

Purification of spores may exacerbate their dissemination to some extent by removing adhesive contaminants and maximizing the spore concentration. However, even in a crude state, dried microbial agents have long been considered especially hazardous. Experiments mimicking laboratory accidents have demonstrated that simply breaking vials of lyophilized bacterial cultures creates concentrated and persistent aerosols (4, 8). The potential for propagating disease with crude lyophilized material is illustrated by an outbreak of 24 cases of Venezuelan equine encephalitis throughout three floors of a Moscow virology institute. These infections were caused when vials containing dried infected mouse brain were accidentally broken on a stairwell landing and were spread by air currents and foot traffic (11).

The letter to Senator Leahy found in this search provided the least adulterated and largest quantity of evidence from the 2001 anthrax attacks, allowing subsequent forensic characterization of the recovered material. Among the characteristics of greatest public interest was particle size, which ostensibly could be used for retrodiction of exposure risks and of the methods used to produce the powders. While size analysis of freshly prepared powders may bear signatures of the production process and predict some of their performance characteristics, size determinations for material recovered after it has been deployed must be viewed with circumspection. Particle size distributions are dynamic (13), changing as a powder experiences different conditions upon handling, such as compaction, friction, and humidity, among other factors. The size distribution of a recovered powder represents its state after an unknown period of aging and an unknowable set of conditions experienced during handling. It may not resemble the initial product.

Particles aerosolized from purified powdered spores consist either of individual spores or aggregates of individual spores. The great majority of particles are generally the smallest particles in the population (2), which are single spores in spore powders. This is reflected in the count distribution, which should have a mode of roughly 1 to 2 μm. This size distribution phenomenon has practical safety implications. In essence, even if most of a spore powder is bound in relatively few large particles, some fraction is composed of particles that are precisely in the size range that is most hazardous for transmission of disease by inhalation. For perspective, a crudely ground preparation consisting of only 1 to 10% loose individual spores by mass would contain 10^{10} to 10^{11} loose individual spores in 1 g, considering that moderately purified dried spore preparations contain roughly 10^{12} spores per g (1). These numbers are particularly large when one considers that the acceptable limit of contamination in workspaces is essentially zero and that it is theoretically possible to detect a single viable spore. (The relationship of count to mass distribution can be appreciated by considering that the mass of a particle is proportional to the cube of its radius so that even a moderately sized particle may contain large numbers of spores. See reference 2 for a detailed description of particle size statistics.)

This paper shows that there was successful integration of microbiological methodology with law enforcement and hazmat objectives and procedures. It also highlights some of the basic deficiencies in bioterrorism response at the time described. There was a generally poor understanding of spore powder properties, of the suitability of PPE materials for use with dried microbial agents, and of environmental sampling particulars, including problems posed by poorly characterized sampling methods and microbial backgrounds. Bioterrorism preparedness became a priority, and some progress was made, but some of the most basic problems persist. Preparedness cannot be achieved until a realistic understanding of the threat is generally appreciated and response protocols that are scientifically grounded are established.

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FOOTNOTES

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