Development of an Aerosol Surface Inoculation Method for Bacillus Spores

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A method was developed to deposit Bacillus subtilis spores via aerosolization onto various surface materials for biological agent decontamination and detection studies. This new method uses an apparatus coupled with a metered dose inhaler to reproducibly deposit spores onto various surfaces. A metered dose inhaler was loaded with Bacillus subtilis spores, a surrogate for Bacillus anthracis. Five different material surfaces (aluminum, galvanized steel, wood, carpet, and painted wallboard paper) were tested using this spore deposition method. This aerosolization method deposited spores at a concentration of more than 10^7 CFU per coupon (18-mm diameter) with less than a 50% coefficient of variation, showing that the aerosolization method developed in this study can deposit reproducible numbers of spores onto various surface coupons. Scanning electron microscopy was used to probe the spore deposition patterns on test coupons. The deposition patterns observed following aerosol impaction were compared to those of liquid inoculation. A physical difference in the spore deposition patterns was observed to result from the two different methods. The spore deposition method developed in this study will help prepare spore coupons via aerosolization fast and reproducibly for bench top decontamination and detection studies.

In response to the 2001 Bacillus anthracis spore incidents in the United States, many studies have investigated various technologies to sample media, detect biological agents, and decontaminate materials (3, 9, 10, 17–19, 21). These laboratory studies typically use small coupons and laboratory inoculants to simulate the materials and biological agents that occur in field events. In these studies, test coupons should have reproducible numbers of viable spores on varied surface types. In addition, it is critical that the coupons are prepared so that the inoculation is representative of contamination as it occurs in the field. Liquid inoculation protocols that use suspended spores in aqueous buffers have been the primary methods used to prepare test surfaces for biological agent decontamination and detection studies (15, 16, 20). Liquid inoculation methods offer advantages in that they allow relatively easy control of the number of spores and the contaminated area. However, it is unclear whether surfaces contaminated by liquid inoculation methods are representative of surfaces contaminated with aerosolized Bacillus anthracis spores. Therefore, it is necessary to investigate the impact of various spore deposition methods on Bacillus anthracis decontamination and detection studies. The conventional particle deposition method via aerosolization is comprised of an aerosol generating system, such as a particle nebulizer, to introduce the spores into a chamber and a second chamber to allow the spores to settle onto the target material surfaces. The spore surface concentrations are controlled by varying settling time and the initial aerosolized spore concentration in the chamber. Brown et al. (4–6) tested various methods of collecting samples of Bacillus subtilis spores on porous and nonporous surfaces using this approach. In these studies, surface spore concentrations of 10^2 to 10^6 CFU/cm^2 were achieved. More recently, Baron et al. (2) developed a system to deposit Bacillus anthracis and Bacillus globigii spores on agar plates, achieving concentrations of up to 200 CFU per plate using a particle settling chamber. This system was designed to produce low and predictable surface loadings for spore detection research. Baron and coworkers observed high variability in the surface spore concentrations when using the settling chamber due to inconsistent input of spore particles into the chamber. Further, the particle settling method is time-consuming, usually requiring more than 12 h to achieve the targeted surface concentration, as well as complex and costly with regard to the equipment. A recent study by Heimbuch et al. introduced a new method to deposit spores using a cascade impactor (14). This method is rapid compared to the method using a settling chamber. The surface-deposited number of spores was more than 10^6 CFU/cm^2, with the average coefficient of variation (CV) being 13.6%. The deposited concentration was verified only for glass slides, and it is unclear if this method would work for uneven surfaces such as carpet.

The U.S. Environmental Protection Agency’s (EPA’s) National Homeland Security Research Center conducts biological agent decontamination research. Key to EPA’s decontamination studies is the preparation of contaminated test coupons. Such test coupons consist of various surface materials contaminated with spores at a level of more than 10^6 CFU per coupon and with less than a 50% CV in the number of viable spores. For decontamination and sampling studies, typically several (at least 5) replicate coupons are needed of each material type for each test condition, resulting in a large number of required coupons per test. Further, spores deposited, via aerosolization, may represent the type of contamination likely to be seen during a bioterrorism event (11, 21). In this study, there was...
developed a new method (aerosol impaction) which can de- posit via aerosolization reproducible numbers of viable spores on a wide range of surface types in a relatively short time. This method uses a metered dose inhaler (MDI) filled with Bacillus subtilis spores as the source. The pressurized spore particles, resulting from the MDI, were impacted onto the target surfaces. This new method was tested by determining the total number of viable spores which were deposited on five different materials and the corresponding reproducibility of achieving that number. The five materials tested were industrial carpet, wood, aluminum, galvanized steel, and painted wallboard paper. Spore deposition patterns for samples prepared via the liquid inoculation method and this aerosol impaction method were compared through surface analysis using scanning electron microscopy (SEM). The deposition method and comparison are detailed in this paper.

MATERIALS AND METHODS

Spore preparation. The Bacillus subtilis spores (ATCC 19659; Manassas, VA) in the MDIs were prepared as previously reported by Rastogi et al. (15, 16). Briefly, a seed culture was initiated in tryptic soy broth (TSB) for 24 to 48 h. An aliquot (250 µl per plate) was spread over the surface of large (150-mm) Lab-Lemko (Oxoid Ltd., Hampshire, United Kingdom) agar plates. The medium used for sporulation plates was a mixture of 2 g Lab-Lemko (Oxoid Ltd., Hampshire, United Kingdom) agar, 2 g tryptone (Fisher Scientific, Fair Lawn, NJ), 23 g yeast extract (Becton Dickinson, Sparks, MD), 1% MnCl₂, Sigma Chemical Co., St. Louis, MO), and 2 g agar (Becton Dickinson, Sparks, MD). The plates were incubated at 37°C for 10 to 14 days, and sporulation was monitored using a microscope (BX50; Olympus, Miami, FL). Once more than 90% of cells had sporulated, spores were harvested by flooding the agar surface with 25 ml of chilled deionized water for 4 plates sequentially (elution from one plate was used for the next plate) and dislodging them from the agar surface with a sterile spreader. Plate rinsates were then collected, pooled, and filtered to remove agar. The filtrate was washed three times by centrifugation and resuspension with chilled deionized water. Following the final centrifugation, the spore preparation was reconstituted in chilled deionized water and heat shocked at 65°C for 30 min. Bacillus subtilis spores (ATCC 19659; Manassas, VA) for the liquid inoculation method were prepared using modified ASTM method E 2197-02 (1). One mil- lilitre of a Bacillus subtilis culture grown overnight in TSB was used to inoculate 100 ml of 1:10-diluted Columbia broth (Difco, BD, Franklin Lakes, NJ) amended with 0.1 mM MnSO₄. Following incubation at 35°C ± 2°C for 72 h, the spore suspension was washed 10 times by centrifugation and resuspension with chilled deionized water. After the final wash, the pellet was resuspended in chilled deionized water and heat shocked at 80°C for 30 min. The concentration of spores in the final preparation was determined by serial dilution and plating onto tryptic soy agar (TSA; Difco, BD, Franklin Lakes, NJ). The plates were incubated at 35°C ± 2°C for 18 ± 2 h.

Spore particle characteristics from the metered dose inhaler. Spore particle size distributions from the MDIs containing both viable and nonviable Bacillus subtilis spores (0.5% [w/v] in ethanol) were measured with an electrical low-pressure impactor (ELPI; model 2334; Dekati Ltd., Tampere, Finland) (12). This instrument measures the particle size and concentration by charging them with a corona discharge. The charged particles then pass through a cascade impactor, which collects particles based on their aerodynamic diameter. When these particles land on a stage of the impactor, their charge is measured by internal electrometers connected to the individual impactor stages. The MDIs were actuated into a 1-liter chamber with a 10-liter-per-minute makeup flow of filtered air to that matched for operation of the ELPI. The measured data from the ELPI were converted to particle cross-sectional area information using both aerodynamic diameter and physical diameter to allow comparison with results from previous studies (12).

Deposition materials and setup. For bench scale experiments, a method was developed to deposit spore particles on targeted test surfaces. MDIs were prepared by the aerosol science laboratory at Edgewood Chemical and Biological Center (Aberdeen Proving Ground, MD). Each MDI contained Bacillus subtilis spores in an ethanol (analytical reagent grade; Mallinkrodt Inc., Paris, KY) solution and propellant. The spore concentration in an MDI was 0.05 or 0.5% (w/v) (approximately 10⁵ and 10⁶ CFU per puff, respectively). Each MDI provided approximately 200 doses. During experimentation, the number of doses from each MDI was tracked to ensure that no more than 150 doses were dispensed from any one MDI.

A small cylindrical acrylic chamber was designed and custom fabricated by the EPA, located in Research Triangle Park, NC, to deposit spores, aerosolized using an MDI, onto solid substrates. The chamber is composed of five major parts: (i) a 12-cm-long cylindrical body with a 5-cm inner diameter, (ii) a lid for the MDI actuator, (iii) a lid with distance-adjustable knob, (iv) 3 center aligning knobs, and (v) a substrate holder with an axial-distance-adjustable bar. The diagram of this chamber is shown in Fig. 1. A laser pointer was used to align the center of the MDI adapter nozzle with the center of the substrate. The center of the substrate holder was aligned with the laser pointer using three center aligning knobs as shown in Fig. 1. Deposition of Bacillus subtilis spores using this device was initially characterized for two different spore-concentration MDIs (0.05 and 0.5% [w/v]) and three different distances (3.4, 4.3, and 5.3 cm) between the MDI actuator nozzle and the coupon (aluminum stub). After selection of the appropriate spore concentration and distance, deposition using this device was tested for five different surface materials (test materials are summarized in Table 1). Each material was cut to an 18-mm-diameter disc and attached to an aluminum stub (Ted Pella Inc., Redding, CA) using double-sided carbon tape (Ted Pella Inc., Redding, CA). These coupons were then stored in the laboratory for 48 to 72 h to cure the adhesives before sterilization. The potential spore loss due to the presence of carbon tape during extraction was evaluated, and the results (data unpublished) showed no difference in the recoveries due to the carbon tape.

Substrates were transported into and out of the chamber using a stainless steel gripper (long-handled pin stub SEM gripper, 130 mm; Ted Pella Inc., Redding, CA). All surface materials, transport plates, and grippers were sterilized by autoclaving (standard gravity cycle and 121°C for 60 min) before spore deposition.

Spore deposition. The blank substrate (the sterilized coupon) was positioned inside the particle deposition chamber as shown in Fig. 1. The distance between the coupon surface and the MDI nozzle was adjusted using a depth micrometer. The different thicknesses of the various coupon substrates (e.g., aluminum, carpet versus carpet, carpet versus wood, carpet versus aluminum) were be adjusted for each substrate type. After the alignment, each coupon was puffed upon once by activating the MDI canister. The aerosol-impacted coupon was then immediately removed from the chamber using a sterilized gripper and transported to a circular stainless steel transporting disc that was specifically designed to hold seven 18-mm stubs. Stubs were then secured by inserting the pin portion of the stub into a hole in the disc. After all stubs for a test were in place, the disc was placed into a glass petri dish and immediately transported for analysis to the microbiology laboratory. All tests were composed of 3 to 5 aerosol-impacted replicate samples and 2 procedural blanks (not exposed to the MDI or liquid inoculation) per transporting disc/petri dish.

Liquid-inoculated coupons were also prepared to compare spore patterns to aerosol impacted coupons using SEM. The same surface materials were attached to the carbon stubs using a double-sided carbon tape (Ted Pella, CA) and the spores were suspended in distilled water at a concentration of 10⁶ CFU/ml and one 100-µl droplet of this solution was applied to the material surfaces using a micropipette. For all materials except wood, the liquid inoculum remained on the surface until the water was evaporated. The liquid inoculum rapidly disappeared from the wood coupon surface, as it intercalated into the voids of this material.

Extraction and counting. Aerosol-impacted and liquid-inoculated coupons were singly and aseptically removed from the petri dishes and placed in 50-ml sterile vials with 10 ml BBL peptone-buffered water (BD Becton, Dickinson, and Company, Franklin Lakes, NJ) containing 0.01% Tween 80 (Fisher Scientific, Pittsburgh, PA). The vials were then sonicated for 10 min and vortexed for 2 min to dislodge the spores from the coupon surface and suspend them in the aqueous buffer. Following extraction, 10-fold serial dilutions were performed, as needed, by adding 0.1 ml of the sample to 0.9 ml of peptone-buffered water using a micropipette. Appropriate dilutions were spread in triplicate (0.1 ml each) onto Trypticase soy agar (BD Becton, Dickinson, and Company, Franklin Lakes, NJ) plates and incubated at 37°C. These spread plates were counted manually after approximately 18 h. The surface spore concentration reported for each sample was determined by averaging the results from triplicate subsamples. Extraction bias was tested to measure the potential loss of spores during the extraction procedure. Test tubes, which contained extraction solution with or without a coupon, were directly inoculated with Bacillus subtilis spores. Five tubes per individual test material were tested. The test results showed that the loss due to the presence of coupon materials was not statistically significant.

Scanning electron microscopy analysis. Sample surfaces were probed using SEM to observe the differences in the particle loading due to the two coupon
inoculation methods, aerosol impaction and liquid inoculation. A personal scanning electron microscope (PSEM; Aspex, Delmont, PA) was used for the analyses. Spore-inoculated/impacted surfaces were coated with ~150 Å of carbon using a carbon coating system (model 108A; Cressington Scientific Instruments, Watford, England, United Kingdom) to reduce sample surface charging before analysis. The SEM was operated in both the secondary electron emission (SE) mode and the backscattered emission (BE) mode and was optimized to identify spore particles on various surface types. The operational conditions were magnifications of ×17 to ×1,000, zero degrees of tilt, 12- to 16-mm working distances, and a 20-kV accelerating voltage.

RESULTS

Characteristics of spore particles from the MDI. The number of spores (both viable and nonviable) from a set of MDIs was determined using an ELPI (electrical low-pressure impactor). The spore size distribution data for multiple puffs from an 0.5% (wt/wt)-spore-concentration MDI are shown in Fig. 2.

Deposition distance determination. Spore deposition was evaluated at several distances between the MDI actuator nozzle and the target surface using MDIs with two spore concentrations (0.05 and 0.5% [wt/wt]). Three distances were tested, and the results are summarized in Table 2. This “distance test” was conducted with aluminum stubs (a diameter of 18 mm) to determine the distance and MDI spore concentration needed to achieve at least $10^6$ CFU per coupon. Each of the distances tested resulted in coupons having spore loads higher than the target concentration. The results also showed that spore concentrations were not dependent on the deposition distance within the tested distances. The 0.5% (wt/wt)-spore-concentration MDIs produced approximately 5 to 20 times more viable spores on the surface than did the 0.05% (wt/wt)-spore-concentration MDIs. The aerosol-impacted areas from 0.5% (wt/wt)-spore-concentration MDI tests were more visibly distinguishable from the background aluminum surface than were the areas from the 0.05% (wt/wt)-spore-concentration MDI. The deposition areas were circular with diameters of approximately 0.8, 1.0, and 1.2 cm for 3.4-, 4.3-, and 5.3-cm distances, respectively. An MDI with 0.5% (wt/wt) spores and a distance of 3.4 cm was selected for further testing with other surface materials because of its lower CV and higher spore deposition than those of the 0.05% (wt/wt)-spore-concentration MDI and the other distances.

Spore deposition onto surfaces. The method of depositing spores onto surfaces was tested on five different surface materials. Each individual test was conducted with 3 to 5 replicate

| Test material               | Supplier                      | Specification                                                                 |
|-----------------------------|-------------------------------|-------------------------------------------------------------------------------|
| Aluminum stub               | Ted Pella Inc., Redding, CA   | Scanning electron microscope pin stub, 18 mm by 3.15 mm (diam by thickness)    |
| Galvanized steel            | East Coast Metal, Durham, NC  | 18 mm by 0.6 mm (diam by thickness)                                          |
| Industrial carpet           | The Home Depot, Cary, NC      | Mannington Integra HP, 18 mm by ~6 mm (diam by thickness)                     |
| Painted wallboard paper     | The Home Depot, Cary, NC      | Georgia Pacific sheetrock facing painted with Painter’s Select (True Value)   |
|                             |                               | Interior PVA drywall primer (PVA-1 white) and then interior flat finish EZF-1 White Acabado paint, 18 mm by ~0.5 mm (diam by thickness) |
| Bare structural wood, fir  | The Home Depot, Cary, NC      | 18 mm by ~5 mm (diam by thickness)                                           |
samples, and the same set of tests was conducted multiple times (5 to 10 times depending on the surface type) with different MDIs and operators. The individual test results are shown in Fig. 3 and the combined deposition results from the multiple test sets are summarized in Table 3. The column “No. of MDIs” in Table 3 shows the number of MDIs used for testing deposition on each of the surface types. The variations of spore deposition within each test set are also shown in Fig. 3. The overall CV in Table 3 of individual surface materials is less than 50%, and the coefficients of variance from an individual test shown in Fig. 3 were also within 50% with the exception of two tests, one with aluminum (51%) and one with wood (52%) coupons. No contamination was observed on procedural and laboratory blanks during any tests.

Surface spore analysis. Spores were deposited onto five surface materials using the aforementioned aerosol impaction and liquid inoculation methods. The materials’ surface characteristics were then probed using SEM with the SE mode, except for the painted wallboard paper surfaces in Fig. 5, which were probed with the BE mode. Secondary electrons are emitted from the surface of a feature; thus, the SE image is more sensitive to a particle’s surface morphology. The BE signal increases monotonically with atomic number so that BE images can reveal compositional differences within a single particle (features with higher atomic numbers appear brighter) (13). The BE mode was used for the painted wallboard paper surfaces because the spores on the painted wallboard paper surfaces could not be clearly distinguished using SE mode due to the similar shapes of the painted wallboard paper surfaces and spores. The samples in Fig. 4 and 5 (wood and painted wallboard paper, respectively) were prepared using aerosol impaction and liquid inoculation. The samples in Fig. 6 show the liquid-inoculated galvanized steel surfaces with two different magnifications (×50 and ×300). SEM images of liquid-inoculated and aerosol-impacted carpet coupons are shown at a ×25 magnification on the left and a ×100 magnification on the right in Fig. 7 and 8, respectively. All aerosol-impacted coupons show a center-crowded circular pattern of spores, but liquid inoculated-coupons show varied patterns depending on the surface types. SEM images (Fig. 4) of the wood coupons show similar patterns for both aerosol impaction and liquid inoculation.

**DISCUSSION**

Characteristics of spore particles from the MDI. Carrera et al. demonstrated that the output of the MDIs (0.05% [wt/wt]) used in this study could be particles containing single spores or particles containing multiple spores (7). In the first set of measurements, they used optical microscopy to determine particle area. They determined, by visual inspection, that particles having average cross-sectional areas between 0 and 1 \( \mu \text{m}^2 \), and 2 and 5 \( \mu \text{m}^2 \), or 2 and 5 \( \mu \text{m}^2 \) contained 1, 2, or 3 to 5 spores, respectively. Carrera and coworkers further confirmed the number of spores per particle for the 0.5% (wt/wt)-spore-concentration MDIs used in this study. First, the particle frequency as a function of particle area.
(calculated using the ELPI-determined aerodynamic diameter) was determined using an ELPI instead of optical microscopy. The data are shown in Fig. 2.

The data were then converted to the percentage of particles containing 0 or 1, 1 or 2, or 3 to 5 spores using the same methodology developed by Carrera et al. discussed above (7), and the converted data are shown in Fig. 9. As seen in Fig. 9, >65% of the particles contained single spores when using the cross-sectional areas calculated with the ELPI-determined aerodynamic diameter. It is difficult to do a direct comparison with the data from the work of Carrera et al. because physical diameters (measured by optical microscopy) result in larger cross-sectional particle areas. A comparison was attempted by converting the aerodynamic diameters to physical diameters using the bulk dry density of *Bacillus subtilis* spores (1.52 g ml⁻¹) (8). After this conversion, the number of particles containing single spores greatly increases to almost 90%, as shown in Fig. 9. This larger value may be in part due to artifacts of the particle diameter conversion and the ELPI analysis. When the aerodynamic particle sizes are converted to physical particle sizes, the 0.66-μm cut point becomes 0.54 μm, causing a larger number of smaller particles to be counted. In addition, since the ELPI impactor plates were not analyzed by optical microscopy, it is impossible to determine if the particles collected on this 0.66-μm stage are single spores or spore fragments. A lower percentage of particles containing single spores were expected due to the higher concentration of spores in the MDI (0.5 versus 0.05% [wt/wt]).

**Spore deposition onto surfaces.** As shown in Table 3, the average number of deposited spores was higher than 10⁷ CFU per coupon for all five material surfaces and the CV for each material was less than 50%. The results in Fig. 3 and Table 3 indicate that the relative standard deviations in the numbers of spores per puff were similar between and within MDIs.

These results can be compared to the spore deposition study by Heimbuch et al. (14). Heimbuch et al. used a cascade impactor to deposit dried spores onto glass slides by impaction. The highest concentration of spores deposited onto a glass slide coupon reported by Heimbuch et al. was 3 × 10⁵ CFU/cm². This spore surface concentration is 10 to 100 times less than the concentrations achieved using the method described in this paper. In order to achieve similar spore surface concentrations using the method reported in the work of Heimbuch et al., long impaction times may be needed (on the order of hours). The method of Heimbuch et al. showed more reproducibility (the average CV was 13.6% within an experiment) than did that in the current study (the average CV range was from 14 to 47% across multiple experiments). However, it is difficult to directly compare the results due to the different surface types used in the two studies. If the CVs determined for similar materials are examined (galvanized steel, painted wallboard paper, and wood), comparable reproducibilities (the average CV of 14% from Table 3) are observed.

**Surface spore analysis.** The aerosol-impacted and liquid-inoculated samples in Fig. 4 show a single layer of spores deposited onto the wood surfaces. Both samples showed deposition of spores into the crevices of the wood surface. The deposited surfaces were probed with SEM, and penetration of spores could not be confirmed. However, the liquid inoculum soaked into the wood surface, which implies that the liquid

![FIG. 4. SEM images (SE mode, ×1,000 magnification) of wood coupon with aerosol impaction (left) and liquid inoculation (right).](image-url)
medium facilitates the transportation of spores through the wood crevices.

Spore deposition patterns were, however, different for the liquid-inoculated and aerosol-impacted painted wallboard paper surfaces. The liquid-inoculated samples have concentrated spore bands on the outer edge of the inoculated area. The SEM image in Fig. 5 was taken using BE mode, in which image signals increase monotonically with atomic number. Materials with higher density are imaged more brightly in the BE mode SEM image. Because the spores are less dense than are painted wallboard paper surface material, the spores appear darker in the image. The thick dark line across the SEM image in Fig. 5 (left) is a concentrated area (layer) of spores. This high spore population on the outer edge of the liquid-inoculated area is likely caused by spore migration to the outer edge of the liquid droplet during water evaporation. The spore deposition (right in Fig. 5) for the aerosol-impacted area created a center-crowded pattern, which is produced by the spray action from the MDI actuator nozzle. This center-crowded pattern of spore deposition was observed for all other aerosol-impacted surface materials.

The deposition patterns on aerosol-impacted galvanized steel and painted wallboard paper were similar. However, the liquid-inoculated galvanized steel sample demonstrated a unique spore deposition pattern. The SEM images in Fig. 6 show spore deposition on a liquid-inoculated galvanized steel sample. The left image in Fig. 6 shows a curved line (arrows) that separates the inoculated area (left side) from the bare galvanized steel surface (right side). Further, this image shows an area (circled) of agglomerated spores. This spore agglomeration was confirmed by higher-magnification SEM imaging, shown in the right panel of Fig. 6. The phenomenon of spore agglomeration was observed across the spore-inoculated area of the galvanized steel surface.

The most significant difference in the spore deposition patterns was observed on the carpet surfaces, shown in Fig. 7 and
8. The liquid inoculation method created large spore agglomerations (circled areas in Fig. 7) on the carpet surface. When a spore-containing liquid droplet was inoculated onto the carpet surface, the liquid droplet was not soaked into the carpet surface. The droplet was instead preserved for an extended period of time on the carpet surface while the liquid was evaporating. This slow and steady water evaporation may result in spore agglomerations on top of the carpet fibers. Spores deposited by the aerosol impaction method appear on the individual carpet fiber surfaces, as seen in Fig. 8. Also, similarly to spore distribution on other aerosol-impacted surfaces, the spores on the carpet fiber surface were concentrated in the center of the coupon.

These SEM analyses show that there is a physical difference in the spore deposition patterns resulting from the two methods. The spore deposition patterns from the aerosol impaction method developed in this study, a center-crowded pattern, were similar for all surface types. The liquid inoculation method created various forms of spore-agglomerated areas on the surfaces: thick linear spore layers on painted wallboard paper, spore piles on galvanized steel, and spore agglomerations on carpet fibers. It is possible that these spore-agglomerated areas that formed on the test surfaces via liquid inoculation may cause misrepresentative results for decontamination or sampling studies, compared to results from aerosol impaction. Therefore, this aerosol impaction method is inherently useful for biological agent decontamination and detection studies.

In summary, a novel spore deposition method has been developed using an MDI as a viable spore-dispersal source. This method can produce high spore concentrations (more than $10^7$ CFU per coupon) on small (18-mm-diameter) test coupons with various surface types in minutes. The ability to vary aspects of this method such as initial MDI concentration and deposition distance allows the user to generate a wide spore concentration range and deposition areas on various surface types. This method is not limited to the use of an MDI; any metered and pressurized spore particle generator can potentially be used with appropriate verification. The limitation of this method is the small spore deposition area (less than 2.5
cm²) on coupon surfaces; hence, this method is not effective for spore deposition onto large coupons.

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FIG. 9. The percentages of the total number of particles from MDI outputs containing 1, 2, or 3 to 5 spore particles.