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ORIGINAL ARTICLE

Evaluation of sampling tools for environmental sampling of bacterial endospores from porous and nonporous surfaces

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Abstract

Aims: Having and executing a well-defined and validated sampling protocol is critical following a purposeful release of a biological agent for response and recovery activities, for clinical and epidemiological analysis and for forensic purposes. The objective of this study was to address the need for validated sampling and analysis methods called out by the General Accounting Office and others to systematically compare the collection efficiency of various swabs and wipes for collection of bacterial endospores from five different surfaces, both porous and nonporous. This study was also designed to test the collection and extraction solutions used for endospore recovery from swabs and wipes.

Methods and Results: Eight collection tools, five swabs and three wipes, were used. Three collection/preservation solutions were evaluated: an ink jet aerosol generator was used to apply *Bacillus subtilis* endospores to five porous and nonporous surfaces. The collection efficiencies of the swabs and wipes were compared using a statistical multiple comparison analysis.

Conclusions: The ScottPure® wipe had the highest collection efficiency and phosphate-buffered saline (PBST) with 0·3% Tween was the best collection solution of those tested.

Significance and Impact of the Study: Validated sampling for potential biological warfare is of significant importance and this study answered some relevant questions.

Introduction

Following the dispersal of anthrax through the US Postal Service in 2001, there was a heightened awareness of the lack of standardized protocols for effective environmental sampling. While there is a general recognition that samples needed for analysis must be collected carefully, there is also little expectation – or experience – that samples are being handled consistently. For example, the *Manual of Clinical Microbiology*, 7th edn states that 'specimens arriving in the laboratory may be improperly selected, collected and transported' (Miller and Holmes 1999). Many different sampling scenarios arise for response and restoration activities, clinical and epidemiological monitoring and forensics. The sampling tools used and the collection, transportation and preservation of the samples before they arrive at the laboratories are all critical to delivering uncompromised samples whose analysis will be usable for decision making (Miller and Holmes 1999; Budowle *et al.* 2005).

Prior to the attacks of 2001, there had been a handful of studies evaluating swabs and wipes for collection of environmental samples (Favero *et al.* 1968; Kirschner and Puleo 1979). After the attacks, scientists from the CDC published a paper on the relative effectiveness of sampling methods, and indicated that their sampling research should serve as a baseline for future sampling efforts which should include epidemiologic data (CDC, 2002). There have been additional studies evaluating swabs and wipes for collection of environmental samples (Buttner *et al.* 2001, 2004; Carlson *et al.* 2001; CDC, 2002; Sanderson *et al.* 2002; Teshale 2002; Rose *et al.* 2004; Budowle *et al.*

2006; Hodges *et al.* 2006; Brown *et al.* 2007). In response to the CDC urging, the paper by Teshale *et al.* developed recommendations for sampling guided by epidemiological data (Teshale 2002). In addition to establishing standard procedures for sample collection, quality assurance guidelines for laboratories analysing the samples are also necessary (Budowle *et al.* 2003), consistent with strong recommendations of the GAO that the entire sample collection and analysis process needs to be validated.

Surface sampling of unknown substances of concern requires a standard protocol to efficiently collect samples, to prevent contamination and loss of the sample, and to provide samples for forensic analysis. The overall goal of this study was to systematically determine the collection and recovery efficiency of various swabs and wipes to collect and preserve biological materials from five different surfaces, both porous and nonporous. Swabs are typically used for small smooth surfaces such as computer keyboards and wipes are more commonly used for larger surface areas such as table tops, floors and walls (CDC, 2002). This study determined the best collection and extraction solution of the three tested and the best swab and/or wipe of the eight tested for collecting bacterial endospores dried onto porous and nonporous surfaces. We report both colony forming units per millilitre (CFU ml^{-1}) and per cent recovery.

In this study, sampling protocols were developed for collection of bacterial endospores deposited and dried onto porous and nonporous surfaces. Liquid samples were chosen so that they would adhere to the surfaces, thus giving a better account of the bacteria being recovered. Bacterial endospores of an anthrax surrogate, *Bacillus subtilis* ATCC® 49760, were applied to porous and nonporous surfaces using an ink jet aerosol generator (IJAG). The surfaces included commercial carpet, polyester upholstery fabric, plastic laminate counter-top, sealed red oak wood flooring and a computer monitor screen. The sample squares with deposited *B. subtilis* were sampled with seven different swabs or wipes, prewetted with a phosphate-buffered saline with 0·3% Tween (PBST) solution. An eighth device, the calcium alginate swab, was eliminated in initial testing because it fell apart with sampling. The endospores were recovered from the sampling swabs and wipes in PBST solution by mixing with a vortex. Recovery of endospores from sampling devices was followed by enumeration with plate counts.

Collection and extraction solutions were also evaluated to determine the optimum solution to collect, extract and preserve the samples. The three solutions sterile E-pure® water, PBS and PBST were tested. It is recognized that PBST may not be the best collection solution when using downstream analytical techniques that are affected by the presences of salt in the sample. Depending on the specific need, multiple samples may have to be collected in different collection solutions for different types of analysis. As the purpose of this study was to look at collection and recovery of endospores, the sample collection solution used was the one that allowed for the greatest recovery from the sampling devices – the PBST. Water and PBS were eliminated early in the study because of the difficulty recovering the endospores from the sampling devices without the aid of a surfactant.

The endospores were in PBST buffer to facilitate the dispersal of a known quantity of endospores with the IJAG. According to a study by Buttner *et al.* (Buttner *et al.* 2001) endospores suspended in a buffer with a surfactant do not adhere to surfaces well. However this was the only means of generating reproducible dispersal through the IJAG. Rose *et al.* (2004) reported results in per cent recovery by premoistened swabs with a recovery method similar to this study. They reported higher per cent recovery (9·9–43·6%) but was based on their per cent recovery upon mixing with a vortex and scraping endospores from control surfaces (5×5 cm stainless steel plates). Their endospores were suspended in ethanol (Rose *et al.* 2004). Our per cent recovery was based on the theoretical deposition of endospores, based upon the endospore counts reported by the IJAG as they were deposited onto the surfaces. In 1979, a study was done by Kirschner and Puleo on a wipe-rinse technique for quantitative evaluation of contamination on large surfaces. They used a PBST solution and enumerated endospores with plate counts. Their study showed that a polyester-bonded cloth was found to be superior to cotton for sampling from simulated spacecraft surfaces (Kirschner and Puleo 1979).

Experimental designs for this study were developed by Pacific Northwest National Laboratory statisticians to ensure an optimal sampling protocol.

Materials and methods

Bacterial endospore sample preparation

Bacillus subtilis ATCC® 49760 obtained from the American Type Culture Collection, Manassas, VA, USA was used as a surrogate for *B. anthracis* in this study. This surrogate is easily cultured and uniformly dispersed. The Gram-positive, endospore-forming bacterium was cultured in Bacto® tryptic soy broth (TSB) without dextrose (Becton Dickinson and Company, Sparks, MD, USA). The culture was started from a −80°C, 10% glycerol-preserved freezer stock. Approximately 10 μl of freezer stock was added to 3·0 ml of sterile TSB in a 10-ml snap-cap tube. The tube was incubated in a shaker incubator at 30°C for *c.* 14 h at 150 rev min^{-1}. Following the 14-h

incubation, 150 μl of the vegetative cells were spread-plated onto nutrient sporulating medium (NSM) agar plates, inverted and incubated for 3–5 days in a 37°C incubator. NSM contains 3 g l^{-1} tryptone, 3 g l^{-1} yeast extract, 2 g l^{-1} Bacto-agar, 23 g l^{-1} Lab-Lemco agar and 1 ml 1% $MnCl_2{\cdot}4H_2O$ in 1 l of E-pure® water. The sporulating cultures were checked for endospores microscopically and harvested when >95% endospores were present in the culture. The endospores were washed from the plates with 10 ml of sterile E-pure® water using a sterile, disposable inoculating loop to scrape the endospores from the agar. They were then centrifuged – 121 RCF (×***g***) (2 min), 3020 RCF (×***g***) (3 min) and 12 100 RCF (×***g***) for 10 min to facilitate separation of the endospores from the vegetative debris. The samples were decanted and washed four to five times with sterile E-pure® water to remove the vegetative cell debris. Microscopic evaluation was used to determine the number of washes needed to obtain a stock with minimal vegetative debris resulting in >95% endospores. The endospores were stored in water at 4°C for the duration of the study. The endospore count, based upon plate counts on TSA agar, was $7{\cdot}2 \times 10^9$ CFU ml^{-1}.

Endospore deposition method development

In an attempt to achieve uniform, reproducible distribution of cells over the testing surface, an IJAG was used for endospore deposition. A series of preliminary experiments determined the best method for reproducible deposition. Absorbance measurements using a bromocresol purple dye were used to verify that the IJAG was delivering reproducibly. As bacterial endospores tend to clump, especially in buffers, breaking up these clumps was necessary to decrease deposition variability. Several different pretreatments of the endospore stock in water were evaluated in an effort to improve reproducibility in sample-to-sample deposition. These pretreatments included (i) sonication of the endospore stock for 5, 10, 15 or 20 min, (ii) diluting the stock, (iii) filtering through a 5-μm Millipore® filter, (iv) adding ethylenediaminetetraacetic acid to a final concentration of 0·1, 1·0, and 3·0 mmol l^{-1} concentration respectively or (v) adding Tween (0·01% final concentration) as a surfactant. Microscopic observations were made of the treated endospore stocks to check for clumping. In addition, viability studies were done concurrently to verify that the endospores were not being compromised by the various treatments. Plate counts were done and compared to the original stock solution plate counts. Control plates were evaluated by applying 200 droplets of endospores with the IJAG directly onto TSA plates, incubated overnight and counted. The results showed an average endospore count of 40·6% based upon the 200 droplets deposited. Droplets from the IJAG may contain more than one endospore.

Early testing showed that deposits of at least 150 000 droplets would be required for measurable recovery with all of the sampling tools from all of the surfaces. A deposition of 200 000 droplets was chosen for the enumeration of recovered endospores. An endospore deposition protocol was established for the endospore collection, recovery, viability and reproducibility testing. The *B. subtilis* (200 000 droplets) were deposited onto nonporous and porous surfaces using the IJAG.

Collection solutions

Three collection solutions were evaluated: sterile E-pure® water, PBS (phosphate buffered saline) and PBST. The collection solutions were first tested on stainless steel and vinyl surfaces as these surfaces would allow for easy recovery. A multiple comparison analysis was done on the three collection solutions. The statistical analysis showed PBST was the most efficient collection solution for all sampling tools tested except for the Dacron®/polyester swab, in which case PBST was not statistically different from water. Therefore, PBST was selected as the collection solution to use for the remainder of the study.

Sampling from nonporous and porous surfaces

The endospore-collection protocol was first developed using glass slides as the test surface because the endospores were easily and consistently recovered. *Bacillus subtilis* endospores were applied to $10{\cdot}2 \times 10{\cdot}2$ cm surfaces with an IJAG, recovered with wipes or swabs and enumerated using plate counts. PBST (pH 7·2) was used to prewet the swabs and wipes prior to sampling and to store the endospore samples. Once the protocol was developed on the glass slides, the porous and nonporous test surfaces were added to the study. The three nonporous surfaces tested were plastic laminate countertop (no. 4763-60, Wilsonart International, Temple, TX), finished oak wood flooring (Model no. 95-200; Cryntel, Hollywood, FL, USA) and a computer monitor screen (Dell). The computer screen was taped off into 5 cm squares for sampling and the spore application was adjusted accordingly (150 000 spores applied). The computer monitor was activated so that the electrostatic field of the screen would be taken into account. The smaller squares (5 *vs* 10·2 cm) were chosen to allow for more samples to be taken across the monitor screen. The IJAG nozzle was shortened prior to deposition onto the computer monitor so that the IJAG and the computer monitor would fit within the biological safety cabinet for sample deposition.

The two porous surfaces evaluated were 100% polyester upholstery fabric- (manufactured in the USA) and commercial carpet (Style: Southern Plains, no. 14624; Interface, Inc. Atlanta, GA, USA). The commercial carpet was new and had been factory treated with an antibacterial agent. Preliminary tests indicated that the antibacterial agent did not interfere with bacterial endospore recovery. All surfaces were autoclaved before endospore deposition, except the computer monitor screen. It was cleaned with 70% ethanol, rinsed with sterile E-pure® water, and allowed to dry in a biological safety cabinet.

Collection tools

Eight sampling tools were evaluated initially, including both swabs and wipes. Sterile swabs included cotton swabs (Fisher no. 14-959-92B), polyurethane foam swabs (Fisher no. 14-960-3H), Alpha® (Texwipe, Kernersville, NC) swabs – polyester head (Fisher no. 18-385), Dacron® (INVISTA, Wichita, KS)/polyester swabs (Fisher no. 14-959-90), and calcium alginate swabs (Fisher no. 4-959-80). Cotton swabs are generally not recommended because they may interfere with PCR (CDC, 2002), but were evaluated in this study because of their ease of use and ready availability. Wipes included cotton wipes (TexWipe 304 – Fisher no. 18-308B), HS II Cleanroom Wiper (TexWipe 3210 – Fisher no. 18-390), and ScottPure® rayon/polyester CR Class 100 Critical Task Wipers (VWR no. 21908-010; Kimberly-Clark, Dallas, TX). All sampling tools were autoclaved before sampling. The calcium alginate swabs were removed from the study after the first surface test because they disintegrated with sampling.

Sample collection and extraction protocol

Sterile techniques were used for all sampling. Deposited endospore samples were collected from the surfaces by moving a sterile, premoistened swab or wipe over the entire sample surface, 10 passes in a horizontal direction and 10 passes in a vertical direction. An S-shaped stroke from side to side in a swath about 2 cm wide was used while traversing the sample surface during each pass. Care was taken to overlap the previous pass to ensure thorough coverage of the surface. The swabs were rotated slowly while making each pass and held at a 45° angle, so the full length of the swab head contacted the surface. The swabs were placed into tubes containing 2 ml of collection solution and mixed with a vortex for 1 min to release the endospores from the swabs. The swabs were removed from the tube after rolling them on the inside edge of the tube to extract as much liquid as possible. Plate counts of each sample were used to determine the endospore concentration in CFU ml^{-1}.

The wipes were folded into quarters before sampling and one quarter was used for each five passes across the surface. The wipe was carefully rolled up from the backside of the quarter with sample on it and dropped into a 50 ml polypropylene centrifuge tube containing 20 ml of the extraction solution. It is recognized that first responders would not fold the wipes this many times but the purpose of this study was to evaluate collection/recovery of the various sampling tools. The sample handler wore nitrile gloves and changed them between each sample collection. The tubes containing the wipes were mixed at the highest speed on a vortex for 2 min. The wipes were removed from the tubes with sterile forceps and twisted into a tight ball around the tongs of the forceps while pressing against the inner tube wall to extract as much liquid as possible. The liquid wipe samples were then centrifuged for 20 min at 12 100 RCF ($\times g$) and 4°C. Ten millilitres were removed from the top portion of each tube to decrease the dilution factor. Plate counts of a representative number of the 10 ml aliquots that were removed showed the endospore count in the remaining liquid was not being significantly impacted. Dilutions were made from the remaining sample and plated onto TSA for endospore enumeration (CFU ml^{-1}). The plates were incubated overnight at 30°C before counting. Control plates with no sample were enumerated as a blank.

For each surface, data were collected in 10 replicate sets with at least one set completed per day. Each set included data from all seven collection tools used to collect endospores from the surface. A balanced, randomized, complete block design was used to determine the order in which each device would be tested within each set. In this way, unanticipated effects of the endospore deposition, day-to-day variability and the recovery process on plate counts were minimized.

Results

This study was designed to test the collection/extraction solutions used for endospore recovery from swabs and wipes, and to determine the best sampling tools for collection of bacterial endospores.

The sample preparation/application to the various surfaces that was determined to be optimum was a sonication time of 15 min, dilution of the endospore stock in water and addition of Tween at a final concentration of 0·01%. This included sonicating the endospore stock for 10 min prior to making a 1 : 100 dilution in water and five additional minutes before adding Tween and spraying/depositing with the IJAG. Endospore counts on TSA plates showed that sonicating the 1 : 100 dilution of bacterial endospores for 15 min before deposition with the

IJAG resulted in a more reproducible deposition onto TSA plates. The average counts were 75·7 ± 6·18 (SD = 20·6 and CV = 0·27). These results were comparable to or better than those for the pipette drop method.

For each surface, the different tools were compared using the recovered CFU ml^{-1}. Results for the swabs and wipes are provided in Fig. 1. Roughly speaking, if two boxes have a large degree of overlap, the difference in the average CFU ml^{-1} is not significantly different. If two boxes have a large amount of space between them, the difference in average CFU ml^{-1} is significantly different. If two boxes have a small gap between them or they overlap slightly, a significant difference may or may not be present.

Of the swabs, the polyurethane foam swab has the highest average recovery (CFU ml^{-1}) for all of the surfaces tested (Fig. 1). For plastic laminate and commercial carpet, the difference in average CFU ml^{-1} between the polyurethane foam swabs and the device with the next highest average recovery are statistically significant at the $P = 0{\cdot}05$ level. For the other surfaces, the difference is not significant at the $P = 0{\cdot}05$ level. The difference in average CFU ml^{-1} between the polyurethane foam swab and the other swabs is not always statistically significant. However as the polyurethane swab has the best average recovery across all surfaces tested, it is recommend as the best performing swab in our study.

Of the remaining three swabs, the results are less clear. For example, cotton swabs have the second highest average CFU ml^{-1} for plastic laminate and oak flooring surfaces, but they have the lowest recovery for upholstery and carpet. The Alpha® swabs consistently rank second or third in terms of average CFU ml^{-1} and the Dacron/polyester swabs consistently rank third or fourth. In some cases, statistically significant differences were observed between the average recovery of cotton, Alpha® and Dacron/polyester swabs (e.g. plastic laminate), and in other cases, the differences were not significant (e.g. commercial carpet).

Of the wipes, no single wipe outperformed the others for all surfaces. For the nonporous surfaces, the average recovery between the three wipes was close (Fig. 1). The ScottPure® wipe had the highest average recovery for the laminate counter top and computer monitor, while the HSII had the best recovery for oak flooring. However, no statistically significant difference between any of the wipes was observed.

For the porous surfaces (Fig. 1) the ScottPure® wipe had the highest average CFU ml^{-1} for both upholstery and carpet. The difference in recovery between the

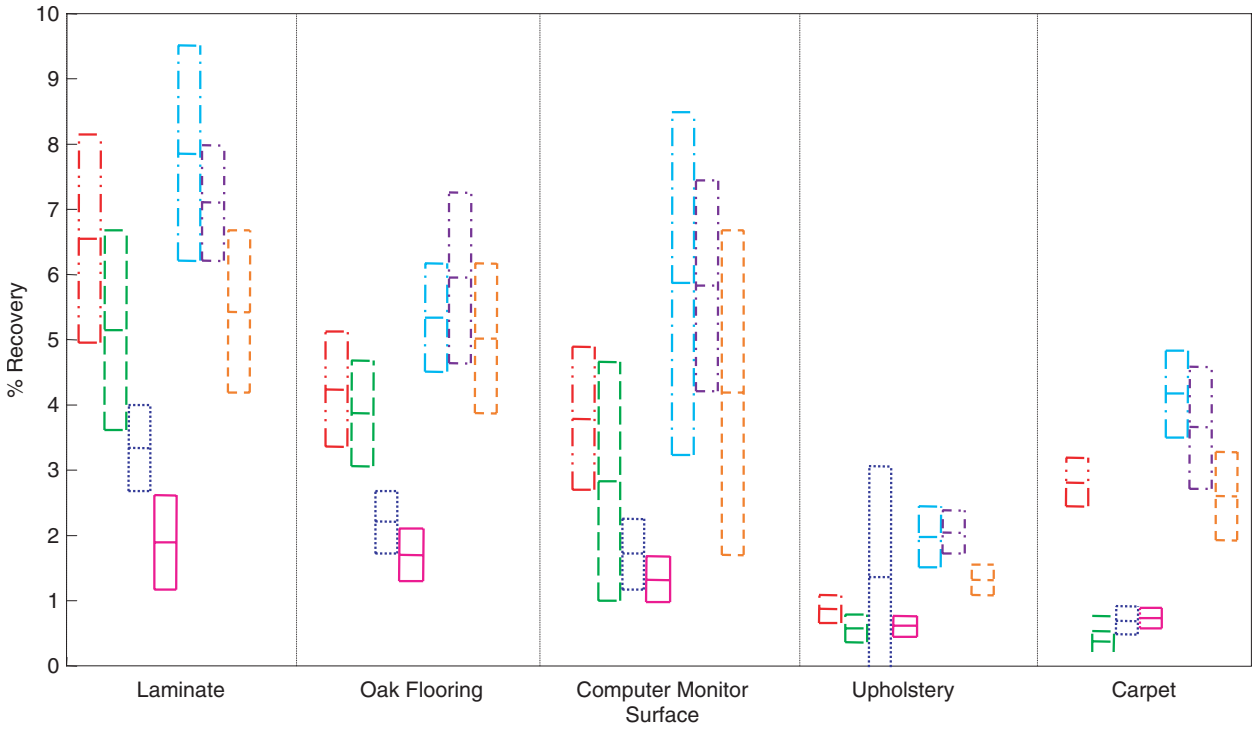

Figure 1 Recovery of *Bacillus subtilis* ATCC® 49760 endospores from different surfaces with swabs and wipes. The average recovery for a given device (CFU ml^{-1}) is plotted as a line in the centre of its corresponding vertical box. The top and bottom ends of each box represent the 95% confidence interval on the average recovery (CFU ml^{-1}). –·–, Polyurethane foam swab; – – –, Cotton swab; ·····, Alpha swab; ——, Dacron/polyester swab; –·–, ScottPure wipe; –·–·–, HSII wipe; – – –, Cotton wipe.

Table 1 Summary of sample collection results

Surface	Best swabs	Best wipes
Plastic laminate	Polyurethane	ScottPure®, HSII, cotton
Oak flooring	Polyurethane, cotton	HSII, cotton, ScottPure®
Computer monitor	Polyurethane, Alpha®	ScottPure®, HSII, cotton
Upholstery	Polyurethane, Alpha®, Dacron®	ScottPure®, HSII
Carpet	Polyurethane	ScottPure®

All collections were into phosphate-buffered saline with 0·3% Tween.

ScottPure® wipe and the HSII wipe was statistically significant for carpet, but not for upholstery. These results suggest that all wipes perform similarly for the surfaces tested in this study. A summary of the sample collection results can be found in Table 1.

To compare swabs with wipes in Fig. 1, results were converted to per cent recovery (because of different collection solution volumes for swabs *vs* wipes). Figure 1, plots the average and 95% confidence limits on the per cent recovery for all collection tools and all surfaces in this study.

For all surfaces, the wipes tend to have a higher per cent recovery than the swabs. While the 95% confidence intervals plotted in Fig. 1 do not suggest that this difference is always statistically significant, the average CFU ml^{-1} is consistently higher for all wipes suggesting that wipes outperform swabs under the conditions of this study.

Discussion

The sampling device needed for surface collection is typically determined by the type of surface – swabs for small, difficult to sample surfaces and wipes for larger surfaces. The ScottPure® wipe was the best performer for most surfaces except oak flooring, in which case HSII, cotton and ScottPure® were not significantly different. For swabs, the polyurethane swab had the highest collection/recovery efficiency from all the surfaced tested. PBST was the collection/extraction solution used for all surfaces.

The bacterial endospores in this study were enumerated by plate counts in CFU ml^{-1}. It is acknowledged that while quantitative PCR would have provided additional information, it also measures the DNA of both viable and nonviable endospores. Consequently, PCR would not have provided an accurate comparison to the endospore counts.

Sampling from each of the selected porous and nonporous surfaces presented challenges. For example, the IJAG had to be modified (shortened) to deposit samples onto 5-cm taped-off squares of the computer screen. Activated computer monitor screens have an electrostatic field that attracts bacterial endospores, making endospore collection from them difficult. Entrainment into very porous surfaces, such as carpet, makes endospore recovery more difficult than from nonporous surfaces.

The sample-collection results from all surfaces were analysed by comparing the average number of CFU ml^{-1} across the different collection tools. This study suggests that wipes are more efficient collection tools on the small porous and nonporous surfaces tested.

We note that our per cent recovery appears quite low based upon the estimated number of endospores deposited by the IJAG droplet counter. In particular, per cent recovery was calculated by estimating the number of CFUs per IJAG droplet, where the typical IJAG droplet size was estimated to be 50 microns in size. As the original endospore stock was $7{\cdot}2 \times 10^8$ CFU ml^{-1}, based upon volume, this equates to *c.* 47 CFU $droplet^{-1}$. In this way, it was estimated that the IJAG was depositing *c.* 200 000 droplets or $9{\cdot}4 \times 10^6$ CFU ml^{-1} onto each $10{\cdot}2 \times 10{\cdot}2$ cm sample surface (the computer monitor screen was marked off into 5 cm squares, and only 150 000 droplets were deposited).

If the estimated and actual number of endospores deposited were approximately the same, then we should have observed a high per cent recovery for our control samples. An average of 40·6% recovery was observed for our control plates in this study (TSA plates, four per day, based on 200 droplets), suggesting the actual number of endospores deposited was significantly lower than expected. Endospore adherence to the nozzle wall of the IJAG and endospores not adhering to the targeted surfaces are two likely reasons for this discrepancy. Endospores also adhere to porous and nonporous surfaces differently. While our per cent recovery numbers are low, the comparative study conducted here still indicates the best sampling tools for each surface.

This study evaluated the collection efficiency of a limited number of swabs and wipes and collection solutions. A standardized sampling protocol needs to be established and accepted for future sample collection.

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