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Recovery Efficiencies of Anthrax Spores and Ricin from Nonporous or Nonabsorbent and Porous or Absorbent Surfaces by a Variety of Sampling Methods*

ABSTRACT: The 2001 anthrax letter cases brought into focus the need to establish the most effective environmental sampling procedures. Results are presented from two studies aimed at establishing the best procedures for everyday surfaces likely to be contaminated after the release of environmentally stable bioaggressive agents, as exemplified by anthrax spores and ricin. With anthrax spores, contact plates, with mean retrieval rates of 28–54%, performed better than other methods by a wide margin for flat nonporous, nonabsorbent surfaces. They also proved best on flat porous, absorbent materials, although recoveries were low (<7%). For both agents, dry devices (swabs, wipes, Trace Evidence Collection Filters) had universally poor retrieval efficiencies with no significant differences between them. Among moistened devices (wipes, swabs, and Sample Collection and Recovery Devices), wipes were generally best, albeit with considerable cross-over among individual readings (highest mean recoveries for anthrax spores and ricin 5.5% and 2.5%, respectively, off plastic).

KEYWORDS: forensic science, bioforensic detection, bioterrorism, deliberate release, recovery efficiency, anthrax spore, ricin, surfaces

The topic of optimal bacteriological sampling techniques is as old as the microbiology of public health and hygiene. Conventional swabbing of surfaces, for example, dates back to at least 1917 (1). The 1950s saw a surge of interest in quantitative determination of bacterial contamination of surfaces, primarily as related to post-war improvements in the hygiene of food and dairy premises, processing equipment, preparation areas, utensils and containers, and even certain foods themselves, such as the skin of dressed poultry for predicting shelf life. Sampling methods were basically divisible into swabbing, rinsing, and agar contact. Angelotti et al. (2), reviewing the numerous investigations in the 1950s "*conducted to fill the need for simple, reliable bacteriological tests to determine quantitatively the sanitary quality of food contact surfaces*" which "*resulted in the development of various swabbing, rinsing and agar contact methods*," concluded that there was no one method available that was applicable to the diversified surfaces encountered in the business of monitoring food hygiene.

Rinse tests, where the contaminated surface is immersed in a sterile fluid and agitated to suspend contaminating micro-organisms, are not relevant to this paper. Under the topic of swabbing in these former studies, the relative efficiencies of cotton (absorbent), unmedicated ribbon gauze and calcium alginate wool swabs were compared. In theory, by dissolving in the suspension fluid after swabbing, alginate swabs would free all entrapped bacteria and therefore perform better than cotton swabs, and some publications did report higher recoveries with alginate swabs (3–5). However, others presented evidence that alginate swabs retrieved fewer organisms than cotton swabs (2,6) or that alginate was inhibitory to some microorganisms (7). Tredinnick and Tucker (4) found that gauze swabs performed more poorly than either cotton or alginate ones but Barnes (6) had more ambivalent comparative results. Nonabsorbent cotton swabs had very low recoveries (6).

Early contact agar methods took the form of agar slices delivered from a syringe (8) and a later modification (9) in which the syringe was replaced with sausage casings, the surface contact agar-paper strip (10) or its muslin equivalent (11), the direct surface agar plate method (12), and the RODAC (replicate organism direct agar contact) plate (13,14). All these were basically for sampling flat, smooth surfaces, although the muslin agar strip was designed to allow some flexibility on surfaces which were not perfectly flat.

As reviewed elsewhere (15,16), numerous other sampling methods were devised and suggested over time for examining specific surfaces ranging from hospital carpets to the surfaces of spacecraft. One study (17) found that, for the large surface areas of spacecraft, far greater efficiencies could be achieved by using small polyester-bonded clean room cloths than by conventional cotton swabs. More recently a cellulose sponge-based biological sampling kit (BiSKit) has been designed and demonstrated to have greatly enhanced recovery efficiencies for large surface areas as compared with traditional swabs (18).

The aftermath of the "anthrax letter" events in the last quarter of 2001 inevitably involved extensive environmental sampling of equipment and premises to assess levels of contamination, to supply forensic indicators of events that led to the contamination, and

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to determine the effectiveness of decontamination efforts. Questions arose as to the efficiency of detection using standard swabs and alternative sampling procedures, culminating in a call by the General Accounting Office (GAO) for validation of procedures chosen (19). That report (19) supplied some analyses of the differing results obtained with different sampling procedures in the numerous postal facilities tested in the months after the "anthrax letter" events. A more detailed analysis of the comparative isolations by swabs, wipes, and HEPA vacuum socks in one particular facility was supplied by Sanderson et al. (20). Efficiency of recovery of known numbers of contaminating *Bacillus anthracis* spores by swabs made of different materials (cotton, macrofoam, polyester, and rayon) from one surface type (steel coupons) was addressed by Rose et al. (16).

A number of information gaps that became apparent in the aftermath of the "anthrax letter" events have been filled by two studies reported here. These were carried out independently by separate operators. The aim of the first study was to expand the database for anthrax spores and ricin on recovery efficiencies of a number of methods from representative nonporous and porous surfaces. The second study dealt specifically with the retrieval efficiency of contact plates as compared with swabs for anthrax spores contaminating nonporous and porous surfaces. The motivation behind study 2 was the forensic need for rapid, quantitative detection of anthrax spores in hundreds of samples after a deliberate release event, with many of the samples also carrying substantial levels of natural environmental bacteria. Together, the results of the two studies contribute substantially to meeting the need for validating sampling methodology for environmentally stable agents of potential bioaggression.

Materials and Methods

Sampling Systems and Surfaces

In study 1, plastic (1 cm^2 plastic block), as representative of nonporous surfaces, and wood (untreated pine) and cotton cloth pieces, representing porous or absorbent surfaces, were contaminated in triplicate with 100 μL volumes of a suspension of *B. anthracis* spores at specific concentrations ranging from 10^3 to 10^6 cfu/mL. The surfaces were allowed to dry overnight in a biosafety cabinet. Retrieval of the spores was done with (i) polyester (Dacron©, Curtis Matheson Scientific, Houston, TX) swabs, either dry or premoistened with phosphate-buffered saline (PBS) containing either 0.1% Tween-20 (PBST) or 0.1% Triton-X, (ii) 2 inch by 2 inch gauze Kendall© wipes (The Kendall Company, Mansfield, MA), similarly either dry or premoistened with PBS and one or other of Tween-20 or Triton-X, (iii) Sample Collection and Recovery Devices (SCRD paddles—ASD BioSystems, Danville, VA) premoistened with PBST, and (iv) dry Trace Evidence Collection Filters (TECF—3M, Saint Paul, MN).

After being rolled over the contaminated surfaces, swabs were transferred to sterile 5 mL polypropylene tubes containing 1 mL of PBST. After use, SCRD paddles were transferred to 2 mL PBST in 50 mL conical tubes; Kendall wipes were transferred to 1 mL PBST in 50 mL conical tubes. TECFs were inserted into 50 mL conical tubes and 3 mL of PBST were added. In all cases, the tubes were vortexed for 30 sec and 200 μL spread over duplicate blood agar (sheep) plates (Remel Inc., Lenexa, KS). Colonies were counted after overnight incubation at 36 ± 1°C.

In study 2, concerned with comparing the merits of contact plates and premoistened cotton swabs for quantitating anthrax spore contamination on a range of surfaces, the surface under test was spotted in triplicate with ten 5 μL drops of spore suspension diluted to ±1 cfu/μL and allowed to dry. Retrieval was done by rolling premoistened swabs across each of the three contaminated sites and by application of contact plates with blood agar (BA), trimethoprim-sulfamethoxazole-polymyxin blood agar (TSPBA), or polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar to duplicate contaminated surfaces. Controls consisted of the same inocula in triplicate applied directly to BA plates. After the appropriate incubation period colonies were counted. As there were no significant differences in counts on the three types of media, the counts from all media were included in the comparisons of the two recovery systems here.

The surfaces used in study 2 were plastic (petri dishes), glass (microscope slides), tin plate metal sheet, and formica desk top, as representative of nonporous surfaces, and brick, synthetic cloth, as used for covers on notice boards and partitions between office areas, and synthetic office carpet representing porous or absorbent surfaces.

Strains and Spores

In the first study, concerned with direct comparison of recovery methods from three surface types, spores of the Sterne vaccine strain of *B. anthracis* were used. In the second study, principally aimed at refining the contact plate for quantitative retrieval of anthrax spores, spores from the Ames and Vollum strains, LSU 158, a bovine isolate from Zambia, and LSU 62, a bovine isolate from Hungary (via Poland), were used. Spores were prepared as described previously (21).

Ricin

In addition to assessing retrieval of anthrax spores in study 1, the effectiveness of the polyester swabs, SCRDs and Kendall wipes for collecting ricin, as representative of an environmentally stable toxin, was examined. One hundred microliter volumes of 1000, 100, 10, and 1 μg/mL solutions of ricin (ricin agglutinin II; Vector Laboratories, Burlingame, CA) were pipetted onto three areas of each material type and allowed to dry in the biosafety cabinet overnight. Transfer of the sampling devices to PBST was the same as for the anthrax spores, with 400 μL being taken for determination of recovered ricin. Determination was done by antigen capture enzyme-linked immunosorbent assay with an affinity purified goat anti-ricin polyclonal for capture and a mouse monoclonal specific to ricin as the detector. A standard curve generated with the commercial toxin was included with each assay. The limit of detection of this assay was in the order of 5 ng/mL (background + 3 × SD). All samples were run at three dilutions and calculation of antigen concentration based on an exponential association formula.

Results

The relative recovery efficiencies of anthrax spores from the different surfaces by the different methods are summarized in Table 1, Fig. 1 (study 1) and Table 2, Fig. 2 (study 2). While wide ranges in individual readings underscored the need for tests to be carried out at least in triplicate, the means of triplicates revealed clear trends which could be supported statistically (Tables 1–3).

Although there was no point of direct comparison between the two studies, recoveries with moist swabs (polyester in study 1 and cotton in study 2) from nonporous, nonabsorbent surfaces (plastic, glass, desktop formica, and metal) were not significantly different ($p = 0.11$), indicating generally equivalent performances

TABLE 1—*Recovery efficiencies (to two significant figures) for* Bacillus anthracis *Sterne strain spores and ricin, expressed as percent of known inocula, from three surface types by six sampling methods (study 1).*

		Dry Swab		Moist Swab		Dry Wipe		Moist Wipe		SCRD		TECF	
	Parameter	*B. anthracis*	Ricin	*B. anthracis*	Ricin	*B. anthracis*	Ricin	*B. anthracis*	Ricin	*B. anthracis*	Ricin	*B. anthracis*	Ricin
Surface	*n=	12	12	24	24	12	12	24	24	12	12	12	12
Plastic	Mean	2.3	1.0	5.5	2.5	0.9	0.6	6.6	2.5	4.1	2.2	1.0	0.5
(petri dish)	SD	0.7	0.5	2.3	1.5	0.6	0.4	4.0	1.7	2.4	1.2	0.9	0.4
	CV (%)	30	46	42	61	63	66	60	66	58	56	84	79
Wood	Mean	0.3	0.3	2.5	2.1	0.2	0.2	6.0	1.4	3.6	1.5	0.1	0.1
(untreated pine)	SD	0.1	0.2	1.6	1.9	0.2	0.1	3.5	1.5	2.2	0.9	0.1	0.1
	CV (%)	56	77	63	88	70	67	58	106	62	58	57	83
Cotton cloth	Mean	0.6	1.4	2.0	1.1	0.9	0.6	4.0	2.0	1.7	1.5	0.7	0.9
	SD	0.4	0.6	1.2	0.6	0.5	0.4	1.9	2.6	1.1	0.7	0.4	0.7
	CV (%)	71	45	61	50	55	56	46	127	61	45	56	81

SD, standard deviation; CV, coefficient of variation.
*n, number of tests included in the four inoculum levels. Each test result is the average of triplicate readings.

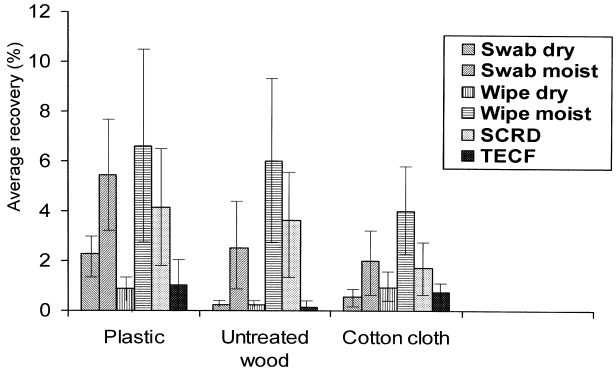

FIG. 1—*Recoveries of* Bacillus anthracis *spores for all inoculum sizes in study 1.*

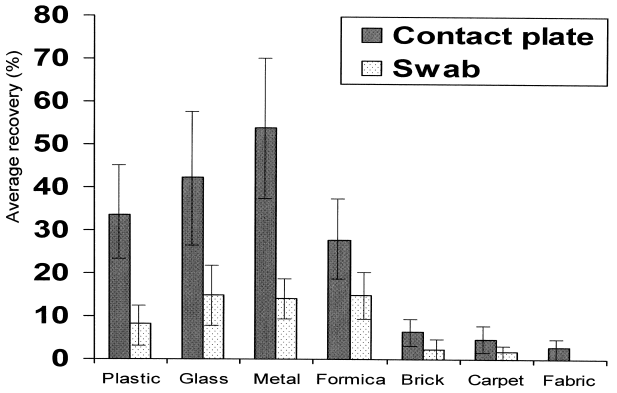

FIG. 2—*Comparison of mean recovery rates (% of original contamination) of anthrax spores by contact plates and premoistened swabs (study 2).*

TABLE 2—*Recovery efficiencies (percent of known inocula to nearest whole number) for spores of four strains of* Bacillus anthracis *from six surface types using contact plates and premoistened cotton swabs (study 2).*

	Parameter	Contact	Swabs
Surface	*No. of Tests	11	11
Plastic	Mean	34	8
	SD	11	4
	CV (%)	33	45
Glass	Mean	42	15
	SD	16	6
	CV (%)	37	39
Desktop formica	Mean	28	15
	SD	9	6
	CV (%)	32	37
Metal	Mean	54	14
	SD	16	4
	CV (%)	30	28
Carpet	Mean	5	2
	SD	2	2
	CV (%)	53	95
Brick	Mean	6	2
	SD	4	3
	CV (%)	61	144
†Synthetic cloth	Mean	3	0
	SD	3	-
	CV (%)	111	-

SD, standard deviation; CV, coefficient of variation.
*Each test result is the average of triplicate readings for four strains.
†Only done for one strain.

in the two studies. Based on this, contact plates, with mean retrieval rates of 27–54%, performed better than other methods by a wide margin for flat nonporous, nonabsorbent surfaces (Table 2). Although technically speaking contact plates performed better than other methods on porous, absorbent materials (carpet, brick, and synthetic cloth), actual recoveries by this method were low (<7%).

The degrees of significance of differences between the different recovery methods for both anthrax spores and ricin are summarized in Table 3. In study 1, dry devices (swab, wipe, TECF) had universally poor retrieval efficiencies with no significant differences between them. Among the moistened devices, apart from wipes versus swabs for detecting ricin, moist wipes were significantly more efficient than moist swabs and SCRDs and the latter two proved of similar efficiencies. There was, however, considerable cross-over when individual readings were viewed.

The comparison of surfaces in Table 4 highlights the significantly greater retrieval efficiencies from hard, nonporous surfaces (plastic, glass, metal) as compared with porous, absorbent surfaces (untreated wood, cloth, carpet, brick), with an element of reduced efficiency from formica in the hard, nonporous group. With contact plates, although retrieval rates from the porous surfaces (carpet, brick, cloth) were far lower than those from the nonporous surfaces (Table 2), in comparative terms, contact plates were still significantly more efficient than swabs for the porous surfaces ($p < 0.001$).

TABLE 3—*Comparisons of methods. Overall recoveries from all surface types (both studies).*

Method		*B. anthracis*					Ricin				
		More Effective		*Significance of Differences			More Effective		*Significance of Differences		
1	2	Method	†Factor	S/NS	No. of Paired Means	*p*-value	Method	†Factor	S/NS	No. of Paired Means	*p*-value
Tween	Triton	–	–	NS	24	0.814	–	–	NS	24	0.39
Dry swab	Moist swab	Moist swab	3.2	S	12	<0.001	Moist swab	2.4	S	12	0.003
Contact plates	Moist swab	Contact plates	3	S	66	<0.001	Not done	–	–	–	–
Dry swab	Dry wipe	–	–	NS	12	0.181	–	–	NS	12	0.06
Dry swab	TECF (dry)	–	–	NS	12	0.074	–	–	NS	12	0.16
Moist swab	Moist wipe	Moist wipe	1.75	S	24	<0.001	–	–	NS	12	0.88
Moist swab	SCRD (moist)	–	–	NS	11	0.789	–	–	NS	12	0.47
Dry wipe	Moist wipe	Moist wipe	4–7	S	12	<0.001	Moist wipe	4.9	S	12	0.01
Dry wipe	TECF (dry)	–	–	NS	12	0.350	–	–	NS	12	0.76
SCRD (moist)	TECF (dry)	SCRD	2–4	S	12	<0.001	SCRD	3.5	S	12	<0.001
Moist wipe	SCRD (moist)	Moist wipe	1.7	S	11	0.026	–	–	NS	12	0.56

*Student's *t*-test for comparison of means. S/NS, significant/not significant at the 95% confidence level.
†Factor by which the overall means differ.

TABLE 4—*Comparisons of surfaces.*

Study	Methods	Surface Type		*B. anthracis*			Ricin		
		1	2	Highest Recovery From	*Significance of Difference *p*-value	S/NS	Highest Recovery From	*Significance of Difference *p*-value	S/NS
1	Swab (dry and moist),	Plastic	Untreated wood	Plastic	0.03	S	N/A	0.056	NS
	wipe (dry and moist),	Plastic	Cotton cloth	Plastic	0.002	S	N/A	0.21	NS
	SCRD, TECF	Cotton cloth	Untreated wood	N/A	0.52	NS	N/A	0.54	NS
2	Cotton swab (moist),	Plastic	Glass	N/A	0.72	NS		–	–
	contact plate	Plastic	Metal	N/A	0.75	NS		–	–
		Plastic	Formica	N/A	0.20	NS		–	–
		Metal	Formica	Metal	0.04	S	Not done	–	–
		Plastic	Brick	Plastic	<0.001	S		–	–
		Plastic	Synthetic carpet	Plastic	<0.001	S		–	–
		Brick	Synthetic carpet	N/A	0.88	NS		–	–

N/A, not applicable.
*Student's *t*-test for comparison of means. S/NS, significant/not significant at the 95% confidence level.

TABLE 5—*Comparisons of inoculum size on recovery efficiency (study 1).*

B. anthracis				Ricin			
Inoculum (cfu)		*Significance of Difference		Inoculum (μg)		*Significance of Difference	
Level 1	Level 2	S/NS	*p*-value	Level 1	Level 2	S/NS	*p*-value
10^5	10^4	NS	0.53	100	10	S	0.003
10^5	10^3	NS	0.06	100	1	S	0.03
10^5	10^2	S	0.01	100	0.1	S	<0.001
10^4	10^3	NS	1.0	10	1	NS	0.13
10^4	10^2	NS	1.0	10	0.1	S	<0.001
10^3	10^2	NS	0.076	1	0.1	S	<0.001

*Student's *t*-test for comparison of means. S/NS, significant/not significant at the 95% confidence level.

An analysis of overall recoveries from all surface types by all methods (Table 5) indicated that the level of contamination on the surface only influenced the recovery efficiency to a minor extent in the case of anthrax spores, with statistical significance only becoming apparent with the most widely separated inoculum levels. With ricin, recovery efficiency was more influenced by the degree of surface contamination.

The highest recovery achieved for ricin was 2.5% of the original inoculum off plastic with moist wipes and moist polyester swabs but this was not significantly greater than the 2.2% with SCRDs (Table 1). As with anthrax spores, dry swabs, dry wipes, and TECFs (also dry) performed less well than the moist devices (Table 3) and, again as with spores, ricin recoveries off porous or absorbent surfaces (untreated wood, cloth) were less than off the nonporous, nonabsorbent (plastic) counterpart ($p = 0.025$). Coefficients of variation were consistently high.

Discussion

Where comparable, the recovery efficiencies of bacteria off nonporous, nonabsorbent surfaces found in the studies presented here are lower on the whole than those noted in other reports and the coefficients of variation (CV—an expression of the variation or spread about the average) are generally relatively high (Table 6). This is mostly attributable to the methods used to prepare the test surfaces or to determine the recovery rates. In two of the other studies, for example (3,4), known volumes of bacterial suspensions

TABLE 6—*Previous reports of recovery rates by various sampling procedures.*

Reference	Surface	Agent	Method	Recovery (%)	CV (%)
2	Nonporous china	*Bacillus globigii*	Cotton swab	30–44	25.4
			Alginate swab	11–26	60.6
			Agar syringe	33–51	28.5
			Surface rinse/agar	71–91	15.9
3	Dairy plant surfaces	Natural contaminants	Cotton swab	50–67	Not known
			Alginate swab	90–91	Not known
4	Dairy plant surfaces	Natural contaminants	Ribbon gauze	33	41
			Alginate swabs	86	19
6	Drinking glass	*Bacterium coli*	Nonabsorbent cotton swab	9.1	37
			Absorbent cotton swab	4–0–61	16–65
			Alginate swab	19–57	22–63
			Ribbon gauze	32	92
12	China coated with food matter	*Bacillus globigii*	DSAP	88–101	7.1
		Micrococcus pyogenes var. *aureus*		51–97	11.5
	Glazed porcelain	*Bacillus globigii*		91–97	Not known
	Unglazed porcelain			71–84	
	Painted wood			0	
	Unpainted wood			0	
	Plastic			90–96	
	Stainless steel			90–94	
	Plastic wrapping film			45–59	
	Paper picnic plates			4–9	
	Cotton fabric			2–4	
16*	Steel coupons	*Bacillus anthracis*	Cotton swab	24–63	35.0
			Macrofoam swab	30–64	25.4
			Polyester swab	4–17	38.3
			Rayon swab	1–24	68.7
17	Stainless steel spacecraft surfaces	Natural contaminants	Cotton swab	75.2	12.3
			Polyester cloth wipe	90.4	15.9
			Cellulose cloth wipe	72.0	47.6
20	U.S. postal facility after "anthrax letter" events	*Bacillus anthracis*	Dry swab	14	Not applicable
			Wet swab	54	
			Premoistened wipe	87	
			HEPA vacuum sock	80	
This study*	Nonporous surfaces (plastic, glass, metal, desktop formica)	*Bacillus anthracis*	Moist cotton swab	6.2–24	28–45
			Moist polyester swab	2–10	41
			Moist wipe	2–13	60
			SCRD	2–8	58
			Contact plates	12–76	30–37
	Porous surfaces/materials (brick, cotton cloth, synthetic carpet, untreated wood)		Moist cotton swab	0–12	95–144
			Moist polyester swab	0–6	60–63
			Moist wipe	1–8	46–58
			SCRD	0.5–4	61–62
			Contact plates	1.5–15	53–61

CV, coefficient of variation.
*Values listed are the best obtained with the conditions determined as optimal.

were simply added to swabs and a comparison made between direct plate counts of the suspensions and the counts obtained from plating out the swabs. Others (2,12) spread their inocula within water or milk onto their test surfaces and allowed the surfaces to dry for just 10 min at ambient temperature and relative humidity. Barnes (6) apparently did not attempt to dry her glasses after inoculation. The method used for spacecraft surfaces (17) is hard to decipher. The generally higher recoveries of Rose et al. (16) with cotton swabs than our own is probably attributable to the different inocula used (5×10^5 cfu in the tests of Rose et al. versus a range from 50 to 10^5 cfu per test surface in our studies here).

Previous data on bacterial recoveries from porous or absorbent surfaces are limited (12) but comparable with the results of the two studies reported here. It is unsurprising that recovery efficiencies from these types of material are low and the variability of recovery is very large (very high CVs).

Although recovery levels of ricin were low with the highest achieved being 2.5% of the original inoculum off plastic, the results show that it is possible to demonstrate the presence of this toxin on everyday surfaces in the event of a release.

It is hard to know how the inoculation procedures used are representative of the situations existing following a deliberate release event. Bacteria and spores dried onto surfaces are quite firmly attached by hydrophobic and possibly other forces and the degree of attachment varies greatly with the material of which the surface is made. Following the deliberate release event, the agents are likely to be resting "gently" on the surfaces with limited attachment forces and recoveries by any of the methods discussed might be expected to be higher than suggested by the results presented here. Nevertheless, most pertinent are the relative efficiencies and, at least for flat surfaces, particularly nonporous ones, the contact plate offered significant advantages in speed of test, ease of use and efficiency of recovery for anthrax spores. In other circumstances, for example, curved or uneven surfaces, premoistened wipes would, in general, be the choice for large flat areas where contact plates are not possible or feasible. At present there is no

ideal, nondestructive method for sampling porous or absorbent surfaces.

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