MICROBIOLOGICAL METHODS

Method Modification (2004.08) to Field Testing of Visible Powders on a Variety of Nonporous Environmental Surfaces: Field Study

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The RAMP® Anthrax Test Cartridge for detecting *Bacillus anthracis* was validated for use in the field for detection of *B. anthracis* spores in visible powder residues on 7 nonporous environmental surfaces. Six teams of trained first responders and civil support personnel in Class C personal protective equipment sampled visible powder residues on plastic, stainless steel, ceramic tile, wood, rubber, sealed concrete, and food-grade painted wood and analyzed the samples on the RAMP Anthrax Test System. The accuracy for each surface was at least 97% and the overall average was 98.8%. The overall average false-positive rate was 1.79% and false-negative rate was 1.07% for all surfaces. There were no significant differences between surfaces or between spore levels.

Bacillus anthracis is a large, Gram-positive, nonmotile, aerobic, spore-forming rod and is found in infected animals or infected animal products such as wool, undercooked meat, and soil associated with livestock (1). Anthrax is the disease caused by *B. anthracis*. It is possible to treat anthrax if it is diagnosed in the early stages of infection. The RAMP® Anthrax Test Cartridge is an immunochromatographic test strip intended for the screening of environmental samples for the presence of *B. anthracis* spores, the causative agent of anthrax disease. A positive test result indicates the presence of *B. anthracis* at or above the detection limit.

The RAMP Anthrax Test Cartridge was previously certified as an AOAC *Performance-Tested Method*SM (PTM; Certification No. 070403) and adopted as a First Action AOAC *Official Method*SM (**2004.08**) for use in the laboratory for detection of *B. anthracis* spores in bulk powders (2, 3). This report describes the results of a field validation study to extend the utility of the method for use in the field for detection of *B. anthracis* spores in visible powder residues on 7 environmental surfaces.

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The recommendation was approved by the Methods Committee on Microbiology and Extraneous Materials as Revised First Action. *See* "Official Methods Program Actions," (2006) *Inside Laboratory Management*, November/December issue.

Field Study

The field study was designed to demonstrate the reliability of the RAMP Anthrax Test Cartridge to detect *B. anthracis* spores in visible residual powders on a variety of nonporous surfaces using the draft ASTM-AOAC Standard, "*Standard Practice for Bulk Sample Collection and Swab Sample Collection of Visible Powders Suspected of Being Biological Agents from Nonporous Surfaces*" (4), and AOAC *Official Method*SM **2004.08** (2). Six teams comprised of civil support personnel (CSTs) and first responders participated as field collaborators in this study. Bruce Harper and colleagues in the Life Sciences Division of Dugway Proving Ground were designated as the "lead" laboratory and were responsible for the preparation and distribution of powder samples and monitoring of the study. The trained field collaborators were arranged into teams consisting of 3 members. Most teams elected to rotate responsibilities as Sampler, Facilitator, and RAMP Operator. One team determined that they would not change position in the field, so they did not rotate. The Sampler is the technician responsible for collecting the samples and the Facilitator is the technician supporting the Sampler in the hot zone by manipulating the sampling and collection materials, according to the draft ASTM Standard (4). The RAMP Operator is the technician responsible for processing the RAMP sample according to *Official Method*SM **2004.08** (2). Where necessary, a Dugway laboratory technician was assigned as a third team member and participated only as a Facilitator. In addition, each team was assigned a Dugway technician to act as Study Observer. The role of the Study Observer was to apply the powder samples to the surfaces and to make notes of observations or protocol deviations during the study.

All team members and Dugway personnel received training on the draft ASTM Standard, the RAMP Anthrax Test System, and safety. In addition, a limited trial run was performed during which collaborators could ask questions. The study was performed in trailers equipped as BSL-2 laboratories at Dugway Proving Grounds, with 2 teams per

trailer and a partition separating each of the 2 teams. RAMP testing was performed in a separate room from the sampling to simulate hot and warm zones in the field. All field collaborators and Study Monitors wore Class C personal protective equipment (PPE) and followed appropriate decontamination procedures.

Study Design

Two levels (1.0 and 0.01 g) of *B. anthracis* Sterne (BA) and *B. thuringiensis* Kurstaki (BT) in powder form were sampled from 7 nonporous surfaces: plastic, stainless steel, ceramic tile, wood, rubber, sealed concrete, and food-grade painted wood. Each team sampled ten 1.0 g BA powdered samples, ten 0.01 g BA powdered samples, two 1.0 g BT powdered samples, and two 0.01 g BT powdered samples on each of the 7 surfaces. Once the bulk powder and dry swab samples were collected according to the draft ASTM Standard Method A, the residual powder on each surface was sampled with a microbrush and analyzed according to the RAMP method ("Visible Powder" test sample preparation) as part of Method B of the draft ASTM Standard. In the case of an invalid RAMP result, the microbrush collection and RAMP analysis were repeated once. The study was carried out over the course of 1 1/2 weeks.

Sample Preparation

BA spore samples were grown in Leighton-Doi medium according to procedures outlined in the Life Sciences Division, Dugway Proving Ground Standard Operating Procedure-WDL-BIO-154. BT spore samples were pruchased from Certis USA, Columbia, MD. For each surface type, sixty 1.0 ± 0.05 g portions of BA spore powder were weighed out; twelve 1.0 ± 0.05 g portions of BT spore powder were weighed out; sixty 0.010–0.013 g portions of BA spore powder were weighed out; and twelve 0.010–0.013 g portions of BT spore powder were weighed out. All sample weights were recorded. The samples were randomized and coded for each team/surface combination.

Sample Collection and Analysis

On the day of analysis, each sample was applied to a 30 × 30 cm (12 × 12 in.) surface. The sample was applied to the surface by carefully spilling the sample onto the surface in a manner that minimizes aerosolization, at the same time spreading the sample across the surface as it is dropped, so that the sample is not in a pile. Each of the 6 analyst teams, wearing Class C PPE, collected twelve 1.0 g samples and twelve 0.01 g samples using a sterile plastic laminated card and a sterile swab and following the instructions in the draft AOAC-ASTM Sample Collection Standard by collecting the bulk sample first and then following the RAMP method instructions of collecting a sample using a microbrush. All samples were analyzed according to the RAMP method (**2004.08**; 2).

AOAC Official Method 2004.08
RAMP® Anthrax Test Cartridge

First Action 2004
Revised First Action 2006

(Intended for laboratory use for presumptive detection of *B. anthracis* spores in environmental samples and field use for presumptive detection of *B. anthracis* spores in visible powders on nonporous environmental surfaces. Not to be used for human clinical diagnostic purposes.)

Caution: The testing of samples for the presence of vegetative *B. anthracis* or *B. anthracis* spores should be carried out under appropriate and current containment and handling procedures as required by governmental policy and/or regulation.

A. Apparatus

Materials supplied in kit (Response Biomedical Corp., 8855 Northbrook Ct, Burnaby, BC, Canada V5J 5J1; Tel: +1-604-681-4101, Fax: +1-604-412-9830; www.responsebio.com):

(**a**) *TriContinent MiniPet™*.—70 μL.

(**b**) *Disposable powder sampling microbrushes*.—Twenty-five.

(**c**) *Lot card*.

(**d**) *Package insert*.

(**e**) *Marking pen*.

Materials provided by user:

(**a**) *Pipet*.—With disposable sterile tips; capable of pipeting 10 μL.

(**b**) *Reader*.—RAMP environmental reader.

(**c**) *Printer*.—Small 40 character serial printer (such as the Citizen iDP 3110) and accessories (recommended, but not required).

(**d**) *Personal computer*.—With RS-232 connector (recommended, but not required).

(**e**) *Biohazardous waste container*.

B. Reagents

Reagents supplied in kit (Response Biomedical Corp.):

(**a**) *RAMP anthrax test cartridges*.—Twenty-five.

(**b**) *Anthrax assay tips*.—Twenty-five; packaged with test cartridges.

(**c**) *Anthrax sample buffer vials*.—170 μL; twenty-five.

C. Preparation of Test Suspension for Field Use

Visible powders.—(*1*) Obtain anthrax sample buffer vial from kit. With lid on, hold lid of vial and quickly flick wrist in downward motion to ensure that no liquid is retained in the vial lid.

(*2*) Remove a dry microbrush from its container, holding it by the handle so as not to touch the sampling end.

(*3*) Lightly touch the dry tip of the microbrush to the surface of the powder and gently roll the microbrush, taking care not to pick up too much powder.

(*4*) Open vial, dip microbrush into buffer, and stir for 10 s. Avoid foaming the buffer.

(*5*) Pull microbrush from buffer and rotate against inside of vial to remove excess liquid.

(*6*) Discard microbrush as biohazardous waste.

D. Preparation of Test Suspension for Laboratory Use

Liquid suspension.—(*1*) Obtain anthrax sample buffer vial from kit. With lid on, hold lid of vial and quickly flick wrist in downward motion to ensure that no liquid is retained in the vial lid.

(*2*) Open sample buffer vial. Using a sterile pipet tip, remove 10 μL liquid suspension and transfer to sample buffer vial. Avoid foaming the buffer.

E. Determination

(**a**) *Reader set-up*.—(*1*) Turn on reader.

(*2*) If not previously done, remove lot card from pouch and insert lot card for the test cartridge lot being used into the lot card slot below the keypad on the reader. Once the lot card information has been uploaded, return lot card to its pouch. (*Caution*: Avoid touching the contacts at the end of the lot card.)

(*3*) Press [Enter] to select RUN TEST on the RAMP reader.

(*4*) Enter sample ID (user defined up to 20 alphanumeric characters), and if the user ID feature has been enabled, select or enter user ID.

For detailed information regarding the RAMP reader or lot card operation, refer to the RAMP environmental reader operator's manual.

(**b**) *Analysis procedure*.—(*1*) Open a kit pouch containing a test cartridge and assay tip. Place the test cartridge on a clean, dry, level surface.

(*2*) *Firmly* place the single use assay tip on the 70 μL TriContinent MiniPet. Check to confirm that there is a pink dot on the inside surface of the assay tip.

(*3*) If required, write the sample ID on the test cartridge with the marking pen provided.

(*4*) Fully depress the MiniPet plunger and insert the assay tip into the sample buffer vial containing the suspected agent specimen, close to the bottom of the vial (not touching).

(*5*) Holding the vial at eye level, gently release the plunger to fill the assay tip. Avoid pressing against the bottom of the vial, which may block the tip.

(*6*) Mix the sample by *slowly* pressing and releasing the plunger 10 times (2 s per cycle), taking care each time to eject all of the liquid into the vial and to draw only liquid and no air into the assay tip. This will prevent foaming.

(*7*) Check that the liquid is fully mixed by confirming that the pink dot is no longer visible on the inside of the assay tip.

(*8*) Fully depress and gently release the plunger to fill the assay tip with liquid (no foam).

(*9*) Position the filled assay tip directly over the sample well on the test cartridge and fully depress the plunger to dispense the entire liquid into the sample well. (Disregard any remaining droplet within the assay tip.) Remove and dispose of assay tip.

(*10*) Insert the test cartridge into the reader and then press until firm resistance is felt. *Note*: Do not try to hold onto or force the test cartridge into the reader once resistance is felt.

(*11*) The reader accepts the test cartridge and begins timing the test development process. Within approximately 15 min, when the test is complete, the reader will scan the test cartridge, perform data analysis, and report the result from the RAMP anthrax test cartridge on the LCD display.

(*12*) Remove the used test cartridge from the reader when prompted to do so by the reader LCD display. Dispose of the test cartridge and sample vial.

References: *J. AOAC Int*. **88**, 202(2005); **89**, 1622(2006).

Results

A summary of the results of the field study is shown in Table 1. Overall, the method was >98% accurate. Out of 1008 samples, there were 8 incidents of errors or invalid results that gave the correct result upon repeat sampling and analysis. Of 840 BA samples, there were a total of 9 false-negative results (1.07%), 3 at the high level and 6 at the low level. Of 168 BT samples, there were 3 false-positive results (1.79%). A total of 14 analysts among the 6 teams performed the RAMP analyses. Six analysts accounted for all of the false-positive and -negative results. The raw data sorted by analyst and surface and sample types are shown in Table 2.

It was proposed to analyze the RAMP results for effects of analyst, surface type, inoculum level, and interactions between these parameters. The standard statistical model proposed in this evaluation was:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4z_1 + b_5z_2 + b_6z_3 + b_7z_4 + e$$

where x_1 = analyst effect (random effect). x_2 = 0, If low inoculum (0.01 g); 1, if high inoculum (1.00 g). x_3 = Surface type: –3, If plastic; –2, if steel; –1, if tile; 0, if wood; 1, if rubber; 2, if concrete; 3, if food-grade wood. $z_1 = x_1x_2$ = Interaction (analyst/spore level). $z_2 = x_1x_3$ = Interaction (analyst/surface type). $z_3 = x_2x_3$ = Interaction (spore

Table 1. Summary of collaborative study data

All surfaces	Correct analyses	Total analyses	Correct analyses, %
B. anthracis Sterne (1.0 g)	417	420	99.29
B. anthracis Sterne (0.01 g)	414	420	98.57
B. thuringiensis Kurstaki (1.0 and 0.01 g)	165	168	98.21
Overall correct	996	1008	98.81

Table 2. Field study data by analyst and sample and surface types

			Fraction correct answer (correct results/No. of analyses)				
Surface	Team	RAMP analyst	BA 1.0 g[a]	BA 0.01 g	BT 1.0 g[b]	BT 0.01 g	Total
Plastic	1	A	5/5	3/3	1/1	NA[c]	9/9
		B	3/5[d]	5/7[d]	1/1	2/2	11/15[d]
	2	C	4/4	2/2	2/2	1/1	9/9
		D	6/6	8/8	NA	1/1	15/15
	3	E	6/6	6/6	2/2	1/1	15/15
		F	4/4	4/4	NA	1/1	9/9
		G	NA	NA	NA	NA	—
	4	H	4/4	4/4	1/1	NA	9/9
		I	6/6	6/6	1/1	2/2	15/15
		J	NA	NA	NA	NA	—
	5	K	10/10	10/10	2/2	2/2	24/24
	6	L	10/10	10/10	2/2	2/2	24/24
		M	NA	NA	NA	NA	—
		N	NA	NA	NA	NA	—
Total			58/60[d]	58/60[d]	12/12	12/12	140/144[d]
Stainless steel	1	A	7/7	4/4	1/1	NA	12/12
		B	3/3	6/6	1/1	2/2	12/12
	2	C	1/1	3/3	1/1	1/1	6/6
		D	9/9	7/7	1/1	1/1	18/18
	3	E	NA	NA	NA	NA	—
		F	8/8	6/6	1/1	1/1	16/16
		G	2/2	4/4	1/1	1/1	8/8
	4	H	6/6	2/2	1/1	1/1	10/10
		I	NA	NA	NA	NA	—
		J	4/4	7/8[d]	1/1	1/1	13/14[d]
	5	K	10/10	10/10	2/2	1/2[d]	23/24[d]
	6	L	4/4	2/2	2/2	2/2	10/10
		M	6/6	8/8	NA	NA	14/14
		N	NA	NA	NA	NA	—
Total			60/60	59/60[d]	12/12	11/12[d]	142/144[d]
Rubber	1	A	9/9	6/6	1/1	2/2	18/18
		B	1/1	4/4	1/1	NA	6/6
	2	C	4/4	5/5	1/1	2/2	12/12
		D	6/6	5/5	1/1	NA	12/12
	3	E	NA	NA	NA	NA	—
		F	10/10	10/10	2/2	2/2	24/24
		G	NA	NA	NA	NA	—
	4	H	NA	NA	NA	NA	—
		I	5/5	4/4	1/1	1/1	11/11
		J	5/5	6/6	1/1	1/1	13/13
	5	K	9/10[d]	10/10	2/2	2/2	23/24[d]
	6	L	NA	NA	NA	NA	—
		M	8/8	7/7	2/2	1/1	18/18
		N	2/2	3/3	NA	1/1	6/6
Total			59/60[d]	60/60	12/12	12/12	143/144[d]

Table 2. (*continued*)

Surface	Team	RAMP analyst	Fraction correct answer (correct results/No. of analyses)				Total
			BA 1.0 g[a]	BA 0.01 g	BT 1.0 g[b]	BT 0.01 g	
Sealed concrete	1	A	6/6	7/7	2/2	1/1	16/16
		B	4/4	3/3	NA	1/1	8/8
	2	C	10/10	10/10	1/2[d]	2/2	23/24[d]
		D	NA	NA	NA	NA	—
	3	E	NA	NA	NA	NA	—
		F	4/4	2/2	1/1	1/1	8/8
		G	6/6	8/8	1/1	0/1[d]	15/16[d]
	4	H	4/4	4/4	NA	NA	8/8
		I	6/6	6/6	2/2	2/2	16/16
		J	NA	NA	NA	NA	—
	5	K	10/10	10/10	2/2	2/2	24/24
	6	L	10/10	10/10	2/2	2/2	24/24
		M	NA	NA	NA	NA	—
		N	NA	NA	NA	NA	—
Total			60/60	60/60	11/12[d]	11/12[d]	142/144[d]
Ceramic tile	1	A	4/4	2/2	1/1	1/1	8/8
		B	6/6	8/8	1/1	1/1	16/16
	2	C	8/8	7/7	1/1	2/2	18/18
		D	2/2	3/3	1/1	NA	6/6
	3	E	10/10	8/10[d]	2/2	2/2	22/24[d]
		F	NA	NA	NA	NA	—
		G	NA	NA	NA	NA	—
	4	H	7/7	8/8	2/2	1/1	18/18
		I	NA	NA	NA	NA	—
		J	3/3	2/2	NA	1/1	6/6
	5	K	10/10	9/10[d]	2/2	2/2	23/24[d]
	6	L	NA	NA	NA	NA	—
		M	NA	NA	NA	NA	—
		N	10/10	10/10	2/2	2/2	24/24
Total			60/60	57/60[d]	12/12	12/12	141/144[d]
Wood	1	A	10/10	10/10	2/2	2/2	24/24
		B	NA	NA	NA	NA	—
	2	C	NA	NA	NA	NA	—
		D	10/10	10/10	2/2	2/2	24/24
	3	E	NA	NA	NA	NA	—
		F	10/10	7/7	2/2	1/1	20/20
		G	NA	3/3	NA	1/1	4/4
	4	H	7/7	6/6	NA	2/2	15/15
		I	NA	NA	NA	NA	—
		J	3/3	4/4	2/2	NA	9/9
	5	K	10/10	10/10	2/2	2/2	24/24
	6	L	NA	NA	NA	NA	—
		M	10/10	10/10	2/2	2/2	24/24
		N	NA	NA	NA	NA	—
Total			60/60	60/60	12/12	12/12	144/144

Table 2. (*continued*)

Surface	Team	RAMP analyst	Fraction correct answer (correct results/No. of analyses)				Total
			BA 1.0 g[a]	BA 0.01 g	BT 1.0 g[b]	BT 0.01 g	
Food-grade painted wood	1	A	4/4	4/4	2/2	2/2	12/12
		B	6/6	6/6	NA	NA	12/12
	2	C	2/2	3/3	NA	1/1	6/6
		D	8/8	7/7	2/2	1/1	18/18
	3	E	9/9	8/8	2/2	1/1	20/20
		F	1/1	2/2	NA	1/1	4/4
		G	NA	NA	NA	NA	—
	4	H	NA	NA	NA	NA	—
		I	2/2	2/2	NA	NA	4/4
		J	8/8	8/8	2/2	2/2	20/20
	5	K	10/10	10/10	2/2	2/2	24/24
	6	L	8/8	6/6	NA	2/2	16/16
		M	NA	NA	NA	NA	—
		N	2/2	4/4	2/2	NA	8/8
Total			60/60	60/60	12/12	12/12	144/144
Overall total			417/420	414/420	83/84	82/84	996/1008

[a] BA = *Bacillus anthracis* Sterne strain.
[b] BT = *Bacillus thuringiensis* Kurstaki strain.
[c] NA = None analyzed.
[d] Presence of false-positive or -negative results in that sample set.

level/surface type). $z_4 = x_1x_2x_3$ = Interaction (spore level/surface type/analyst).

Detection of the low level, 0.01 g, vs the high level, 1.0 g, was not significantly different for either BA or BT spores. Hence, there is no need for variables x_2, z_1, z_3, or z_4. The technicians were not isolated by test group (team), so no analyst effect could be measured statistically. Hence, variable x_1 drops out of the equation, as well as z_2, so the full model is $y = b_0 + x_3$.

Because there are so few negative readings and so many positive ones, converting the data to proportions or percentage values would be less reliable than would a χ^2 test. Therefore, the hypotheses are:

H0: The surface types cause no difference in detection.

HA: At least one surface type differs from the others.

Set $\alpha = 0.05$.

$$\chi^2 = \frac{1}{\overline{p}\overline{q}} \sum_{i=1}^{m} n_i \cdot (p_i - \overline{p})^2$$

where n_i = sample size of the ith surface type; $\overline{p}$ = average proportion of correct identifications from all surface types; p_i = correct identification from the ith surface type; $\overline{q} = 1 - \overline{p}$ = average proportion of incorrect identifications of spores from all surface types.

Low-level BA

$\chi^2 = 10.47$, not significant at $p > 0.10$

There is no significant difference between the 7 surfaces at the low level of BA.

High-level BA

$\chi^2 = 8.73$, not significant at $p > 0.10$

There is no significant difference between the 7 surfaces at the high level of BA.

Low-level BT

$\chi^2 = 5.12$, not significant at $p > 0.10$

There is no significant difference between the 7 surfaces at the low level of BT.

High-level BA

$\chi^2 = 6.07$, not significant at $p > 0.10$

There is no significant difference between the 7 surfaces at the high level of BT.

All levels BA and BT

$\chi^2 = 7.89$, not significant at $p > 0.10$

There are no significant differences between spore detection and correct identifications among low- and high-level spores or surface types.

Reliability

The probability of obtaining a false negative out of 100 tests is:

$$\text{Correct proportion} = 0.9881 = e^{-\lambda(\text{No. of tests})} = e^{-\lambda(100)}$$
$$-\lambda(100) = -0.01076$$

Hence, 0.0001 = λ, or 1 in 10 000.

Discussion

The data demonstrate that the RAMP Anthrax Test Cartridge accurately and precisely detected BA spores in visible residual powders on nonporous surfaces and that the method can be performed well by trained technicians in Class C PPE. The false-positive and -negative rates were very low (1.79 and 1.07%, respectively).

Finally, although the specific analyst effect could not be isolated, clearly there appears to be an indication that it is important. For example, one analyst had 4 false negatives in 20 samples. Given that the probability of a false negative for any analyst in 20 samples is 0.0006, 4 false-negative results would have a probability of 1.28×10^{-13}, which indicates that the technique was faulty, not the device. A second analyst had 2 false negatives out of a sample size of 10, again indicating faulty technique. It can not be identified whether the faulty technique is related to sampling of the surface or performance of the RAMP test. In either case, the results emphasize the importance of proper training.

Recommendations

It is recommended that Method **2004.08** be modified to include field use for detection of *B. anthracis* spores in visible powder residues on a variety of nonporous environmental surfaces.

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Aaron Decker and Jeremy McLean, 101st WMD CST, Boise, ID

Andrew Lesson, Kevin Merrill, Tauseef Badar, and Gary Hoffman, Chemical Biological Incident Response Force, U.S. Navy, Indian Head, MD

Dan Bartle, Mark Hall, Michael Piland, and Malcolm Trigg, Florida HazMat, Orlando, FL

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(4) ASTM Draft (2006) "Standard Practice for Bulk Sample Collection and Swab Sample Collection of Visible Powders Suspected of Being Biological Agents from Nonporous Surfaces," ASTM, West Conshohocken, PA