SUPPLEMENTAL INFORMATION FOR "EVALUATION OF PCR SYSTEMS FOR FIELD SCREENING OF *BACILLUS ANTHRACIS*"

Inclusivity and Exclusivity DNA and Suspicious Powder Panels for PCR System Testing

The AOAC SMPR 2010.003, "Standard Method Performance Requirements for Polymerase Chain Reaction (PCR) Methods for Detection of *Bacillus anthracis* in Aerosol Collection Filters and/or Liquids"[1] guided the use of inclusivity and exclusivity *Bacillus* strains for DNA-based testing. Our inclusivity DNA panel (Table S1) does not include BA02 or BA14 because these 2 strains lack the pXO2 plasmid, which is the target for the PCR assays that we tested. Our exclusivity DNA panel (Table S2) does not include BANN13 or BANN14 because these strains contain a plasmid homologous to pXO2, which would generate an expected misleading positive result for pXO2. Suspicious powders used for testing are listed in Table S3; these were selected based on AOAC SMPR 2010.004[2] and interviews with first responders.

Limited Testing for Detection of Ricin Toxin

We evaluated the performance of 5 hand-held PCR instruments for detection of *Bacillus anthracis*, as described in the main body of the paper. Four of these instruments also had commercially available assays for detecting ricin, a protein toxin produced in the seeds of the castor plant *Ricinus communis*.[3] The PCR assays for "ricin" are actually assays to detect *R. communis* DNA. In addition to the statistically based testing of *B. anthracis* described in the main body of the paper, we performed limited testing of ricin as follows.

Instruments and Assays

The 4 instruments and assays tested were:

- FilmArray®: Biothreat pouch
- RAZOR® EX: 10-threat pouch
- T-COR 4™ : Ricin assay
- POCKIT™: Ricin assay

Assays were performed according to the manufacturer's instructions. See the main body of the paper for brief descriptions of the instruments.

Samples

Pure ricin toxin was obtained from Vector Laboratories (Burlingame, CA). Three crude ricin preparations were extracted from castor seeds and prepared at PNNL as described by Wunschel et al:[4] (1) crushing castor seeds to produce a mash (CM), (2) extracting crushed castor seeds with acetone to remove oil (AE), and (3) precipitating protein from crushed castor seeds with acetone (AP). Following are very brief summaries of each of the ricin preparation methods.

Table S1. *Ba* inclusivity panel, with strain numbers taken from the AOAC inclusivity panel (SMPR 2010.003)[18]

Strain Number	*Genotype*	*Strain*	*Characteristics*
BA1	7	Canadian bison	pXO1+, pXO2+, VNTR genotype group A1a
BA3	29	PAK-1	pXO1+, pXO2+, VNTR genotype group A2
BA4	51	BA1015	pXO1+, pXO2+, VNTR genotype group A3a
BA5	62	Ames	pXO1+, pXO2+, VNTR genotype group A3b
BA6	67	K3	pXO1+, pXO2+, VNTR genotype group A3c
BA7	68	Ohio ACB	pXO1+, pXO2+, VNTR genotype group A3d
BA8	69	SK-102 (Pakistan)	pXO1+, pXO2+, VNTR genotype group A4
BA9	77	Vollum 1B	pXO1+, pXO2+, VNTR genotype group A4
BA10	82	BA1035	pXO1+, pXO2+, VNTR genotype group B1
BA11	80	RA3	pXO1+, pXO2+, VNTR genotype group B2
BA12	NA	2002013094 (240)	pXO1+, pXO2+, VNTR genotype group C
BA13	8	Pasteur	pXO1-, pXO2+; VNTR genotype group A1a
BA15	23	Turkey #32	pXO1+, pXO2+, VNTR genotype group A1b

Table S2. *Ba* exclusivity panel, with strain numbers taken from the AOAC exclusivity panel (SMPR 2010.003)[17]

Number	*Species*	*Strain*	*Characteristics*	*Source*
BANN1	*B. cereus*	S2-8	pXO1-, pXO2-	LANL
BANN2	*B. cereus*	3A	pXO1-, pXO2-	CRP
BANN3	*B. thuringiensis*	HD1011	pXO1-, pXO2-	LANL
BANN4	*B. thuringiensis*	97-27	pXO1-, pXO2-	BEI
BANN5	*B. thuringiensis*	HD682	pXO1-, pXO2-	CRP
BANN6	*B. cereus*	E33L	pXO1-, pXO2-	BEI
BANN7	*B. cereus*	D17	pXO1-, pXO2-	LANL
BANN8	*B. thuringiensis*	HD571	pXO1-, pXO2-	LANL
BANN9	*B. thuringiensis*	Al Hakam	pXO1-, pXO2-	CRP
BANN10	*B. cereus*	ATCC 4342	pXO1-, pXO2-	CRP
BANN11	*B. cereus*	FM1	pXO1-, pXO2-	LANL
BANN12	*B. cereus*	G9241	pBCXO1+[a], pXO2-	BEI
BANN15	*B. thuringiensis*	subsp. *Israelensis,* HD 1002	pXO1-, pXO2-	LANL
BANN16	*B. thuringiensis*	subsp. *Kurstaki,* HD 1	pXO1-, pXO2-	LANL
BANN17	*B. thuringiensis*	subsp. *Morrisoni,* HD 600	pXO1-, pXO2-	LANL
BANN18	*B. coagulans*	ATCC 7050	pXO1-, pXO2-	ATCC
BANN19	*B. mycoides*	ATCC 6462	pXO1-, pXO2-	ATCC
BANN20	*B. megaterium*	ATCC 14581	pXO1-, pXO2-	ATCC

[a]Contains a plasmid homologous to the pXO1 plasmid found in *Ba*.

Table S3. Commonly encountered suspicious powders tested with and without *Ba* Ames spores

Powder Type	*Powder Brand*	*Powder Type*	*Powder Brand*
Brewer's yeast powder	Swanson Premium	Toothpaste powder with fluoride	The Country Gent
Dipel dust	Green Light	Baking powder (aluminum free)	Rumford
Milk powder	Peak	Antacid (calcium carbonate)	TUMS Ultrastrength 1000
Infant formula	Enfamil Gentlease	Baking soda	Arm & Hammer
White flour	Bob's Red Mill Organic	Epsom salt	Target
Coffee creamer (nondairy)	Coffeemate Original	Gym chalk (magnesium carbonate)	No brand name
Instant pectin	Real Fruit Pectin	Borax	20 Mule Team
Acetaminophen	Simply Right Healthcare Extra Strength	Talc	Pinaud Clubman
Powdered sugar	Domino	Road dust	ISO 12101-1, A1 Ultrafine
Cornstarch	Argo	Kaolin	Making Cosmetics
Polyethylene glycol 3350 (PEG)	MiraLAX	Popcorn salt	Diamond Crystal

Table S4. Limited testing of pure ricin and crude ricin preparations using *Ricinus communis* DNA assays for each PCR system. Data are reported as number of positive results/total tests performed.

Sample	*FilmArray® BioThreat Panel*	*RAZOR® EX Ten™ Target Screen*	*T-COR 4™ Ricin assay*	*POCKIT™ Ricin assay*
Pure ricin (0.1 μg/mL)	0/3	0/3	0/3	0/3
Castor seed acetone protein precipitate (AP) (0.1 mg/mL)	3/3	1/3	5/5	5/5
Castor seed acetone extract (AE) (0.1 mg/mL)	3/3	2/2N	3/6	5/5
Crushed castor seeds (CM) (0.1 mg/mL)	3/3	3/3	5/6	5/5

N = no results reported by the instrument for the third sample.

(1) CM: Soak seed in NaOH, rinse in cold water, then air dry and peel the seed hull. Crush the peeled seeds with a mortar and pestle.
(2) AE: Prepare crushed seeds as for CM, then cover with acetone in a flask, and stir for 72h. Filter through a double layer of filter paper, dry, and scrape the dried material into a vial.
(3) AP: Prepare 5 grams of castor seeds as for CM but add 10 mL phosphate-buffered saline when grinding with a pestle. Centrifuge the resulting slurry at 10,000 x *g* for 20 min at 4°C, which results in 3 layers. Recover the middle (aqueous) layer. Add acetone to approximately 6 times the aqueous volume and freeze overnight. Centrifuge the next day at 10,000 x *g* for 20 min, decant the acetone, and air-dry the precipitate.

We tested each of the instruments 3 times with pure ricin and 3 to 6 times with the 3 crude castor seed preparations (CM, AE, and AP). Pure ricin was tested at 0.1 μg/mL. The castor seed preparations were tested at 0.1 mg/mL. Results are shown in Table S4.

Ricin Assays: Pure Toxin and Crude Preparations

None of the instruments detected the pure ricin preparation, which was expected since ricin is a protein toxin and the "ricin" PCR assays are designed to detect *R. communis* DNA.

Both the FilmArray® and the POCKIT™ returned positive results in all replicates with the 3 different castor seed preparations, demonstrating that all 3 preparations contained *R. communis* DNA. The RAZOR® EX and the T-COR 4™ returned some false-negative results, and 1 of the RAZOR assays did not go to completion.

Crude ricin preparation instructions, particularly the CM and AE methods, can be readily found in the so-called "anarchist literature" (eg, refs 5-8). Therefore, it is important that first responders be able to identify the presence of ricin in such preparations. Although PCR, which detects specific DNA sequences (here, DNA from the *R. communis* plant), is not a direct test for the presence of the protein ricin, these results demonstrate that PCR can be used to detect crude ricin preparations.

References

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4. Wunschel DS, Melville AM, Ehrhardt CJ, et al. Integration of gas chromatography mass spectrometry methods for differentiating ricin preparation methods. *Analyst* 2012;137(9): 2077-2085.
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