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Comprehensive Laboratory Evaluation of a Highly Specific Lateral Flow Assay for the Presumptive Identification of Ricin in Suspicious White Powders and Environmental Samples

David R. Hodge, Kristin Willner Prentice, Jason G. Ramage, Samantha Prezioso, Cheryl Gauthier, Tanya Swanson, Rebecca Hastings, Uma Basavanna, Shomik Datta, Shashi K. Sharma, Eric A. E. Garber, Andrea Staab, Denise Pettit, Rahsaan Drumgoole, Erin Swaney, Peter L. Estacio, Ian A. Elder, Gerald Kovacs, Brenda S. Morse, Richard B. Kellogg, Larry Stanker, Stephen A. Morse, and Segaran P. Pillai

Ricin, a heterodimeric toxin that is present in the seeds of the *Ricinus communis* plant, is the biothreat agent most frequently encountered by law enforcement agencies in the United States. Even in untrained hands, the easily obtainable seeds can yield a highly toxic product that has been used in various types of threats, including "white-powder" letters. Although the vast majority of these threats are hoaxes, an impediment to accurate hazard assessments by first responders is the unreliability of rapid detection assays for ricin, such as lateral flow assays (LFAs). One of the complicating factors associated with LFAs is the incorporation of antibodies of poor specificity that cross-react with near-neighbors or with

David R. Hodge, PhD, is Program Manager, Research and Development; Kristin Willner Prentice is a Support Contractor, Booz Allen Hamilton, Inc.; Jason G. Ramage, MS, MBA, is Staff Scientist; and Segaran P. Pillai, PhD, is Chief Medical and Science Advisor; all in the Chemical and Biological Defense Division, Science and Technology Directorate, US Department of Homeland Security, Washington, DC. Samantha Prezioso is a Microbiologist, Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia. Cheryl Gauthier, MA, is Director; Tanya Swanson is Supervisor; and Rebecca Hastings is a Bacteriologist; all at the Bioterrorism Response Laboratory, Massachusetts Department of Public Health, State Laboratory Institute, Jamaica Plain, Massachusetts. Uma Basavanna, PhD, is Select Agent Manager (Microbiologist), USAMRIID, Fort Detrick, Maryland. Shomik Datta, PhD, is a contractor; Shashi K. Sharma, PhD, is Research Microbiologist; and Eric A. E. Garber, PhD, is Research Chemist; all in the Office of Regulatory Science, Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland. Andrea Staab, PhD, and Denise Pettit, PhD, were, at the time this article was written, with the Virginia Division of Consolidated Laboratory Services, Richmond. Dr. Staab is currently a Microbiologist, CBR Concepts and Experimentation Branch, Naval Surface Warfare Center, Dahlgren Division, Dahlgren, Virginia. Dr. Pettit is Assistant Director, North Carolina State Laboratory of Public Health, NC Department of Health and Human Services, Raleigh. Rahsaan Drumgoole, MPH, is Emergency Preparedness Branch Manager, and Erin Swaney is Bioterrorism Coordinator, both at the Texas Department of State Health Services, Emergency Preparedness Branch, Austin, Texas. Peter L. Estacio, PhD, MD, MPH, is Chief Medical Scientist and Lead Team Physician, Environmental Health & Quality Assurance, Lawrence Livermore National Laboratory, Livermore, California. Ian A. Elder, PhD, is a Chemist, US Department of Homeland Security, Washington, DC. Gerald Kovacs, PhD, was, at the time this article was written, CBRN Director at BARDA. Brenda S. Morse, PhD, is on the LRN Technical Review Committee, and Richard B. Kellogg, MPH, is LRN Liaison, both in the Laboratory Preparedness and Response Branch, Division of Preparedness and Emerging Infections, National Center for Emerging and Zoonotic Infectious Diseases, CDC, Atlanta. Larry Stanker, PhD, is Acting Research Leader, Foodborne Toxin Detection and Prevention Research Unit, USDA Agricultural Research Service, Albany, California. Stephen A. Morse, PhD, is Associate Director for Environmental Microbiology, National Center for Emerging and Zoonotic Infectious Diseases, CDC, Atlanta.

plant lectins that are capable of nonspecifically cross-linking the capture and detector antibodies. Because of the compelling and critical need to promote the interests of public safety and public health, the Department of Homeland Security conducted a comprehensive laboratory evaluation study of a commercial LFA for the rapid detection of ricin. This study was conducted using comprehensive inclusivity and exclusivity panels of ricin and near-neighbor plant materials, along with panels of lectins and "white-powders," to determine the specificity, sensitivity, limits of detection, dynamic range, and repeatability of the assay for the specific intended use of evaluating suspicious white powders and environmental samples in the field.

Ricin (*Ricinus communis* Agglutinin II, RCA-II, RCA_{60}, or ricin D) is a protein found primarily in the endosperm of the seed of the castor plant (*R. communis),*[1] where its concentration among cultivars has been shown to vary from 1.9 to 16 g/kg of seed.[2] It is one of the most toxic and easily produced plant toxins known to man. Ricin is a Type II ribosome-inactivating protein (Type II RIP) belonging to the A-B family of toxins (dimeric) and consisting of 2 functionally different subunits—a 267 amino acid, 32 kDa A chain, and a 262 amino acid, 32 kDa B chain—linked by a single disulfide bond.[3] The B chain is a lectin that binds to galactose-containing glycolipids and glycoproteins present on the surface of cells, facilitating the entry of ricin into the cytosol.[4] The A chain is an *N*-glycosidase that inhibits protein synthesis by irreversibly inactivating eukaryotic ribosomes through removal of a single adenine residue from the 28S RNA loop contained within the 60S subunit.[5] This interaction prevents chain elongation of polypeptides and leads to cell death. One molecule of ricin ($K_{CAT} = 1{,}500\ min^{-1}$) is sufficient to inactivate 1,500 ribosomes/minute, leading to rapid inhibition of protein synthesis and cell death.[6,7]

In addition to its ribosome-inactivating activity, ricin also has lipase activity that is targeted to glycerophospholipids and triglycerides.[8] Ricin is highly toxic through a variety of exposure routes. In humans, the LD_{50} is estimated to be 5 to 10 μg/kg body weight by inhalation and 1 to 20 mg/kg body weight (ca. 3 to 6 seeds) by ingestion.[9,10] A dose-dependent latent period after poisoning limits prompt diagnosis of ricin exposure, and irreversible toxin internalization renders postexposure therapy problematic.[11]

Castor seeds also contain a closely related lectin termed *R. communis* agglutinin I (RCA-I or RCA_{120}), which is also a Type II RIP but is about 1,000 times less toxic than RCA_{60}.[4,12] RCA_{120} causes agglutination and lysis of mammalian red blood cells.[4,13] It is a tetramer consisting of 2 ricinlike heterodimers held together by noncovalent forces.[4] Each heterodimer consists of an A chain (32 kDa) linked to a galactose-binding B chain (37kDa), both of which are similar in structure to the A and B chains of RCA_{60}. The homologies between the A chains and B chains of RCA_{120} and RCA_{60} are 90% and 84%, respectively.[14] Because of this homology, RCA_{120} cross-reacts with nearly all antibodies reactive to RCA_{60}.[13] RCA_{120} and RCA_{60} are present in approximately equal concentrations in the seeds of *R. communis* cultivar Hale.[13] However, these proteins are products of distinct genes; it has been suggested that the ricin gene evolved first and then duplicated to give rise to the agglutinin gene.[15]

There are 2 variants of ricin that are referred to as ricin D and ricin E.[16] Several studies have suggested that large seed cultivars of *R. communis* contain ricin D and RCA_{120}, while small seed cultivars contain ricin D, ricin E, and RCA_{120}.[17,18] Ricin D and ricin E have been shown to differ in amino acid sequence, isoelectric point, affinity to Sepharose 4B, and cytotoxicity to cultured cells.[15,17] The A chains of ricin D and E are similar. However, ricin E is a hybrid protein in which the B chain of ricin E is composed of the N-terminal half of ricin D and the C-terminal half of the B chain of RCA_{120}.[16] The difference in toxicity may reflect reduced binding of saccharides to the high-affinity site in the B chain of ricin E.[19] It has been proposed that the gene encoding ricin E arose by recombination between the ricin D and RCA_{120} genes.[20]

R. communis is indigenous to the southeastern Mediterranean region, eastern Africa, and India, but it is now widespread throughout temperate and subtropical regions of the world.[21,22] The plant is cultivated for its seeds, which are a source of commercial products such as castor oil. More than 1,481,600 tons of castor seeds were produced in 2009.[22] The plant is also grown as an ornamental, and seeds are available at nurseries and on the internet. Because of the wide availability of the seeds and source plants, as well as the ease of production, stability, and lethal potency, ricin is considered a bioterrorism threat. Ricin is the most common agent used in biocrimes; it is also one of the most prevalent agents involved in WMD (weapon of mass destruction) investigations by the Federal Bureau of Investigation (FBI).[11] Recent attempted uses of ricin by various extremists and radical groups have heightened concerns regarding its potential for use in urban terrorism.[11,23]

Ricin has been classified by the Centers for Disease Control and Prevention (CDC) as a Category B agent for public health preparedness efforts.[24] First responders are often faced with unidentified white powders and are called on to test in the field for the presence of ricin and other

potential biological agents of concern. Samples purportedly containing ricin have been sent to laboratories belonging to the Laboratory Response Network (LRN) for analysis, but only a small percentage of them have been found to contain ricin.

The purpose of this study was to conduct a multicenter evaluation of the sensitivity, specificity, reproducibility, and limitations of a lateral flow assay (LFA) for ricin (Ricin BioThreat Alert® [BTA] Test Strip, Tetracore®, Inc., Rockville, Maryland) that could be used in the field or in the laboratory to screen for ricin.[25] To ensure an unbiased evaluation, blinded samples were provided to testing sites that perform assays on a regular basis. The primary aims of this evaluation were to determine the probability of false-positive results (assay is positive but the analyte is not present) and false-negative results (assay is negative but the analyte is present at amounts at or above the limit of detection [LOD]). The overall goal of this project was to be able to provide information to the first responder community on the reliability and robustness of this assay in order to enable appropriate and effective medical decisions by public health workers and others, avoid unnecessary disruption of civil society, and reduce the economic impact of a ricin attack.

Materials and Methods

Assay Evaluation Study

Ricin BioThreat Alert® Test Strips (catalog number TC-8008-025) and BioThreat Alert® Readers (catalog number TC-3001-001) were obtained from Tetracore®, Inc. (Rockville, Maryland). The performance of the LFA and Reader were evaluated at 5 test sites: CDC; the Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (CFSAN); the Massachusetts Department of Public Health; the Texas Department of State Health Services; and Virginia's Division of Consolidated Laboratory Services. Samples for analysis were prepared at Lawrence Livermore National Laboratory, coded, and shipped (on cold pack) to the participating laboratories, where they were diluted and analyzed according to a standard protocol. LFA results were read both visually and with the BioThreat Alert® Reader according to directions provided by the manufacturer—that is, between 15 and 30 minutes after adding the sample (150 µL) to the lateral flow strip. Samples with readings of ≤125 were considered negative. The BioThreat Alert® Reader measures the ratio of incident light and absorbing light intensity on the surface of the lateral flow strip. As an example, if you used 100 cd/m^2 incident light and 0.25 cd/m^2 is absorbed on the surface, the resulting ratio (ie, 0.0025), converted into a BioThreat Alert® Reader value by the instrument, is expressed without units.

The study consisted of 7 phases, which are enumerated below. At least 5 negative control and 2 positive control LFAs were run at each test site during each phase of the study.

Phase 1: Repeatability Study

The repeatability of the LFA was determined using purified RCA_{60}. Stock solutions of RCA_{60} were prepared at concentrations of 0.2 µg/mL and 0.4 µg/mL in phosphate-buffered saline containing 0.1% (v/v) Tween-20 and 0.1% (w/v) bovine serum albumin (PBSTB) and shipped to the testing sites, where they were centrifuged for 3 minutes at 3,000 rpm in a microfuge. Operators diluted the stock solutions 1:10 in BTA buffer, supplied by Tetracore®, Inc., along with the BTA Strip, and, after mixing, 150 µL of the diluted toxin was added to each lateral flow strip (final concentration 20 ng and 40 ng/mL [3 and 6 ng/assay], respectively). Twelve replicates of each concentration were tested by 2 operators per site at each of 4 sites (24 total replicates/site), except at CDC, where 24 replicates of each concentration were tested by a single operator. A total of 120 LFAs were run at each ricin concentration.

Phase 2: Inclusivity Panel

In order to determine whether this assay could detect ricin in castor beans from diverse geographic locations, crude extracts of 18 *R. communis* cultivars (Table 1) were prepared, as described below. The extracts were diluted in PBSTB to a final concentration of 13.2 µg protein/mL and

Table 1. *Ricinus communis* Cultivars ($N=18$) Used for Testing

Country	*ARS Accession Number*[a]	*Seed Weight (g/100 seeds)*[b]
Afghanistan	212115	29.05
Argentina	219767	20.79
Brazil	241368	25.19
China	436592	32.46
Cuba	208839	16.23
El Salvador	197048	37.51
Former Soviet Union	257654	25.20
India	183470	22.56
Iran	247100	22.56
Mexico	165446	32.47
Morocco	253621	29.11
Pakistan	217539	27.69
Peru	215770	36.69
Puerto Rico	209132	18.53
Syria	181916	16.54
Turkey	167342	36.02
United States (Hale)	642000	27.48
US Virgin Islands	209326	17.72

[a]Seeds were obtained from the Plant Genetics Resource Conservation Unit, US Department of Agriculture, Agriculture Research Service, Griffin, Georgia.

[b]Seed size is based on a visual observation after the seed has been harvested and can be confirmed using 100 seed weights. Those accessions with 100 seed weights ranging from 10 g to 19 g are small seeds, 20 g to 29 g are medium seeds, and >30 g are large seeds (B. Morris, ARS, USDA, personal communication).

then shipped to the test sites. Before testing, the tubes were vortexed and centrifuged for 3 minutes at 3,000 rpm in a microfuge. The extracts were subsequently diluted 1:2 with BTA buffer, and, after mixing, a 150-μL volume was added to each test strip. The final protein concentration of the diluted extract was 6.67 μg/mL (1 μg/assay). Each cultivar was tested once per test site.

Phase 3: Informational Panel

In order to determine whether the assay was specific for RCA_{60}, solutions of ricin A and B chain, RCA_{120}, several ricin vaccine candidates, and other commercially available purified Type II RIPs (or those in crude extracts) were prepared (1.32 μg protein/mL) in PBSTB for testing. The protein solutions were shipped to the test sites, where they were vortexed and centrifuged for 3 minutes at 3,000 rpm and then diluted 1:2 in BTA buffer. After mixing, a 150-μL volume containing 100 ng of protein was added to each LFA. Each sample was tested once per site.

Phase 4: Lectin Panel

Stock solutions of 66 lectins (see list of lectins below) were prepared in PBSTB at a lectin concentration of 1.32 μg protein/mL and shipped to test sites, where they were subsequently vortexed and centrifuged for 3 minutes at 3,000 rpm and diluted 1:2 in BTA buffer, and a 150-μL volume containing 100 ng of lectin was added to each LFA. Each lectin was tested once per site.

Phase 5: Near-Neighbor Panel

Crude extracts were prepared from the seeds or leaves of 35 near neighbors of *R. communis* and *Abrus precatorius.* The extracts were diluted in PBSTB to an extract protein concentration of 13.2 μg/mL and shipped to the test sites, where they were subsequently vortexed and centrifuged for 3 minutes at 3,000 rpm and then diluted 1:2 in BTA buffer. A 150-μL volume containing 1 μg extract protein was added to each test strip. Each near neighbor was tested once per site.

Phase 6a: White Powder Panel

Twenty-four of the white powders most commonly encountered by first responders and the LRN were evaluated for their ability to affect the performance of the assay.[26] Five milligrams of each of the 24 white powders (see list below) were sent to the test sites. Operators added 500 μL of BTA buffer, and each tube was vortexed for 10 seconds. The suspension was allowed to settle for at least 5 minutes, and a 150-μL volume of supernatant was removed and added to an LFA. Each powder was tested once per site.

Phase 6b: White Powders Spiked with Castor Bean Extract

The 24 white powders were also evaluated for their ability to interfere with or inhibit the detection of ricin by the assay. Five milligrams of each of the 24 white powders along with a tube of crude extract of the Hale cultivar of *R. communis* (diluted in PBSTB to 66 μg extract protein/mL) was shipped to each test site. Approximately 1% of the extracted protein was ricin, as determined by mass spectrometry. Operators added 450 μL BTA buffer and 50 μl of the Hale extract to each tube containing powder. After mixing on a vortex for 10 seconds, the suspension was allowed to settle for at least 5 minutes, after which a 150-μL volume of the supernatant was removed and added to an LFA (1 μg Hale protein [ca. 10 ng ricin] per test strip). Each powder spiked with extract was tested once per site.

Phase 7a: BioWatch Filter Extract

BioWatch filter extract containing 13.2 μg protein/mL was shipped to each test site, where it was vortexed and centrifuged for 3 minutes at 3,000 rpm. Operators added 250 μL BTA buffer (1:2 dilution). After mixing on a vortex for 10 seconds, the suspension was allowed to settle for at least 5 minutes, after which a 150-μL volume of supernatant (1 μg total protein) was removed and added to an LFA. The filter extract was tested once at each test site.

Phase 7b: BioWatch Filter Extract Spiked with Castor Bean Extract

A 250-μL volume of BTA buffer and 55 μL of the diluted Hale extract were added to a second tube containing BioWatch filter extract. After mixing on a vortex for 10 seconds, the suspension was allowed to settle for at least 5 minutes, after which a 150-μL volume of supernatant containing 1 μg of Hale protein was added to an LFA. The spiked filter extract was tested once per site.

***Ricinus communis* Seeds**

Seeds of 18 accessions (ie, cultivars) of *R. communis* were obtained from J. Bradley Morris, Plant Genetic Resources Conservation Unit, Agricultural Research Service (ARS), US Department of Agriculture (USDA), Griffin, Georgia. *R. communis* exhibits low levels of genetic diversity, and mixing of genotypes has led to minimal geographic structuring of castor bean populations worldwide.[27-29] Thus, our primary goal was to maximize geographic distribution of samples without regard to plant phenotype (Table 1). Nevertheless, the seeds selected did exhibit phenotypic variation with respect to seed size (small, medium, large) and color (brown, tan, reddish-brown). These accessions had been propagated at the Plant Genetics Resources Conservation Unit (50 plants per castor bean accession per 6 m^2 plot).[21] To avoid cross-pollination, the inflorescences were bagged prior to fertilization. At maturity, castor bean plants were hand-harvested, dried at 21°C, 25% relative humidity, for approximately 1 week and threshed. Seeds were counted, weighed, and stored at 4°C for distribution, while seeds for long-term storage were stored at -18°C.

Crude Extracts

Crude extracts of *R. communis* seeds were prepared by modifying a method provided by E. A. E. Garber, CFSAN, FDA. All work involving the potential generation of aerosols was conducted at BSL-2 in a Class II biosafety cabinet. Briefly, the castor beans were weighed and placed in a small coffee grinder, and the edges were taped to minimize aerosols. After grinding to a fine particle size, the ground seed material was carefully transferred to a 50-mL conical centrifuge tube containing phosphate-buffered saline with 0.1% Tween-20 (v/v) (PBST). The ratio of PBST to whole bean weight was 2.25 mL/g of beans. The tube was closed, sealed with parafilm, mixed on a vortex, and placed on a rocker platform for 16 to 19 hours in the dark at 4°C, after which the tubes were centrifuged at 3,000 rpm (GSA rotor) for 4 minutes. After centrifugation, the cloudy brownish middle layer was removed, dispensed into cyrovials, and stored at -80°C until used. The same procedure was used to prepare extracts of other seeds, bulbs, and leaves.

Plant Sources

The number of near neighbors tested was limited by the commercial availability of their seeds. Seeds from near neighbors of *R. communis* were obtained from various sources.[30] *Adriana quadripartita, Jubernardia globifera, Phytolacca americana*, *Plukenetia volubilis, Saponaria officinalis,* and *Trewia nudiflora* were obtained from B & T World Seeds in Aigues-Vives, France. *Fatsia japonica* and *Saponaria officinalis* were obtained from Plant World Seeds in Newton Abbott, Devon, United Kingdom. *Macaranga grandifolia* was obtained from Top Tropicals in Ft. Myers, Florida. Seeds of *Mallotus philippensis* and *Mercurialis annua* were obtained from USDA, ARS, in Pullman, Washington. Leaves from *Acalypha rhomboidea* and *Manihot esculenta* were obtained from the Botanical Gardens in Washington, DC. Seeds of *A. precatorius*, which contain a related toxin—abrin—and its near neighbors were also obtained commercially and tested for cross-reactivity. *Abrus laevigatus*, *A. precatorius*, *Bryonia dioica*, *Canavalia gladiata*, *Canavalia rosea*, *Canavalia virosa*, *Cinnamomum camphora*, *Cucurbita moschata, Dianthus caryophyllus*, *Luffa acutangula, Luffa cylindrica (aegyptica)*, *Lychnis chalcedonica*, *Momordica charantia*, *Phytolacca dioica, Sambucus ebulus*, *Sambucus nigra*, *Senna occidentalis,* and *Trichosanthes kirilowii* were obtained from B & T World Seeds in France. *Abrus schimperi* subs. *Africanus*, *Galactia striata*, and *Galactia wrightii* were obtained from the Desert Legume Project in Tucson, Arizona. *Iris hollandica* bulbs were purchased from American Meadows in Williston, Vermont.

Lectins

The following lectins were purchased from E Y Laboratories, Inc., in San Mateo, California: *A. precatorius, Agaricus bisporus, Aleuria aurantia, Allium sativum, Amaranthus caudatus, Arachis hypogaea, Arum maculatum, Autocarpus integrifolia, Bauhinia purpurea, Bryonia dioica, Canavalia ensiformis, Caragana arborescens, Cicer arietinum, Colchicum autumnale, Cytisus scoparius, Datura stramonium, Dolichos biflorus, Euonymuse europaeus, Galanthus nivalis, Glycine max, Griffonia (Bandeiraea) simplicifolia* Lectin I, *G. (B.) simplicifolia* Lectin II, *G. simplicifolia, Hippeastrum* hybrid, *Iberis armara, Iris* hybrid, *Jacalin, Laburnum alpinum, Lens culinaris, Lotus tetragonolobus, Lycopersicon esculenntum, Maackiaa amurensis* Lectin I, *Maclura pomifera, Mangifera indica, Narcissus pseudonarcissus,* Peanut agglutinin, *Phaseolus lunatus, Phaseolus vulgaris, Phaseolus vulgaris* Agglutinin, *Phaseolus vulgaris* Erythroagglutinin, *Phytolacca americana, Pisum sativum, Psophocarpus tetragonolobus, P. tetragonolobus* Lectin I, *P. tetragonolobus* Lectin II, *Robinia pseudoacacia, Salvia sclarea, Sambucus nigra* Agglutinin I, *Sambucus nigra* Agglutinin II, *Solanum tuberosum, Sophora japonica,* Soybean Agglutinim, *Trichosanthes kirilowii, Trifolium repens, Tulipa sp., Ulex europaeus* Agglutinin I, *U. europaeus* Agglutinin II, *Urtica dioica, Vicia fava, V. graminea, V. villosa, V. radiate,* Wheat Germ Agglutinin, *Wisteria floribunda* Agglutinin, and *W. floribunda* Lectin.

Ricin Proteins

Purified ricin (RCA_{60}), ricin A chain, ricin B chain, and RCA_{120} were purchased from Vector Laboratories in Burlingame, California. Formalin-inactivated ricin toxoid was obtained from Toxin Technologies, Inc., in Sarasota, Florida. Deglycosylated ricin A chain and the vaccine rRTA1-33/44-198 were obtained from Martha Hale (US Army Medical Institute for Infectious Diseases [USAMRIID], Fort Detrick, Frederick, Maryland). RiVax—a candidate ricin vaccine consisting of a recombinant ricin A chain containing residues 1-267 with 2 substitutions, V76M and Y80A, to reduce toxicity[31]—was obtained from P. Legler of the US Navy (NRL, Washington, DC).

Ribosomal Inactivating Proteins

Purified Shiga toxin was obtained from Toxin Technologies (Sarasota, Florida). Crude extracts of their respective seeds or bulbs were used as sources for Agglutinin SNA-II (*S. nigra* Type II-RIP), Lectin TKL-1 (*T. kirilowii* Type II-RIP), mistletoe lectin (*Viscum album)* Type II-RIP [viscumin], and Type II-RIPs from *C. camphora, S. occidentalis, I. hollandica, A. precatorius* [abrin], and *S. ebulus* [ebulin].

White Powders

Powdered milk, powdered coffee creamer, powdered sugar, talcum powder, baking powder, cornstarch, and popcorn salt were purchased from Raley's Grocery Store in Pleasanton, California. Flour, baking soda, baby powder, chalk dust, and powdered infant formula (iron fortified and low-iron formulation) were purchased from Target Stores in Livermore, California. Powdered toothpaste was purchased

from Walmart Pharmacy in Livermore, California. Brewer's yeast was obtained from GNC Stores in Livermore, California. Drywall dust was obtained from Home Depot in Livermore, California. Gamma aminobutyric acid, L-glutamic acid, kaolin, chitin, chitosan, magnesium sulphate, and boric acid were purchased from Sigma-Aldrich Corp., St. Louis, Missouri. *Bacillus thuringiensis* (Dipel) powder was purchased from Summerwinds Nursery in Palo Alto, California.

BioWatch Filters

Thirty BioWatch filters, collected from several metropolitan sites throughout the United States, were extracted by shaking with PBST, and the extracts pooled. The protein concentration of the pooled extract was determined, and the total protein concentration was adjusted to 13.2 μg/mL with PBSTB.

Protein Determination

Protein concentrations were determined using Bradford Assay Reagent (Pierce Chemical Company, Rockford, Illinois) using a standard curve prepared with bovine serum albumin (EM Sciences, Cole-Parmer, Vernon Hills, Illinois).

Select Agent Issues

Since the total amount of purified ricin employed in this study was less than 100 mg and no attempt was made to purify ricin from the extracted material, this project was exempt from the Select Agent Regulation.

Statistics

Standard deviations (SD) were calculated using STDEVPA in Excel 2010.

Results

Negative Controls

A total of 129 negative control LFAs were run during the course of this study. All of these controls were negative when read visually. When the results were read with the Rapid BioAlert® Reader, 88.4% ($n = 114$) gave readings of 0, while 11.6% ($n = 15$) gave readings between 30 and 74. The mean and standard deviation for the readings of all negative controls was 5.19 and 15.1, respectively (Figure 1). The manufacturer's cut-off for a positive reading is 125. Thus, all of the readings for the negative controls were below the cut-off value, yielding a negative result as expected.

Repeatability

The results from 240 LFAs (120 at 3 ng/test strip and 120 at 6 ng/test strip) performed by 9 operators were used to assess repeatability. When read visually, both the control

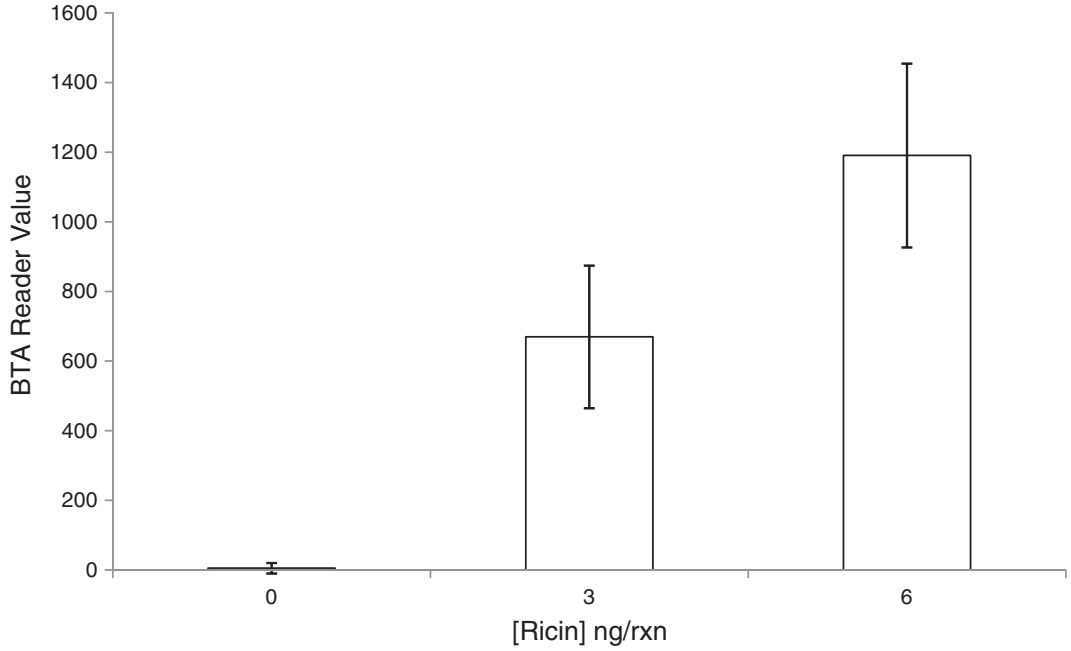

Figure 1. Reader Value as a Function of Ricin Concentration (ng/sample). Results of assays read with the Rapid BioAlert® Reader. LFAs ($N = 240$) were run by 9 different operators to assess repeatability of the assay. The results for the 3 ng/assay ($n = 120$; mean 681, SD 201) and the 6 ng/assay ($n = 120$; mean 1,191, SD 264) are shown. In addition, all readings for the negative controls ($n = 129$) were below the cut-off value of 125, yielding a negative result as expected.

Table 2. Results of Repeatability Study Using BioThreat Alert® Reader by Performance Site

	3 ng Ricin/Assay (N = 120)			*6 ng Ricin/Assay (N = 120)*		
Site[a]	*Mean Reading*	*SD*[b]	*%CV*[c]	*Mean Reading*	*SD*	*%CV*
CDC	685	170	25	1,174	299	25
CFSAN	741	212	29	1,127	177	16
MDPH	612	100	16	1,239	164	13
TDSHS	757	228	30	1,393	298	21
VDCLS	607	216	36	1,021	189	18
All	681	201	30	1,191	264	22

[a]CDC, Centers for Disease Control and Prevention; CFSAN, Center for Food Safety and Applied Nutrition, Food and Drug Administration; MDPH, Massachusetts Department of Public Health; TDSHS, Texas Department of State Health Services; VDCLS, Virginia's Division of Consolidated Laboratory Services.
[b]SD = standard deviation.
[c]CV = coefficient of variation.

and sample lines on all 240 LFAs were positive. When the test strips were read with the Rapid BioAlert® Reader between 15 and 30 minutes after the addition of 150 µL sample to the sample well, the average readings were 681 (SD 201) and 1,191 (SD 264) for 3 ng and 6 ng RCA_{60}/test, respectively; the data are shown in the bar diagram in Figure 1. The mean readings for 3 ng varied across sites, from a low of 607 to a high of 757, while those for 6 ng varied from a low of 1,021 to a high of 1,393. There was also variation in readings within each site. Percent coefficient of variation ranged from 16 to 36 and 13 to 25 for 3 ng and 6 ng, respectively (Table 2). The extent of this variation was dependent on the RCA_{60} concentration and when the test was read within the 15- to 30-minute window (Figure 2). For example, at an RCA_{60} concentration of 5 ng/assay, the readings continued to increase when the test was read every 2 minutes between 15 and 30 minutes; however, at an RCA_{60} concentration of 10 ng/assay, the readings reached a plateau after 20 minutes. Thus, the variation within and between sites for the low concentrations (3 and 6 ng/assay) that were tested could be attributed to the time at which the readings were taken.

A receiver operator characteristic (ROC) curve was plotted with the Phase 1 study data consisting of 240 positive samples and 29 negative controls. In an ROC curve, the true positive rate (sensitivity) is plotted as a function of the false-positive rate (100-specificity) for different cut-off points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the 2 distributions) has an ROC curve that passes through the upper left corner (100% sensitivity, 100% specificity). The ROC curve for ricin lateral flow assay presented in Figure 3 shows 100% sensitivity and 100% specificity for the detection of ricin at a concentration of 3 ng/reaction and above during this phase of the study.

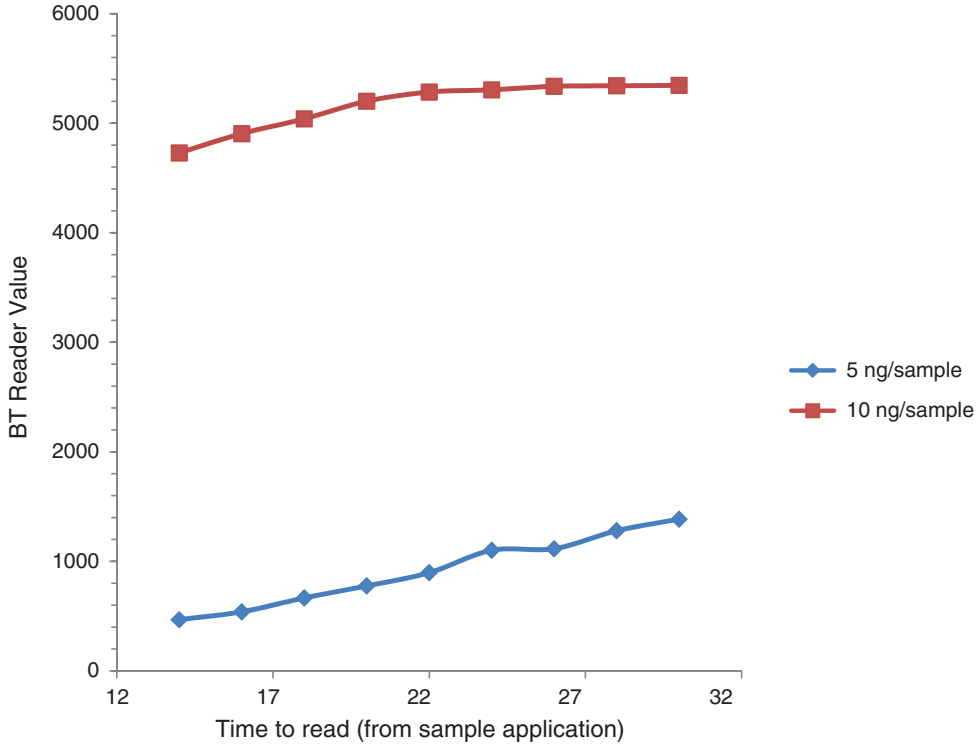

Figure 2. Rapid BioAlert Reader Value as Function of Time to Read. Variations in readings appear to be both time- and concentration-dependent. Shown are the increases between sequential readings every 2 minutes for 5 ng/sample and 10 ng/sample that were observed between 15 and 30 minutes after addition of sample. Color graphics available online at www.liebertonline.com/bsp

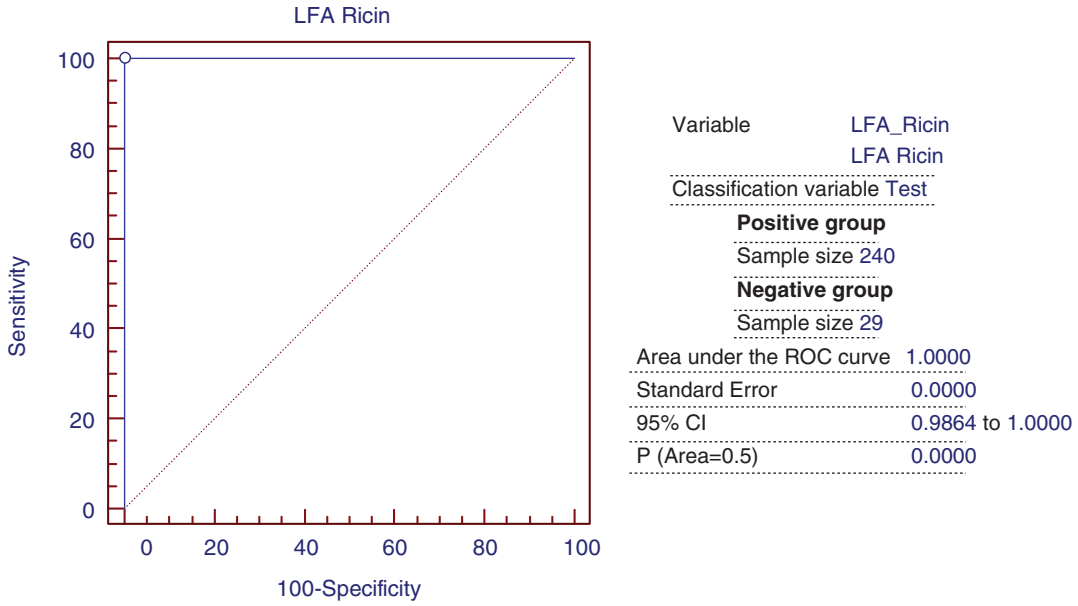

Figure 3. Receiver Operator Characteristic Curve for Ricin LFA. Receiver Operator Characteristic (ROC) curve showing the true-positive rate (sensitivity) as a function of the false-positive rate (100-specificity) for different cut-off points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The ROC curve shows 100% sensitivity and 100% specificity for the detection of ricin at a concentration of ≥3 ng/sample. Color graphics available online at www.liebertonline.com/bsp

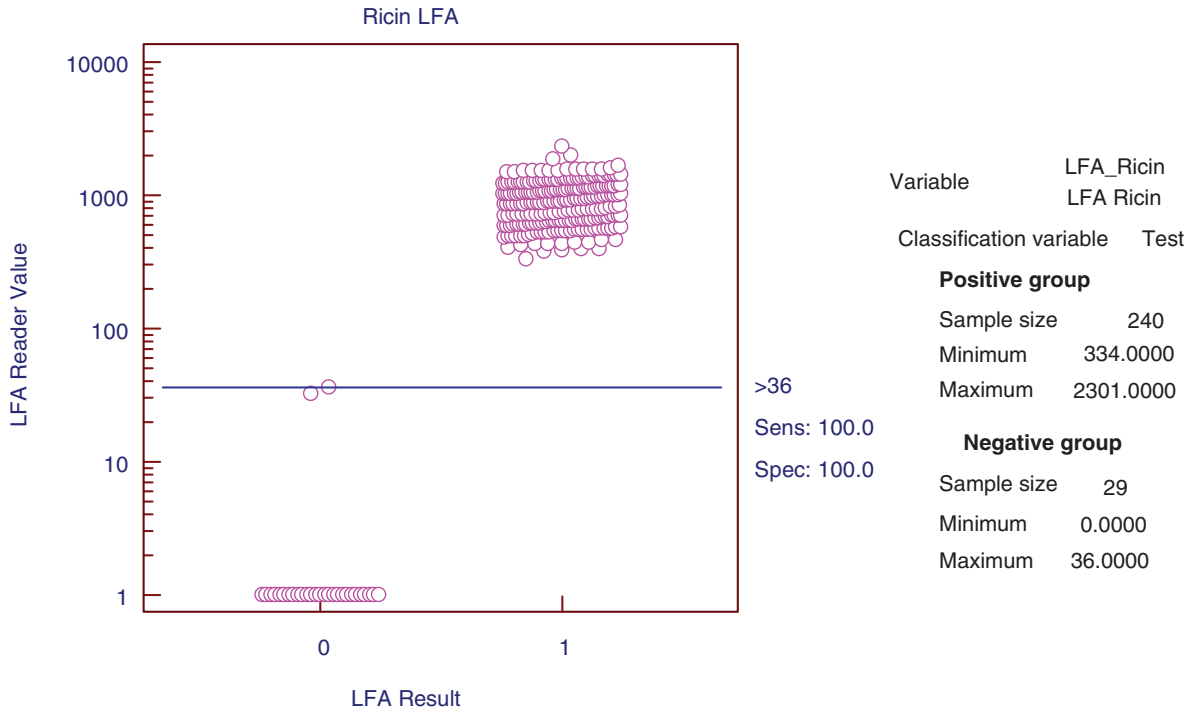

Figure 4. Interactive Dot Plot for Ricin LFA. The data from Phase 1 shown in Figure 3 are depicted as an interactive dot diagram. The values obtained for each of the positive and negative ricin samples, tested at 5 different sites by 9 different operators on at least 2 different days, are shown. The data show that above a cut-off value of 36, the assay has 100% sensitivity and 100% specificity. Color graphics available online at www.liebertonline.com/bsp

The data from the Phase 1 study are also depicted in the interactive dot diagram in Figure 4. This figure shows the value of the LFA Reader for each of the positive and negative samples tested in this study. The samples were tested at 5 different sites by 9 different operators, and, at each site, the samples were tested on at least 2 different days. From this interactive dot diagram, it is clear that above a cut-off value of 36, the assay has 100% sensitivity and 100% specificity. The default threshold cut-off value for the Rapid BioAlert Reader is 125; hence, all the samples were correctly identified in this phase of the testing.

Limit of Detection

Ten replicates each of 5 ng and 10 ng RCA_{60} were run to obtain an estimate of the LOD. Mean readings of 851 (SD 185) and 1,791 (SD 465) were obtained with RCA_{60} concentrations of 5 ng and 10 ng, respectively. These results, together with the data for the negative controls and the 3-ng and 6-ng RCA_{60} concentrations, are presented in Figure 5. Using the manufacturer's recommended cut-off of 125, the assay can detect ≥0.54 ng (ie, ≥3.6 ng/mL).

Inclusivity Panel

Extracts prepared from the seeds of 18 cultivars of *R. communis* (Table 1) were tested at a final protein concentration of 1 µg/150 µL. All LFAs were positive when read visually or with the BioThreat Alert® Reader, suggesting that the assay would give a positive result with ricin-containing extracts from geographically diverse accessions as well as extracts prepared from seeds of different sizes and colors. However, it was not possible to say that the assay results are due solely to the presence of ricin. Table 3 demonstrates that the monoclonal capture antibody employed in this assay reacts with deglycoslyated A chain and therefore recognizes an amino acid–containing epitope on the A chain of RCA_{60}. Furthermore, it does not discriminate between the A chains of RCA_{60} and RCA_{120}. Both proteins are present at similar concentrations in extracts of seeds from the Hale cultivar.[4] Thus, the results with the extracts from the 18 cultivars are likely due to the detection of both RCA_{60} and RCA_{120}.

The assay did not detect formalin-inactivated ricin (ricin toxoid) and the ricin vaccine rRTA 1-33/44-198, but it did detect the vaccine RiVax. The assay did not detect other

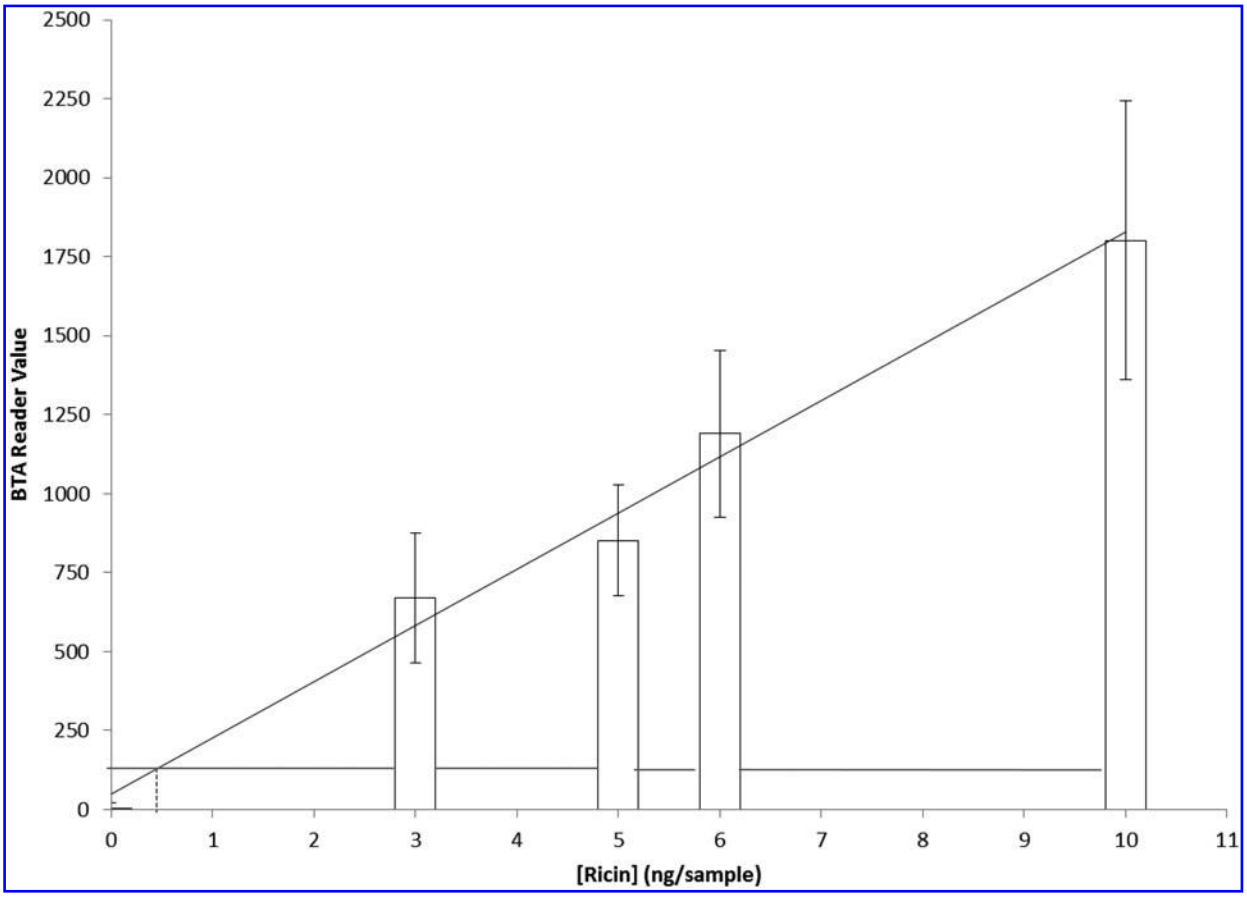

Figure 5. Effect of Ricin Concentration on Rapid BioAlert Reader Values. To determine the LOD, 10 replicates each of 5 and 10 ng RCA_{60} were tested. Mean readings of 851 (SD 185) and 1,791 (SD 465) were obtained with RCA_{60} concentrations of 5 ng and 10 ng, respectively. These results, together with the data for the negative controls and the 3-ng and 6-ng RCA_{60} concentrations are shown. Using the manufacturer's recommended cut-off of 125, the assay could detect ≥0.54 ng of ricin (≥3.6 ng/mL).

Table 3. Reactivity of Ricin BioThreat Alert® LFA with Ricin Derivatives and Type II Ribosomal Inactivating Proteins

Protein[a]	*Mean Reading*	*Result*
RCA_{60} subunits and RCA_{120}		
Ricin A chain	3,127	Positive
Ricin B chain	57	Negative
Ricin agglutinin I (RCA_{120})	3,051	Positive
Ricin vaccine candidates		
Deglycosylated ricin A chain	2,782	Positive
Formalin-inactivated toxoid	0	Negative
rRTA 1-33/44-198 vaccine	0	Negative
RiVax	2,086	Positive
Type II-Ribosome inactivating proteins		
Shiga toxin	0	Negative
Type II-RIP *Abrus precatorius* (abrin)	0	Negative
Type II-RIP *Sambucus nigra*	0	Negative
Type II-RIP *Sambucus ebulus* (ebulin)	0	Negative
Type II-RIP *Cinnamomum camphora*	0	Negative
Type II-RIP *Senna occidentalis*	0	Negative
Type II-RIP *Iris hollandica*	0	Negative
Type II-RIP *Trichosanthes kirilowii*	0	Negative
Type II-RIP *Viscum album* (viscumin)	0	Negative

[a]All proteins were tested at a final concentration of 667 ng/mL.

Type II-RIPs from a variety of eukaryotic and prokaryotic sources.

Lectin Panel

Plant lectins could potentially cause false-positive results by binding to carbohydrate residues on the capture and detector antibodies. In order to investigate this possibility, 66 lectins including wheat germ agglutinin, which has been shown to cause false-positive results,[32] were tested at a concentration of 100 ng/test strip. All of the LFAs ($N=330$) had positive readings for control lines and negative readings for the presence of ricin (both visually and with the Reader). Thus, the lectins, at the concentration tested, did not interfere with the positive control or give false-positive results.

Near Neighbor Panel

Both Type I and II RIPs and lectins are widely distributed throughout the plant kingdom. In order to investigate whether any of these could generate false-positive results with the Ricin BioThreat LFA, extracts (1 µg protein/150 µL) were prepared from the seeds or leaves of 14 near neighbors of *R. communis* and 21 near neighbors of *A. precatorius* and tested. All of the LFAs ($N=175$) exhibited a positive reading for the control line and gave negative readings (both visually and with the Reader) for the sample line.

White Powder Panel

Unknown white powders are often encountered in the field, where they may be tested for the presence of a biothreat agent by law enforcement officers or other first responders. Thus, it is of importance to determine whether any of these powders could interfere with the detection of ricin by this assay. Twenty-four white powders commonly encountered in the field were tested in accordance with the procedures described above. Some of the powders (eg, powdered sugar) were soluble to some degree in the BTA diluent, while others (eg, chitin) were insoluble. However, after settling for at least 5 minutes, 18 of the powder suspensions produced a clear supernatant with a white precipitate, while the remaining 6 were opaque. After testing, none of the powders interfered with the development of the positive control line nor did they give a positive result for the presence of ricin. The powder suspensions were subsequently spiked with an extract of *R. communis* Hale (1 µg protein/150 µL) and retested. All of the LFAs ($N=120$) yielded a positive reading for the presence of ricin when read either visually or with the BioThreat Alert® Reader. However, the presence of the powder reduced the mean reading by 13% to 78% (Table 4) when compared with the mean value for the Hale

Table 4. Effect of White Powders and BioWatch Filter Extracts on the Performance of the Ricin BioThreat Alert® LFA

Powder or Extract	*Mean Reading*[a]	*SD*[b]	*Percent Reduction*
None	4,687[c]	919	—
BioWatch filter extract	4,302	352	8
Flour	4,099	533	13
Chitosan	3,638	879	22
Drywall dust	3,558	566	24
Chitin	3,493	402	25
Baking powder	3,475	603	26
Kaolin	3,457	848	26
Cornstarch	3,323	345	29
Brewer's yeast	3,279	735	30
Powdered infant formula[d]	3,276	359	30
Chalk dust	3,271	587	30
Talcum powder	3,143	610	33
Baby powder	3,118	952	33
Powdered sugar	3,005	360	36
Boric acid	2,793	614	40
GABA[e]	2,730	249	42
Dipel (*B. thuringiensis*)	2,724	221	42
Powdered coffee creamer	2,683	186	43
Baking soda	2,494	542	47
L-Glutamic acid	2,488	310	47
Popcorn salt	2,471	501	47
Magnesium sulfate	2,297	652	51
Powdered toothpaste	1,863	704	60
Powdered infant formula[f]	1,543	417	67
Powdered milk	1,049	190	78

[a]Mean readings were calculated for 5 sites.
[b]SD = standard deviation.
[c]Mean reading was calculated from 49 repetitions of the positive control.
[d]Powdered infant formula, low iron.
[e]GABA = gamma-aminobutyric acid.
[f]Powdered infant formula, iron fortified.

extract alone. The powders having the greatest effect on the reading were powdered milk (78% reduction), iron fortified powdered infant formula (67% reduction), and powdered toothpaste (60% reduction). Flour (13% reduction) had the least effect. The mechanism by which these powders reduced the reading is unclear.

BioWatch Filter Extracts

The 30 BioWatch filters were extracted and pooled in accordance with the procedures described above. The pooled filter extracts were tested in the absence of and after being spiked with Hale extract. The filter extracts alone did not affect the performance of the assay. Positive results were obtained after the addition of Hale extract (1 μg protein/150 μL). When compared to the mean value for the Hale extract alone, the presence of the pooled filter extract reduced the mean reading by 8%.

Discussion

A number of methodologies have been employed for the detection of ricin. In general, they directly measure its toxicity,[33,34] the enzymatic activity of the ricin A chain,[35-37] or the presence of RCA_{60} or ricin A chain.[37-41] All of these methods have limitations. For example, toxicity studies require a suitable animal or cell culture. Measuring enzymatic activity in vitro may not distinguish between RCA_{60} and ricin A chain. Finally, immunologic methods may not distinguish between RCA_{60} and RCA_{120} or their subunits and will not provide information about toxin activity. Many of these methods are complex, requiring expensive equipment, and are performed in a laboratory environment. A few assays have been developed for field screening, but they have not been extensively evaluated.

LFAs or immunochromatographic lateral-flow assays were commercially introduced for pregnancy testing in 1988.[42,43] Also known as "hand-held" assays (HHAs), they are simple to use and require minimal training. HHAs are widely used by law enforcement officers and other first responders for testing suspicious powders in the field. The Ricin BioThreat Alert® Test Strip is a rapid qualitative test to detect the presence of ricin toxin in environmental samples. The test uses a combination of a labeled monoclonal detector antibody and a polyclonal capture antibody to selectively capture and detect the presence of ricin in aqueous samples. The purpose of the current study was to evaluate the performance of one LFA in order to understand its sensitivity, specificity, reproducibility, and limitations for field use and to determine whether this assay could also be used as a screening assay in a laboratory setting.

Using the BioThreat Alert® Reader, we estimated that the Ricin BioThreat Alert® LFA could reproducibly detect > 3.6 ng ricin/mL (0.54 ng/test). This estimate is based on the manufacturer's recommended cut-off of 125 and the data illustrated in Figure 5. The manufacturer's cut-off of 125 is conservative. If, instead, the cut-off is defined as the average negative control signal plus 2 standard deviations, it would be 30.[44] However, employing a cut-off of 30 may be problematic, as 15/129 (11.6%) negative controls gave readings between 30 and 74. For use in the field, sacrificing sensitivity for specificity (ie, a low rate of false-positive results) is tolerable. An LOD value of 10 ng/ml was considered acceptable for the detection of ricin in cosmetics.[32]

The kinetics of this assay, as determined with the Rapid BioAlert Reader, appear concentration-dependent (Figure 2). At low concentrations (≤ 6 ng/test), the readings increase throughout the 15- to 30-minute period recommended by the manufacturer, while at higher concentrations (> 10 ng/test), the readings reach a plateau shortly after 15 minutes or remain relatively constant after 15 minutes. This difference may be due to the time it takes to capture sufficient amounts of labeled antibody-ricin complexes to detect with the Reader.

During the repeatability study, 9 operators from 5 sites tested 240 LFAs (120 at 3 ng ricin/test strip and 120 with 6 ng ricin/test strip). When read visually, both the control and sample lines on all 240 LFAs were positive. However, when the LFAs were read with the Rapid BioAlert Reader according to the manufacturer's directions (ie, between 15 and 30 minutes after the addition of sample), the average readings for each concentration varied between sites (Table 2) as well as within sites as indicated by the coefficients of variation. The kinetics of the assay response may explain the variability in readings at these low ricin concentrations. Operators were running multiple LFAs at each ricin concentration and staggering the addition of sample so that each LFA is read at approximately the same time interval. An examination of the average time-to-read indicates that with 3 ng ricin/test the average time was 19 minutes (SD = 3 minutes, %CV = 15.79), and for 6 ng ricin/test the average time was 19 minutes (SD = 2 minutes, %CV = 11.11). Furthermore, as the data for consecutive readings with 5 ng ricin/assay indicate (Figure 2), a 2- to 3-minute difference in the reading interval could result in a 30% to 50% difference in replicate LFA readings. The 15-minute window during which tests were read and the observed variability of the readings make it problematic to use the readings to quantitate the amount of ricin in a sample, especially low concentrations. However, this is not a problem for field use as the assay is to be used in a qualitative manner as a screening test and any reading above 125 considered positive.

R. communis is a member of the Euphorbiaceae family.[30] Because most castor accessions readily intercross, produce fertile progeny, and have the same chromosome number, castor is now considered to be a single species.[45] It is found across all tropical and semitropical regions of the world, where it is grown as a source of oil or as an ornamental plant.[22] There are a number of repositories of castor

germplasm, which are located in 10 countries and contain a total of 11,300 accessions.[21,22] Cultivars may differ in the size of the plant, color, and size of the seeds. Some of these differences have a genetic basis while others may be the result of the growth and soil conditions. Based on differences in inhalation toxicity of the ricins from 2 cultivars[46] and the large number of ricinlike genes present in the chromosome,[47-49] it has been suggested that ricins extracted from different cultivars might exhibit variations in primary sequence or glycosylation, leading to differences in toxicity and in the limits of detectability.[41] This could be problematic as the Ricin BioThreat Alert® LFA depends on a single monoclonal antibody to initially bind the toxin.

In order to evaluate this possibility, we tested the ability of this LFA to detect ricin in crude extracts from seeds of various sizes and colors from 18 cultivars from various geographic areas (Table 1). The cultivars (ie, accessions) were propagated under the same conditions in the same soil to minimize the effects of growth conditions. Nevertheless, seed weight ranged from 16.23 to 37.51 g/100 seeds, mirroring a difference in the size of the seeds (ie, small, medium, and large). The volume of extraction buffer was adjusted to account for the differences in seed weight. After testing, the BioThreat Alert® LFA results were positive for all of the extracts. The monoclonal capture antibody was shown to recognize an epitope on the ricin A chain; however, differences in glycosylation do not appear to be important as this monoclonal antibody was able to recognize the deglycosylated ricin A chain. Furthermore, the monoclonal antibody binds to the A chains of both ricin D (large seeds) and ricin E (small seeds). Ricin D and E have similar A chains; the difference between the 2 ricins is in the B chain.[16] However, this antibody apparently recognizes an epitope that is shared between the A chains of RCA_{60} and RCA_{120}, which is not surprising considering that they have 90% homology.[14] Thus, the LFA cannot accurately measure the ricin concentration in extracts; only a composite estimate of RCA_{60} and RCA_{120} can be made. Garber[50] observed similar results using the ricin ELISA from Tetracore. Other commercially available tests have similar limitations. For example, the RAMP™ LFA detects the presence of the ricin B chain in addition to the ricin A chain, RCA_{60}, and RCA_{120}.[44]

The Ricin BioThreat Alert® assay did not give false-positive results with extracts from near neighbors or with any of the 66 lectins tested, including wheat germ agglutinin, which had been shown previously by Dayan-Kenigsburg et al[32] to produce a false-positive result in this LFA at concentrations $\geq 15\,\mu g/mL$. However, neither *P. sativum* (pea) or *A. hypogea* (peanut) lectins produced false-positive results at concentrations as high as 1 mg/mL and 250 μg/mL, respectively. The differences between our results and those of Dayan-Kenigsburg et al[32] could be due to differences in the concentration of wheat germ agglutinin as we used a concentration of 0.67 μg/mL, a 32-fold difference. This suggests the need to test higher concentrations of this lectin. We cannot say with certainty that none of the near neighbors will produce a false-positive result because we were limited in the number of *R. communis* near neighbors ($n = 14$) that we were able to obtain. Most of the near neighbors are not found in the United States, and very few seeds are available commercially. For that reason, we obtained leaves for some of the plants for which seeds were not available with the knowledge that the highest concentration of ricin is found in the seeds. In order to expand the near-neighbor panel, we included 21 near neighbors of the legume *Abrus precatorius,* which produces the class II-RIP abrin.

The Ricin BioThreat Alert® LFA has been used in the field to identify the potential presence of ricin in powders and other environmental samples. We evaluated the ability of this LFA to detect the presence of RCA_{60} and RCA_{120} in powders spiked with an extract of the Hale cultivar of *R. communis*. The LFA gave positive results with all of the spiked powders tested. However, most of the powders gave reduced readings when compared to Hale extract alone. It is not clear whether this reduction is due to (1) an inhibition of antigen binding to one of the antibodies; (2) a reduction in the flow rate in the strip (perhaps due to increased viscosity of the solution, which would alter the kinetics of the assay); or (3) binding of RCA_{60} or RCA_{120} by the powder and their subsequent removal during centrifugation. Other investigators have observed flow problems with this LFA when testing viscous solutions.[32]

Because this assay does not discriminate among RCA_{60}, RCA_{120}, and ricin A chain, it can be used only as a qualitative screening assay when testing unknown samples. In order to assess public health risk, positive LFA results must be confirmed at a laboratory with the capacity to specifically identify the presence of ricin.[51] The performance, cost, shelf life, ease of use, and rapidity of results suggests that this test is suitable for field and laboratory use as a screening assay.

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Address correspondence to:
David R Hodge, PhD
S&T BOD STOP 0201
Department of Homeland Security
245 Murray Lane
Washington, DC 20528-0201

E-mail: David.Hodge@hq.dhs.gov