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Research paper

Sample processing approach for detection of ricin in surface samples

Staci Kane[a,1], Sanjiv Shah[b,*,1], Anne Marie Erler[a], Teneile Alfaro[a]

[a] Lawrence Livermore National Laboratory, Livermore, CA, USA
[b] US Environmental Protection Agency, National Homeland Security Research Center, USA

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ABSTRACT

With several ricin contamination incidents reported over the past decade, rapid and accurate methods are needed for environmental sample analysis, especially after decontamination. A sample processing method was developed for common surface sampling devices to improve the limit of detection and avoid false negative/positive results for ricin analysis. Potential assay interferents from the sample matrix (bleach residue, sample material, wetting buffer), including reference dust, were tested using a Time-Resolved Fluorescence (TRF) immunoassay. Test results suggested that the sample matrix did not cause the elevated background fluorescence sometimes observed when analyzing post-bleach decontamination samples from ricin incidents. Furthermore, sample particulates (80 mg/mL Arizona Test Dust) did not enhance background fluorescence or interfere with ricin detection by TRF. These results suggested that high background fluorescence in this immunoassay could be due to labeled antibody quality and/or quantity issues. Centrifugal ultrafiltration devices were evaluated for ricin concentration as a part of sample processing. Up to 30-fold concentration of ricin was observed by the devices, which serve to remove soluble interferents and could function as the front-end sample processing step to other ricin analytical methods. The procedure has the potential to be used with a broader range of environmental sample types and with other potential interferences and to be followed by other ricin analytical methods, although additional verification studies would be required.

1. Introduction

Several ricin contamination incidents (Bozza et al., 2015) since the 2001 anthrax bioterrorism attacks in the US suggest a need for rapid and accurate methods for ricin analysis from environmental samples to complement the methods available for clinical samples. Ricin toxin for bioweapon production can be present in waste material generated during oil production from castor beans (*Ricinus communis*) or from purification and refinement of the castor bean pulp. Ricin toxicity may occur from inhalation (median lethal dose, LD_{50} ~ 21–42 μg/kg), ingestion (LD_{50} ~ 1–20 μg/kg), dermal penetration, or injection (LD_{50} ~ 1–1.75 μg/kg) (Grundmann and Tebbett, 2008). The ricin holotoxin is a heterodimeric, Type 2 ribosome-inactivating protein, consisting of an A- and B-chain, linked by a disulfide bond. The 34 kilodalton (kDa) A-chain with N-glycoside hydrolase activity de-purinates a key adenine residue in the 28S rRNA (ribosome), stops protein synthesis, and ultimately causes cell death. The 32 kDa B-chain is a lectin that is catalytically inactive. However, the lectin mediates specific binding (to carbohydrates) and transport of the holotoxin into host cells, after which the disulfide bond is cleaved, and the A-chain is fully functional. Therefore, the A-chain has extremely low toxicity outside the cell without the B-chain.

Several analytical methods are used to detect ricin, including immunoassays (Koja et al., 1980; Poli et al., 1994) and hand-held devices (Fulton and Thompson, 2007), in vitro cytotoxicity assays (Pauly et al., 2012), cell-based activity assays (Rastogi et al., 2010), mass spectrometric proteomic analysis (Darby et al., 2001), and real-time PCR for *R. communis* deoxyribonucleic acid (DNA) present in the ricin preparation (US Department of Health and Human Services, 2006; Felder et al., 2012). As part of a public health investigation of a white powder incident, many of these approaches were used (Schieltz et al., 2011), as well as a novel mass spectrometry-based activity assay that detected enzymatically active ricin (Kalb and Barr, 2009).

The Time-Resolved Fluorescence (TRF) immunoassay is a potential screening method using ricin-specific antibodies to determine the presence of ricin in environmental samples. TRF has also been used for detection of other toxins and bacterial pathogens (Peruski et al., 2002). The TRF assay is a solid-phase, immunosorbent assay that uses a biotin-labeled capture antibody attached to a streptavidin-coated support and a europium-labeled detector antibody that acts as a reporter for bound

* Corresponding author at: US Environmental Protection Agency, National Homeland Security Research Center, 8801 RR, 1200 Pennsylvania Ave., NW, Washington, DC 20460, USA.
E-mail address: shah.sanjiv@epa.gov (S. Shah).
[1] Authors contributed equally.

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ricin, forming an antibody-ricin-antibody complex (i.e., "sandwich"). Upon excitation, the europium ions are extremely stable and emit fluorescence with a longer lifetime than non-specific background fluorescence, usually > 100 s of microseconds to > one millisecond (Yuan and Wang, 2005), thereby producing a more selective and sensitive assay. The large difference between europium excitation (340 nm) and emission wavelengths (615 nm) and a sharp emission fluorescence peak (full width at half maximum of ~10 nm) further contribute to assay sensitivity. Finally, the Dissociation-Enhanced Lanthanide Fluorescence Immunoassay (DELFIA®; PerkinElmer [PE]) system used for TRF analysis includes an enhancement solution to release europium and stabilize it within surfactant micelles, which further acts to enhance the fluorescence (PerkinElmer Inc., 2015).

Although the TRF assay is an accepted method for ricin detection, it has not been evaluated with the pre- and post-decontamination surface samples expected to result from environmental response to a ricin incident. Recently, high background fluorescence levels have been noted for surface samples after bleach decontamination for the Tupelo, Mississippi (MS) ricin incident (US Environmental Protection Agency, 2013; U.S. Environmental Protection Agency, 2013), leading to unsatisfactory and unreliable results. Specifically, elevated backgrounds were observed and were speculatively attributed to bleach residue or other substances extracted from the surface or from the sampling device with associated wetting buffer. In addition, high backgrounds (~8600 to 10,400 fluorescence counts) were reported from test samples (lacking ricin) prepared by sampling bleach-dried surfaces; these values exceeded typical background fluorescence values of < 2000 counts. It was not clear whether bleach residue, sampling material, sample wetting buffer, or a combination of these components led to high background fluorescence. In addition, environmental samples contain particulates that could lead to assay interferences, including elevated background fluorescence, resulting in inaccurate results.

To mitigate this issue, a sample processing approach was developed and evaluated with both sponge-stick and swab sample types typically used for ricin incidents including post-decontamination surface sampling. These materials were pre-wetted with neutralizing buffer (NB) and tested with and without bleach residue from mock surface sampling. The sample processing procedure also enabled concentration of ricin and an improved detection limit with 10 kDa centrifugal ultrafiltration (UF) devices, which (depending on the device type [0.5 or 2.0 mL]) provided ~12- to 30-fold ricin concentration (based on fluorescence counts). These devices have not reportedly been used for biotoxin cleanup and concentration for bioterrorism incident samples, although UF has been used to concentrate ricin for toxicity studies (Garber, 2008). Based on these findings, the sample cleanup and concentration approach may reduce false negative results in complex environmental samples that could lead to human exposure if contaminated facilities were re-opened prematurely, or to reduce false positive results that could trigger additional unwarranted decontamination activities.

2. Materials and methods

2.1. Immunoassay reagent preparation

For capture antibody, affinity-purified polyclonal goat anti-ricin antibody (CRP, Cat. No. AB-AG_RIC) from the U.S. Department of Defense (DOD) Critical Reagents Program (CRP), now known as the Defense Biological Product Assurance Office (DBPAO), was used. The capture antibody was biotin-labeled using an EZ-Link™ NHS-PEG4 Biotinylation kit (Life Technologies, Cat. No. 21455) following manufacturer's instructions. Absorbance measurements (500 nm) were used with the 4′-hydroxyazobenzene-2-carboxylic acid displacement assay to estimate biotin incorporation following the manufacturer's procedure. Using this method, labeled capture antibody had approximately two or seven biotins per antibody for two different lots. The capture antibody was diluted in DELFIA® Assay Buffer from a 200–400-fold concentrated stock solution just prior to use (final antibody concentration ~200 ng per well).

For detector antibody, Monoclonal Anti-Ricin Toxin A-Chain, Clone RAC18 (BEI Resources, Inc., Manassas, VA; Cat. No. NR-9571 [IgG2aK antibody class]) was used. Detector antibody was europium [Eu]–labeled and quantified by PE Custom Labeling Service (Waltham, MA) with Eu:protein ratios of 3.5:1, 3.9:1, and 1.64:1 for different lots. The labeled detector antibody was diluted in assay buffer 100-fold to 1000-fold (used at ~37 to 370 ng/well, depending on the lot) to determine optimal fluorescence signal in the TRF assay. The optimal dilution was experimentally determined by finding the lowest dilution for which the counts for buffer without ricin were < 2000, and for buffer with ricin (100 pg/well) were 10^4–10^5. The detector antibody was prepared as appropriate aliquots and stored at − 80 °C. For individual experiments, detector antibody aliquots were thawed, diluted appropriately in assay buffer, and filtered through a 0.22-μm Millex-GV filter unit (Millipore, Cat. No. SLGV033RS), just prior to use.

2.2. Sample preparation

Toxin samples were generated from unconjugated *Ricinus communis* Agglutinin II (RCA 60, Ricin; Vector Laboratories, Cat. No. L-1090; Burlingame, CA). Dilutions of ricin holotoxin were made in endotoxin-free 1X-phosphate buffered saline (PBS) (Cat. No. P0300, Teknova, Hollister, CA). Other buffers were also tested to assess TRF performance including PBS with 0.05% Tween-80 (PBST), PBS with 3% Bovine Serum Albumin (BSA; Fraction V, VWR, Radnor, PA; Cat. No. EM2930), and PBST with 3% BSA. These buffers were compared with PBS by making 10-fold serial dilutions in the appropriate buffer from the original ricin stock solution (in PBS) at 100 μg/mL. Dilutions were performed to generate 1 μg/mL to 1 ng/mL concentrations in 1-mL volume (e.g., 100 μL ricin solution added to 900 μL buffer). Since 10 μL of ricin solution were added per well, the final concentrations ranged from ~10 ng to 10 pg ricin per well. Triplicate TRF analyses were performed for each sample replicate (dilution).

Ricin A-chain (Cat. No. L9514, Sigma-Aldrich, St. Louis, MO) was used as a positive control. The A-chain was suspended in 40% glycerol containing 10 mM phosphate, pH 6.0, 0.15 M NaCl, 10 mM galactose, and 0.5 mM dithioerythritol. For initial experiments, the A-chain was diluted in PBS to make a ~1 μg/mL stock; however, poor stability was noted because counts decreased significantly upon storage for even 1 day (data not shown). A-chain dilutions were subsequently made in DELFIA® Assay Buffer (Cat. No. 1244-111) just prior to use, and the dilutions were used within one hr of preparation. Two serial dilutions (~11-fold; 10 μL added to 100 μL) were performed in assay buffer on the plate; fluorescence counts for the three dilutions were used to ensure consistent assay performance between experiments. Solutions containing ricin holotoxin or A-chain were handled and processed in a ducted, Class II biosafety cabinet.

2.3. Preparation of surface coupons with bleach residual

Fresh 10% bleach was prepared using Ultra Clorox® Germicidal Bleach (one part) and autoclaved double distilled water (nine parts). Prior to use, 10 × 10 in. (25.4 × 25.4 cm) stainless steel coupons (20 Gauge 304–2B; Alro Steel, Cat. No. 14812194) were disinfected with 10% bleach, rinsed with water and then 70% isopropyl alcohol, and dried in the biosafety cabinet. For swab sampling, 4 × 4 in. (10.2 × 10.2 cm) sections were taped off on the 10 × 10 in. squares. To mimic bleach decontamination conditions for the Tupelo, MS incident, coupons (placed horizontally) received 10% bleach applied by hand sprayer and were allowed to remain wet for 10 min. Additional bleach was added during the 10 min when areas on the coupons appeared to dry. All coupons were dried overnight prior to sampling. Triplicate samples from bleach-disinfected surfaces, along with one