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Article

Surface Sampling of a Dry Aerosol Deposited Ricin

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Abstract: Sampling of small molecules from both porous and non-porous surfaces poses a significant challenge across biological agents. Particle sizes of toxins are smaller than living organisms and can be extremely toxic at low level concentrations. A small number of studies evaluating sampling efficiencies of commercial off the shelf (COTS) materials have been performed with toxins and proteins. However, they have been limited to non-ricin stimulants with drastically different physical properties than their native counterparts. We have identified a commercially available non-toxic recombinant ricin, complete with both A and B subunits present, which can be recognized by antibodies commonly used to assay native ricin. In evaluating recovery efficiency, we deposited the recombinant ricin by both liquid deposition, and as a dry aerosol. Our studies demonstrated a significant difference in recovery efficiencies from liquid deposited ricin, ranging between 30% and 70%, than from an aerosol generated deposition ranging from below detectable levels to 22%, depending on the contaminated surface and swab material being used. This study demonstrates the necessity for accurate dissemination techniques of sampling technologies for the consideration of use in an environment where suspected toxin contamination is being evaluated.

Keywords: surface sampling; recovery efficiency; bioterrorism; detection; ricin; toxin

1. Introduction

The anthrax attacks of 2001 showcased the non-traditional attempts by which individuals or extremist groups can attempt to cause harm to the United States or other countries around the world. These events led to an increase in defense research and mitigation protocols for biological and toxin contamination which were either non-existent or immature in their development at the time. Surface sampling of biological agents was, and still is, an area of focus that has received significant attention as a result of the 2001 anthrax letters. However, relative to the published data available for surface sampling of biological warfare agents, very little focus has been directed to surface sampling studies of dusty agents, small molecules attached to an aerosolized carrier, and biologically derived toxins such as ricin. This lack of attention is evidenced by a single published report on surface sampling of a ricin-like compound since the 2001 attacks [1]. Ricin is one of the most toxic naturally occurring compounds on Earth with amounts as low as 500 μg being lethal to a human adult. Currently, there is no proven treatment for individuals poisoned by this toxin [2]. The protein resides naturally in the castor bean and accounts for approximately 1%–5% of the bean's total mass [3,4]. Despite the availability and potential use by terrorists, there is little peer-reviewed literature reporting surface sampling data of native ricin. This lack of available information and research is likely due to the toxic nature and subsequent health risk for researchers.

In addition to the toxicity of the native protein, a suspected reason for the lack of published toxin surface sampling data is the inherent difficulty in finding suitable non-toxic simulants. While there are many non-pathogenic organisms within the *Bacillus* genus which are commonly used as *B. anthracis* Ames simulants such as *Bacillus thuringiensis*, *Bacillus atrophaeus*, and *Bacillus subtilis*, as well as non-pathogenic *Bacillus anthracis* strains such as *Ba* sterne and *Ba* delta sterne, until recently the sampling community has been lacking a non-toxic molecule of close homology to ricin to perform sampling studies. Historically, plant oils, egg white albumen, and other ricin-like plant proteins have been utilized as a non-toxic substitute for toxins and dusty agents. Unfortunately, all of these materials have inherent characteristics and properties such as sticking to equipment and not being recognized by antibodies used in ricin assays thus making them poor simulants for the ricin molecule.

Recently, a recombinant non-toxic ricin has become commercially available which overcomes the previous ricin simulant challenges. This protein contains both the A and B ricin subunits which are covalently bonded together significantly decreasing the toxicity of the molecule by rendering the protein incapable of entering the cell. Additionally, a point mutation at the active site of the A subunit ensures that in the unlikely event that the A subunit is able to enter the cell, the molecule will be unable to properly bind to rRNA and inhibit protein synthesis, the mechanism of toxicity of ricin, further ensuring non-toxic characteristics. Additionally, the recombinant ricin used in this study is recognized by commercially available ricin antibodies currently used to detect ricin in biodefense environmental samples at sensitivity levels equivalent to that of native ricin.

Our study evaluates four commercial off the shelf (COTS) swab materials, cotton, rayon, polyurethane (Dacron), and a polyurethane macrofoam, traditionally used in sampling studies, for the ability to recover both liquid deposited and a dry aerosol deposition of recombinant ricin from glass, polycarbonate, vinyl tile, and Chemical Agent Resistant Coating (CARC) painted steel coupons. Traditionally, evaluation of surface sampling materials and sampling efficiencies have been performed using liquid

suspensions of biological agents or toxins despite numerous examples of contaminations and attacks, such as the 2001 anthrax letters, in the form of dry aerosols [5–9]. A recent thorough side-by-side comparison of recovery of liquid deposited and dry aerosol deposited *Bacillus atrophaeus* spores was performed with statistical differences in sampling efficiencies attributed solely to deposition method [10]. To date, only one publication addresses surface sampling of a ricin-like substance, ricin agglutinin II, in which the authors deposited the agent onto surface materials as a liquid suspension and reported rather poor recovery efficiencies of less than 3% [1]. The study presented in this manuscript builds on the previous reports by combining a side-by-side comparison of a non-toxic recombinant ricin molecule deposited onto surfaces by both liquid deposition and a dry deposited aerosol.

2. Results and Discussion

The amount of recombinant ricin recovered from the full submersion control coupons used for calculating recovery efficiencies was 10 ng, 7.1% CV, for each liquid deposition replicate and 10 μg, 6.3% CV, for each aerosol deposition replicate. Recovery efficiencies for all four swab types on all four surface materials were greater when recovering liquid deposited agent than dry aerosol agent from each surface material. In every swab type and surface material comparison between deposition methods, we found there to be a statistically significant difference with a confidence level exceeding 99.99% (p-value equals 0.0001). On glass coupons, Dacron and cotton swabs recovered 70% and 57%, respectively of the liquid deposited material while rayon and macrofoam recovered 55% and 61% respectively (Table 1). These numbers are significantly more than observed with the dry aerosol deposition where Dacron swabs recovered 13% of the aerosolized agent from glass. Cotton and macrofoam recovered 7% and 10% respectively, and rayon failed to recover an amount of material exceeding the background electrochemiluminescent (ECL) signal. On polycarbonate, a similar surface material, Dacron, Cotton, and macrofoam swabs recovered 73%, 59%, and 65% respectively of the liquid deposited ricin while the rayon swab recovered 64% of the liquid deposited material. However, for the dry deposited ricin, Dacron swab recovered only 38% of the agent aerosolized onto polycarbonate, and the cotton, macrofoam, and rayon swabs recovered 13%, 12%, and 8% respectively (Table 1).

Of the two materials with irregular surfaces, CARC painted steel and vinyl floor tile, recovery efficiencies of liquid deposited protein were higher when sampling from the CARC painted steel. Dacron recovered 76% of the agent, Rayon recovered 60%, and Cotton and Macrofoam recovered 50% and 65% respectively of the ricin seeded by liquid deposition. However, the recovery efficiencies of each swab material were much lower with the dry deposited agent. Only 25% of the deposited ricin was recovered with a Dacron swab and cotton, rayon, and macrofoam each failed to recover a measurable amount of protein from the CARC painted steel coupons. On vinyl floor tile, Dacron swabs recovered 70% of the liquid deposited material, macrofoam and cotton recovered 58% and 47% respectively, and rayon swabs recovered 64% of the liquid deposited protein. The recovery efficiencies were significantly lower when sampling dry deposited agent from vinyl floor tile. Dacron swabs recovered 18%, cotton, rayon, and macrofoam failed to recover a measurable amount (Table 1).

Table 1. Evaluation of recovery efficiency of cotton, dacron, rayon, and macrofoam swabs from glass, Chemical Agent Resistant Coating (CARC) painted steel, polycarbonate, and vinyl coupons deposited with liquid and dry aerosolized recombinant Ricin. Each combination of swab, surface material, and deposition method consists of 30 samples composed of three experimental replicates of 10 samples each. In every instance a statistical significance between recovery efficiencies of deposition method was confirmed at a confidence level exceeding 99.99%.

Surface	Swab Material and Deposition Method	% Recovery (SD) [a]	Precision CV (%) [b]	Reproducibility CV (%) [c]
Glass	Cotton			
	Liquid	56.7 (13.6)	24.0	23.3
	Aerosol	7.2 (4.4)	49.1	61.4
	Dacron			
	Liquid	69.5 (10.0)	14.4	7.6
	Aerosol	12.9 (6.6)	51.3	40.9
	Rayon			
	Liquid	54.6 (6.1)	11.1	4.6
	Aerosol	BDL	NA	NA
	Macrofoam			
	Liquid	60.5 (6.7)	11.1	4.3
	Aerosol	9.7 (7.1)	73.3	15.9
CARC-painted steel	Cotton			
	Liquid	49.6 (15.3)	30.9	27.1
	Aerosol	BDL	NA	NA
	Dacron			
	Liquid	76.0 (9.9)	13.1	12.3
	Aerosol	25.2 (8.1)	32.3	6.9
	Rayon			
	Liquid	60.1 (7.4)	12.2	19.3
	Aerosol	BDL	NA	NA
	Macrofoam			
	Liquid	65.3 (5.9)	9	3.1
	Aerosol	BDL	NA	NA
Polycarbonate	Cotton			
	Liquid	58.7 (13.1)	22.3	20.4
	Aerosol	13.1 (9.4)	72.1	30.5
	Dacron			
	Liquid	72.8 (5.4)	7.5	3.5
	Aerosol	38.5 (10.0)	25.9	5.2
	Rayon			
	Liquid	64.4 (10.4)	16.1	18.9
	Aerosol	7.5 (11.3)	149.5	80.5
	Macrofoam			
	Liquid	65.4 (7.1)	10.8	11.3
	Aerosol	12.2 (8.7)	71.7	35.2

Table 1. *Cont.*

Surface	Swab Material and Deposition Method	% Recovery (SD) [a]	Precision CV (%) [b]	Reproducibility CV (%) [c]
Vinyl	Cotton			
	Liquid	47.3 (17)	35.9	32
	Aerosol	BDL	NA	NA
	Dacron			
	Liquid	70.5 (7)	9.9	20.1
	Aerosol	18.0 (8.5)	47.1	26.1
	Rayon			
	Liquid	64.4 (6.2)	11.3	23.4
	Aerosol	BDL	NA	NA
	Macrofoam			
	Liquid	58.3 (7.1)	12.2	8.9
	Aerosol	BDL	NA	NA

[a] Standard deviation of all sampled coupons for each deposition and swab set; [b] CV of % recovery between replicates for each deposition and swab set; [c] CV of % recovery of all sampled coupons for each deposition and swab set. BDL Below Detectable Limit of the ECL Assay (0.5 pg·mL^{-1}).

The high recovery efficiencies of liquid deposited recombinant ricin and sensitivity to ricin assays demonstrate the suitability of this non-toxic surrogate for one of the most poisonous natural toxins on Earth. Unlike previous sampling studies using ricin-like proteins, toxoids, or material such as egg white albumen, we were able to accurately assay a modified recombinant ricin protein in its native form using antibodies capable of detecting native ricin. We have also demonstrated that this recombinant ricin is beneficial to surface sampling studies in that future assessments of equipment and sampling materials can be designed to accurately assess recovery efficiencies of true toxin protein in both liquid and aerosolized form. In addition to surface sampling, efficiencies and efficacies of mitigation techniques and decontamination materials and equipment have been limited to select laboratories capable of handling agents as toxic as ricin. With the use of the non-toxic recombinant ricin, the sampling community has the opportunity to improve on general knowledge and close surface sampling technology gaps which exist between toxins and biological agents. Multiple liquid or aerosol surface sampling assays have been performed on a variety of biological materials [1,5–9,11]. However, liquid and dry deposition side-by-side comparison studies of recovery efficiencies of COTS materials have previously only been performed on *Bacillus* spores [1,5–9,11,12]. Based on the previous side-by-side comparison study with BG spores, the authors were anticipating statistically differing recovery efficiencies between agent deposition methods when comparing identical swabs and surface materials [12]. Although Frawley *et al.* described a low recovery efficiency of a ricin-like protein, it was unexpected to the authors that the recovery efficiency of several of the swab materials on multiple surface types would be below detectable ECL limits when assaying an aerosol deposited ricin considering the sensitivity levels of the ECL assay [1]. Only a single material, Dacron was capable of recovering a relatively significant amount of aerosolized material compared to that of liquid deposited protein. The recovery efficiencies of the 10 μg of recombinant ricin seeded onto the various coupon types of liquid deposited material for all four swab types was between 47% and 76%, depending on the surface material. Dacron swabs had the highest

recovery efficiencies of aerosolized material, recovering 13% to 39% of the 10 ng of aerosolized agent deposited onto the coupons, depending on the surface material, while the other three swab types only recovered up to 10%.

The inability to sample the small molecule agent at levels comparable to that of *B. atrophaeus* spores can be attributed to differences in agent and swab interactions, the amount of material deposited onto the coupons, and the deposition method. Unfortunately, little effort has been placed on understanding the physical and molecular interactions between agent and recovery material and the forces and factors responsible for these interactions are still largely unknown. An argument can be made that the low levels of aerosolized agent could play a role in the low level detection of the ricin. While concentration very likely has an impact on recovery efficiency, it is important to note that the control samples and liquid deposition samples were diluted to the picogram concentrations while the experimental aerosol samples were not diluted due to lower levels of agent recovery. The authors also point out that the recovery efficiency of the Dacron swab was rather significant with the respect to the lower concentration of material deposited onto the coupons as an aerosol. Although there is 1000X less aerosolized material deposited onto the coupons than the liquid deposition method, the recovery efficiencies of the aerosolized material using the Dacron swabs was only 2X–5X better than the recovery efficiencies of the liquid deposited material. It is likely that differences in recovery efficiencies due to the concentration of starting material are insignificant in comparison to the deposition method. Another potential impact on our aerosol deposition recovery efficiencies is the deposition method itself. With the liquid deposition method, the agent is applied to the coupons in few and relatively concentrated locations. As the liquid evaporates, presumably, the agent will concentrate into small droplets until the suspension dries completely into smaller areas than initially deposited as observed with SEM of biological material [12]. This will produce few but significantly higher concentrated overall deposits of agent which is likely to be easier to recover with sampling technologies. However, with an aerosol deposition, an evenly distributed coating of dry agent likely is applied to the entirety of the coupon, as observed with SEM of biological material, and no mechanism is present to concentrate the toxin [12]. This evenly distributed coating of material is not as easily recovered as the localized and more concentrated evaporated liquid depositions. To truly understand the reasoning between differences in recovery efficiencies among deposition methods, sampling materials, and surface substrates, thorough measurements of critical components of agent substrate interactions, such as adhesion forces and concentration of deposited material need to be thoroughly investigated. Not only will this insight better explain the observations of this work and others which have previously been performed, but it could also be used in developing technologies which are more consistent in agent recovery and hazard assessment amongst many different possible classes of surface substrates.

3. Experimental Section

2.1. Swab Description

In this study, four different swab materials were utilized in determining recovery rates of a variety of surface materials. The swabs used were: cotton-tipped (Puritan; Fisher Scientific, Suwanee, GA, USA; Catalog No. 14-959-102), dacron-tipped (FisherBrand; Fisher Scientific, Suwanee, GA, USA;

Catalog No. 14-959-97A), rayon-tipped (Starplex Scientific Inc; VWR, Suwanee, GA, USA; Catalog No. 14211-774), and a polyurethane macrofoam-tipped swab (Critical Swab; VWR, Suwanee, GA, USA; catalog No. 10812-046, discontinued at time of publication).

2.2. Coupon Description

Four unique surface materials served as coupons on which the ricin was deposited: glass, Chemical Agent Resistant Coating (CARC) painted steel, polycarbonate, and vinyl tile. All coupons were cut 3 mm thick, 2 cm × 5 cm by the machine shop on the Aberdeen Proving Ground, Edgewood Area (APGEA). Prior to any deposition, all coupons were sterilized with the use of an autoclave (BetaStar Corporation, Telford, PA, USA).

2.3. Deposition Chamber

In order to determine if aerosol deposition would yield recovery differences in comparison to liquid deposition techniques it was necessary to design an aerosol deposition chamber that could reliably and uniformly deposit the biological test specimen throughout the testing chamber. Two ionizing fans were installed to decrease the static charges within the circular deposition chamber and to continually mix the air during the aerosolization of the ricin. The rotating base of the platform was rotated at a speed such that an individual coupon would not be exposed to any single point in the chamber for a period of time any greater than any other location and which would not create turbulent airflow within the chamber. Further explanation and characterization of the chamber is described in further detail in Edmonds *et al.* 2009 [12].

2.4. Preparation of Dry Coupons

The dry deposited coupons were placed inside a circular aerosol chamber consisting of a rotating platform and removable lid (manufactured in-house with a 30 cm turntable; Barnard LTD, Chicago, IL, USA). Ten coupons of each material per swab type and an additional ten glass control coupons were positioned on the platform in a predetermined deposition zone. When this was completed, the lid was replaced, the rotating platform was plugged in, and the ionizing fans (3 M Mini Air Ionizer, Model 960) were turned on, and left on, during the deposition process. Two milliliters of a 1.06 mg protein/mL stock solution was loaded into a nebulizer (Aeroneb Go 7070 micropump nebulizer, Active Forever, Scottsdale, AZ, USA) and aerosolized onto the coupons through a slit in the top on the chamber lid to achieve a desired 10 ng cm^{-1} of recombinant material as verified with control recovery samples. After the deposition was complete, the fans were turned off and the platform was allowed to continuously rotate overnight as described in detail in Edmonds *et al.* 2009 [12].

2.5. Preparation of Liquid Coupons

For each surface material, ten coupons per swab type and ten glass control coupons were set inside of a category class II type B2 bio-safety cabinet. Each coupon received five 20 microliter drops of a 0.1 mg/mL solution diluted from the stock solution by the addition of 1 X PBS. The coupons were

allowed to air dry inside the bio-safety cabinet with the airflow remaining on and the sash open for a minimum of three hours until all liquid had completely evaporated.

2.6. Sampling

The sampling process was performed identically for the dry deposited and liquid deposited samples. Each of the ten glass control coupons were placed into 50 mL conical tubes containing 10 mL 1 X PBS +0.1% TritonX-100. The additional coupons were broken down into ten coupons per swab type. All swabs were autoclaved and pre-moistened with 100 μL of sterile water prior to sampling. Each coupon was swabbed with a single swab methodically, 5 times along the length, rotated 90 degrees, swabbed 12 times along the width, rotated again 90 degrees and swabbed an additional 5 times along the length. After swabbing, the swab heads were snipped off with sterile wire cutters into individual 50 mL conical tubes each containing 10 mL 0.1% PBS-Triton-X-100.

After the sampling was complete, all of the tubes containing either swab samples or the submerged glass coupon controls were subjected to ten minutes of vortexing using a large area mixer (Glas-Col; Catalog No. 099A-LC1012; Terra Haute, IN, USA). After vortexing, the tubes were then placed in a sonic bath (Branson 5510; Branson Ultrasonics Corporation, Danbury, CT, USA) for an additional ten minutes. At the completion of processing, two milliliters of each sample was collected and reserved for ricin assays.

2.7. Recombinant Ricin

Recombinant ricin (Product Name TST10114, Batch Number AC05001A) used in this study was acquired from Twinstrand Therapeutics Inc., Burnaby, BC, Canada V5A 1W9 [13]. Prior to use, protein was stored at −20 degrees centigrade in 1 X PBS, pH7.4, at a concentration of 1.06 mg protein/mL. Stock solution was diluted with 1 X PBS to achieve desired working solution concentration.

2.8. Ricin Assay

Samples were analyzed in duplicate using a Sector PR 100 (Meso Scale Discovery (MSD); Gaithersburg, MD, USA) and multi-array ricin plates (MSD; Lot No. 594106) [14]. A standard curve generated with the recombinant ricin toxin was run on each plate. A 1X working stock of STAG-labeled anti-ricin detector antibody (MSD; Lot No. 594107) was prepared in MSD antibody diluent (MSD; Lot No. 594107), and 20 μL was added to each well of the plate. One hundred microliters of each sample was then added to the plate. The plate was covered with a plate seal and incubated on a plate-shaker (Labnet International Orbit P4; Edison, NJ, USA) for 60 minutes at 900 rpm. Each well was then washed three times with 200 μL of 1X Phosphate Buffered Saline (Sigma-Aldrich: St. Louis, MO, USA; Catalog No. P3813) using a plate washer (Tecan 96 PW; Durham, NC, USA). One hundred and fifty microliters of 1X MSD T Read Buffer (MSD; Lot No. Y0140206) was then added to each well, and the electrochemiluminescent (ECL) signal was read using the Sector PR 100. The detection limit for this assay is 0.5 pg mL^{-1}.

2.9. Statistics

Ten coupons were used in each experimental replicate and three experimental replicates were performed for each swab and deposition set. Pairwise comparisons between deposition methods were done by performing a Welch's t-test, which allows for unequal sample sizes, data that are not normally distributed, and variances which are not equal. Percent recovery was defined as the amount of material recovered after sample processing relative to the known concentration of material deposited onto the sampling coupons. Coefficient of Variation (CV) is defined as the ratio of the standard deviation to the mean value of recovered material.

4. Conclusions

As with biological agents, it is necessary to assay sampling materials used for toxins with the best available technology and techniques which mimic the environment containing the agent. Although significant developments in understanding surface sampling have occurred since one of the first recorded sampling papers appeared in 1917, researchers continue to employ procedures and ideologies from that time period which may not be suitable in the modern environment of chemical and biological defense [15]. In addition to the well documented anthrax cases in 2001, other instances of anthrax and ricin contamination occurred, including one instance in Danbury, Connecticut where two individuals were sickened from cutaneous anthrax infections originating from animal hides used for drum making, and another report of ricin poisoning of a man in a Las Vegas, Nevada hotel where ricin powder was found [16,17]. In both of these cases, when determining whether areas are safe for reentry and re-occupancy, it is essential to use sampling and recovery materials and methods which have been tested, assayed, and calibrated for their appropriate use which, in these cases along with the anthrax letters of 2001, is a dry aerosol powder. The data presented here suggests a need for further evaluation of recovery materials to gauge their effectiveness of surface sampling of a wide array of agents in order to be prepared in the event of another terrorist attack or unintentional release of a small molecule.

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