Detection of Trichothecene Mycotoxins in Sera from Individuals Exposed to *Stachybotrys chartarum* in Indoor Environments

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ABSTRACT. To date, no study has effectively demonstrated a direct human exposure to mycotoxins in mold-contaminated buildings. Therefore, the authors investigated the presence of trichothecene mycotoxins in sera from individuals exposed to indoor molds (specifically *Stachybotrys chartarum*). Sera from occupants of contaminated (test samples, $n = 44$) and uncontaminated (control samples, $n = 26$) buildings were analyzed using a competitive enzyme-linked immunosorbent assay (ELISA) highly specific for macrocyclic trichothecenes. Twenty-three samples were significantly different ($p < 0.05$) from normal human serum tested in the same manner, whereas only 1 of the control samples tested positive. Mass spectrometry analysis could not confirm the presence of intact *S. chartarum* macrocyclic trichothecenes. The authors hypothesize that this result was caused by uncharacterized ELISA-reactive metabolic breakdown products. Data from this study suggest that trichothecene mycotoxins can be demonstrated in the tissues of certain individuals exposed to *S. chartarum* in contaminated buildings.
<Key words: environmental exposure, mold, mycotoxins, serum, *Stachybotrys chartarum*>

POOR INDOOR AIR QUALITY (IAQ) and its relationship to human health have been the focus of many scientific investigations spanning hundreds of years. The factors that cause poor IAQ include, but are not limited to, poor ventilation/air circulation,[1] the presence of volatile organic compounds of microbial and chemical origin,[2,3] allergens such as animal dander,[4] and water damage/mold contamination. Of these, water damage and ensuing mold growth is one of the most important public issues facing the IAQ field. Problems associated with fungal contamination in indoor environments have long been recognized, and knowledge concerning its effects is vast and well-documented. Fungi have been shown to influence allergic hypersensitivity responses in several animal models[5–7] and in humans.[8,9] Additionally, symptoms of asthma arising from exposure to fungi have been recognized in certain individuals.[10–13] The symptoms of hypersensitivity and asthma are important when considering IAQ, but cannot account for the often-reported severe symptoms of nausea, dizziness, nosebleeds, physical and mental fatigue, and neurological disorders.[14–16] These latter symptoms may be the result of inhalation of airborne mycotoxins. Of the fungi commonly isolated from indoor environments, *Stachybotrys chartarum* is considered one of the most relevant to human health.[17]

Stachybotrys chartarum is known to produce a number of potent mycotoxins, in particular the macrocyclic trichothecenes verrucarins B and J; roridin E; satratoxins F, G,

and H; and isosatratoxins F, G, and H.[18,19] In addition, *S. chartarum* may be associated with human adverse health effects.[15,20–23] The members of the macrocyclic trichothecene family of mycotoxins are known to be potent inhibitors of protein synthesis in eukaryotes.[24–26] *S. chartarum* airborne mycotoxins have been studied in various laboratory settings[27–30] and have been shown to be detrimental to health in several animal models.[31–33] Currently, however, very few studies exist that demonstrate a relationship between exposure to airborne *S. chartarum* mycotoxins and adverse human health effects.[34,35] The aim of this study was to determine whether trichothecene mycotoxins could be detected in the sera of individuals who had been exposed to *S. chartarum* in contaminated indoor environments. Trichothecene mycotoxins detected in exposed individuals would be another step toward demonstrating an association between the presence of *S. chartarum* in buildings and reported human health complaints.

Materials and Method

Human serum samples. All serum samples were received from treating physicians frozen in 1-ml aliquots and de-identified in accordance with the requirements of the Texas Tech University Health Sciences Center Institutional Review Board. Samples were divided into 3 groups: Group 1 comprised samples from individuals with documented indoor *Stachybotrys* exposure ($n = 18$). IAQ firms verified the presence of *Stachybotrys* in air, tape, and bulk samples from buildings in which these individuals resided. Common symptoms included blurred vision, memory loss, fatigue, headache, nausea, loss of balance, cognitive deficits, rhinitis, sinusitis, nosebleeds, rashes, and allergies. More severe symptoms such as hemorrhage and seizures were also reported for certain individuals. Group 2 consisted of samples from individuals with reported exposure to non-identified molds ($n = 26$). Detailed demographics, history, and symptoms were provided for each sample. Symptoms ranged from minor (e.g., congestion, cough, watery eyes) to relatively severe (e.g., nausea, vomiting, dizziness, weakness, numbness). Group 3 sera comprised samples from individuals with no reported symptoms or known mold/mycotoxin exposure ($n = 26$), and hence served as negative controls.

Sample extraction and preparation. Before testing, serum samples were extracted in a manner similar to that described by Garbis et al.[36] and Hedman et al.[37] This was done as a simple and rapid means to remove high-molecular-weight proteins that produced an undesirably high background noise in the enzyme-linked immunosorbent assay (ELISA) (data not shown). Briefly, serum samples were individually aliquoted (200 µl) into sterile 1.5-ml polystyrene microcentrifuge tubes, followed by the immediate addition of 600 µl of high-performance liquid chromatography-grade acetonitrile (Fisher Scientific, Hampton, New Hampshire). Samples were allowed to sit at room temperature for 15 min, after which they were vortexed vigorously for 30 s. They were then centrifuged at 14,500 rpm for 3 min to pellet the precipitated proteins. The supernatants were individually transferred into clean 1.5-ml glass vials. Each sample was evaporated to completion under a gentle stream of dry nitrogen and resuspended in 200 µl prewarmed sterile water. The warming aided in the resuspension of samples. This was the final working solution for the ELISA. The acetonitrile extraction step resulted in negligible, if any, loss of potential trichothecene mycotoxins present. This determination was based on macrocyclic trichothecene-spiked normal human serum (NHS) (CELLect® human serum [Fisher Scientific]) samples that were extracted as already described.

Trichothecene mycotoxin analysis. Samples were analyzed for trichothecenes using the QuantiTox Kit for trichothecenes (EnviroLogix, Portland, Maine) in accordance with the manufacturer's instructions. This competitive ELISA kit incorporates antibodies highly specific for macrocyclic trichothecenes immobilized on polystyrene microtiter wells.[38] We have previously demonstrated that this assay is highly specific for macrocyclic trichothecene mycotoxins, particularly those produced by *Stachybotrys chartarum*.[27] Additional successful applications have been described.[39] All reagents and antibody-coated wells were allowed to equilibrate to room temperature before use. For testing, samples or control mixtures were added to wells in triplicate. Following a 15-minute incubation at room temperature (25° C), the optical densities of the wells were read at 450 nm using an EL-312 microtiter plate reader (Bio-Tek Instruments, Winooski, Vermont).

ELISA interpretation. Data were expressed as percentage inhibitions and relative trichothecene concentrations. Percentage inhibitions were derived from raw data and were based on patient samples, compared with NHS controls run in parallel. The percentage inhibition represents the degree of inhibition the test sample had on the capability of the satratoxin G–horseradish peroxidase (HRP) conjugate to bind to the immobilized antibody. Percentage inhibition values were calculated as done by Schick et al.,[40] using the following equation:

$$\% \text{ inhibition} = 100 \times 1 - [(OD_{450} \text{ sample} - \text{background})/(OD_{450} \text{ control} - \text{background})],$$

where OD_{450} is the optical density at 450 nm.

To obtain relative trichothecene concentrations, an ELISA-based macrocyclic trichothecene standard curve was developed by testing a mixture of 4 macrocyclic trichothecenes (satratoxins G and H, verrucarin A, and roridin A) in equal amounts (see Fig. 1 and Table 1). Satratoxins G and H were purified in our laboratory, using the method described by Hinkley and Jarvis[18] Roridin A and verrucarin A were purchased from Sigma Chemical Co. (St. Louis, Missouri). Dilutions were made

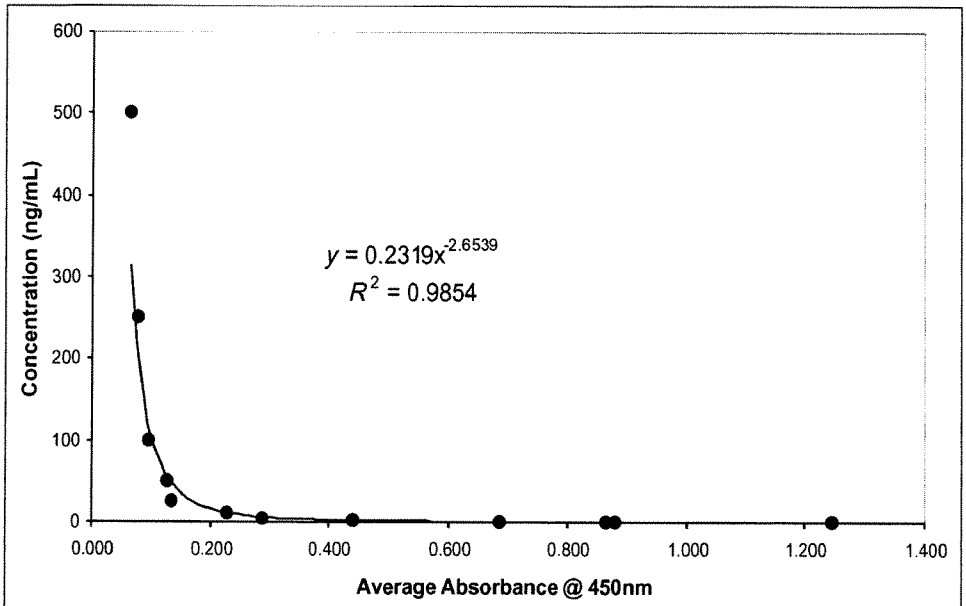

Fig. 1. Macrocyclic trichothecene standard curve based on acetonitrile extracts of normal human serum spiked with roridin A, verrucarin A, and satratoxins G and H.

Table 1.—Average Trichothecene Concentrations Based on Enzyme-Linked Immunosorbent Assay (500–0.1 ng/ml)

Trichothecene concentration (ng/ml)	OD_{450}		% Inhibition	
	Mean	*SD*	Mean	*SD*
500	0.07	0.003	95.9	0.2
250	0.08	0.002	95.2	0.1
100	0.10	0.004	94.0	0.2
50	0.13	0.002	92.0	0.1
25	0.14	0.01	91.4	0.4
10	0.23	0.003	85.5	0.2
5	0.29	0.02	81.9	1.5
2.5	0.44	0.01	72.3	0.3
1	0.69	0.05	57.0	3.2
0.5	0.86	0.04	45.9	2.4
0.25	0.88	0.14	44.9	8.9
0.1	1.25	0.03	21.8	1.8

Note: OD_{450} = optical density at 450 nm. All standard deviations (*SDs*) represent 3 replicates.

in NHS from a concentrated stock solution of the toxins in methanol (250 μg of each toxin), resulting in 12 test concentrations: 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 ng/ml. Each sample was extracted with acetonitrile, as already described, and tested using the ELISA. Average ELISA absorbance (from 3 replicates) at 450 nm were plotted against calculated toxin concentrations to generate a standard curve. Using this curve, an approximate trichothecene amount (in ng/ml) was determined for each sample.

Statistical analysis. Statistical analyses on serum samples were performed using Sigma Stat 3.0 software (SPSS, Inc., Chicago, Illinois). Mean OD_{450} readings of individual test samples were compared with NHS by using either a Student's *t*-test or 1-way analysis of variance (ANOVA). All requirements for normality and equal variance were met for these analyses. Statistical analysis also was performed among groups 1–3 using a Kruskal-Wallis 1-way ANOVA, because normality requirements could not be met. Statistical significance for all analyses was reported as $p < 0.05$.

Mass spectrometry analysis. Mass spectrometry analysis was performed on selected samples using a Sciex API 4000 Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS) system (Applied Biosystems, Foster City, California). A 150 × 4.6-mm Zorbax SB-C8 (5 μm particle size) analytical column (Agilent Technologies, Palo Alto, California) was used for the analyses. The flow rate was set for 0.6 ml/min with an injection volume of 20 μl. Acetonitrile-extracted serum samples were lyophilized and reconstituted in 1 ml methanol. The samples were sonicated and vortexed for approximately 5 min prior to aliquoting into a 96-well polypropylene sample block. Calibration standards (roridins A, E, H, and L-2; verrucarins A and J; satratoxins G and H; and isosatratoxin F) were kindly provided by Dr. Bruce B. Jarvis of the University of Maryland. These standards were prepared at concentrations ranging from 0.100 to 100 ppb in methanol. Samples (standards, test serum samples, and a roridin A-spiked NHS sample) were injected directly into the LC/MS/MS system. They were run in an aqueous mobile phase in which the gradient changed, in 16 min, from 80% acetonitrile to 90% of a 25-m*M* ammonium acetate, 0.5% acetic acid solution. Samples

Table 2.—Competitive Enzyme-Linked Immunosorbent Assay (ELISA) Results for Group 1 (Documented *Stachybotrys* Exposure) Serum Samples

Sample no.	% inhibition†		Relative trichothecene concentration (ng/ml)‡	
	Mean	*SD*	Mean	*SD*
1*	90.8	0.1	42.8	1.1
2*	92.8	0.3	83.6	9.2
3*	18.2	2.1	0.13	0.01
4	0.0	0.0	NA	—
5	11.1	8.9	0.11	0.03
6*	17.6	2.4	0.13	0.01
7	8.5	1.5	0.10	0.004
8*	22.0	1.1	0.10	0.004
9	4.2	3.4	(0.09)§	(0.01)
10*	24.6	10.3	0.17§	0.05
11*	14.0	5.5	0.12§	0.02
12*	13.7	1.4	(0.07)§	(0.003)
13*	14.2	4.5	(0.07)§	(0.01)
14*	14.3	1.4	(0.07)§	(0.003)
15*	18.0	0.6	(0.08)§	(0.002)
16*	21.2	9.4	0.10	0.03
17*	13.3	1.6	(0.07)§	(0.003)
18*	20.9	3.5	0.15	0.02

Notes: *SD* = standard deviation, and NA = not applicable.
*Significantly different from controls (normal human serum [NHS]) when the ELISA was performed.
†Values were based on results obtained using NHS when the ELISA was performed. Values represent triplicate wells.
‡Values were obtained using the trichothecene standard curve shown in Fig. 1.
§Values represent data that fell below the detection limit set on the standard curve. Extrapolated values are shown in parentheses.

Table 3.—Competitive Enzyme-Linked Immunosorbent Assay (ELISA) Results for Group 2 (Reported *Stachybotrys* Exposure) Serum Samples

Sample no.	% inhibition†		Relative trichothecene concentration (ng/ml)‡	
	Mean	*SD*	Mean	*SD*
19	0.31	0.53	(0.06)§	(0.01)
20	0.0	0.0	NA	—
21	0.0	0.0	NA	—
22	0.0	0.0	NA	—
23	7.8	0.9	0.12	0.003
24	7.6	4.6	0.10	0.01
25	5.2	5.8	(0.09)§	(0.02)
26	3.5	3.1	(0.09)§	(0.01)
27	0.44	0.76	(0.08)§	(0.01)
28*	8.0	0.3	0.11	0.001
29	2.9	3.8	(0.09)§	(0.01)
30	4.7	4.3	0.10	0.01
31	1.5	2.1	(0.09)§	(0.01)
32*	10.0	3.4	0.12	0.01
33	3.8	1.3	0.10	0.003
34*	14.7	6.7	(0.08)§	(0.02)
35	8.8	3.9	(0.06)§	(0.07)
36	2.9	2.8	(0.05)§	(0.004)
37*	20.9	8.7	0.10	0.03
38*	21.6	9.4	0.10	0.04
39	4.0	1.8	(0.06)§	(0.003)
40*	8.8	2.6	(0.06)§	(0.01)
41	4.0	4.9	(0.06)§	(0.01)
42*	8.9	1.1	(0.06)§	(0.002)
43*	10.4	0.5	(0.07)§	(0.001)
44*	13.6	3.8	(0.07)§	(0.01)

Notes: *SD* = standard deviation, and NA = not applicable.
*Significantly different from controls (normal human serum [NHS]) when the ELISA was performed.
†Values were based on results obtained using NHS when the ELISA was performed. Values represent triplicate wells.
‡Values were obtained using the trichothecene standard curve shown in Fig. 1.
§Values represent data that fell below the detection limit set on the standard curve. Extrapolated values are shown in parentheses.

were analyzed in multiple reaction monitoring (MRM) MS/MS mode, after which a full scan analysis via electrospray ionization (at 550°C) in positive ion mode was performed. Following the full scan, a precursor ion scan analysis was performed on the serum samples. With precursor ion scan analysis, molecular masses that arise from a common fragment ion could be derived. The logic for this approach was that, because trichothecene mycotoxins are analogs of each other, a common fragment would exist.

Results

Trichothecene mycotoxin analysis. Average ELISA percentage inhibitions and relative trichothecene concentrations for serum samples in groups 1 and 2 are shown in Tables 2 and 3, respectively. More positives (77.7%) were seen in group 1 individuals with known *Stachybotrys* exposure than in group 2 individuals (34.6%). The majority of calculated trichothecene concentrations were low, averaging below or just above the set limit of detection (0.1 ng/ml). Samples 1 and 2 from group 1 demonstrated unusually high concentrations (42.8 and 83.6 ng/ml, respectively). Table 4 shows the results obtained with group 3 (negative controls). Of these, only NC 2 demonstrated a statistically significant ($p < 0.05$) positive response in the ELISA, representing 3.8% of the negative control samples. Trichothecene concentrations for the majority of the negative controls fell below 0.1 ng/ml. Overall, there was a statistically significant difference ($p < 0.05$) between tests (groups 1 and 2) and controls (group 3). Individually, group 1 was significantly different from groups 2 and 3, but groups 2 and 3 were not statistically different from each other. Statistical significance was unchanged, even when the high responders from group 1 (samples 1 and 2) were removed from the statistical analysis (data not shown). Median percentage inhibitions for groups 1, 2, and 3 were 16.3%, 5.8%, and 2.6%, respectively.

Mass spectrometry analysis. Serum samples 1 and 2 from group 1 were subjected to mass spectrometry analysis because of their high trichothecene concentrations. On the basis of the standards used, no detectable compounds were present after MRM analysis in the

Table 4.—Competitive Enzyme-Linked Immunosorbent Assay (ELISA) Results for Group 3 (Negative Controls) Serum Samples

Sample no.	% inhibition[†]		Relative trichothecene concentration (ng/ml)[‡]	
	Mean	*SD*	Mean	*SD*
NC 1	0.0	0.0	NA	
NC 2*	15.0	3.4	(0.08)[§]	(0.008)
NC 3	5.6	2.6	0.11	0.01
NC 4	5.4	2.2	0.11	0.01
NC 5	4.0	2.1	0.10	0.01
NC 6	9.8	3.2	(0.09)[§]	(0.01)
NC 7	2.1	2.0	0.10	0.01
NC 8	1.9	2.8	(0.05)[§]	(0.01)
NC 9	4.8	4.8	0.11	0.02
NC 10	5.3	2.0	0.11	0.01
NC 11	0.0	0.0	NA	—
NC 12	1.4	2.3	(0.08)[§]	(0.01)
NC 13	4.2	3.7	(0.09)[§]	(0.01)
NC 14	0.0	0.0	NA	—
NC 15	2.3	3.3	(0.09)[§]	(0.01)
NC 16	1.1	2.0	(0.09)[§]	(0.01)
NC 17	0.25	0.33	(0.09)[§]	(0.002)
NC 18	0.74	0.80	(0.09)[§]	(0.01)
NC 19	0.71	1.2	(0.08)[§]	(0.01)
NC 20	4.9	2.7	(0.06)[§]	(0.004)
NC 21	10.1	4.9	(0.07)[§]	(0.01)
NC 22	2.1	3.6	(0.05)[§]	(0.01)
NC 23	6.6	6.8	(0.08)[§]	(0.01)
NC 24	3.3	1.2	(0.05)[§]	(0.002)
NC 25	1.3	1.3	0.10	0.01
NC 26	5.1	4.2	(0.06)[§]	(0.01)

Notes: *SD* = standard deviation, and NA = not applicable.
*Significantly different from controls (normal human serum [NHS]) when the ELISA was performed.
†Values were based on results obtained using NHS when the ELISA was performed. Values represent triplicate wells.
‡Values were obtained using the trichothecene standard curve shown in Fig. 1.
§Values represent data that fell below the detection limit set on the standard curve. Extrapolated values are shown in parentheses.

experimental serum samples. Limits of detection for the standards were 0.100 ng/ml for the roridins and verrucarins, 1.00 ng/ml for isosatratoxin F, 2.00 ng/ml for satratoxin H, and 10.0 ng/ml for satratoxin G. Roridin A was positively identified in the spiked control sample, demonstrating that the cleanup methods were successful in extracting the trichothecene mycotoxins. A full-scan analysis of the experimental serum samples between m/z = 155–700 atomic mass units generated several significant peaks. One peak of interest at a mass of 414 had spectral qualities similar to the trichothecene standards (i.e., a similar adduct pattern). Precursor ion scan analysis resulted in the discovery of 2 common fragments with precursor masses of 440 and 484. The 484 mass was the expected mass of verrucarin J, but had a different retention time on the column than the purified standard. These masses were unique to the test serum samples and were not detected in the roridin A-spiked NHS positive control sample.

Discussion

In this study, we were successful in demonstrating the presence of trichothecene mycotoxins in serum samples from individuals exposed to mold (primarily *Stachybotrys*) in water-damaged indoor environments. Our findings indicate that these highly toxic compounds can actually be found in people exposed to these environments and, therefore, have the potential to negatively affect the health of such individuals. This relationship is further strengthened by our previous investigation, which demonstrated that *Stachybotrys* trichothecene mycotoxins can become airborne in a controlled situation and have the potential to do so inside a building.[27]

Through use of an ELISA that incorporated a macrocyclic-trichothecene–specific polyclonal antibody, we detected and quantified trichothecene mycotoxins in the serum samples. Most of the experimental samples demonstrated low percentage inhibitions and trichothecene concentrations, compared with negative and NHS controls (Tables 2–4). One possible reason for this is that trichothecene concentrations in the buildings where the individuals lived/worked were unknown and untested. Concentrations of fungi were known, but this does not accurately assess mycotoxin exposure.[41] We have shown that airborne trichothecene concentrations are low in controlled environments[27] and in mold-contaminated buildings (unpublished data). Therefore, it may be expected that concentrations in naturally exposed humans would be even lower (possibly below the limit of detection of current technology). This is not to say that such low levels pose no human health risk, particularly as it has been shown that these mycotoxins exert biological activity at very low concentrations.[26,42]

Many studies have shown that nonmacrocyclic trichothecenes such as T-2 toxin, verrucarol, nivalenol, and deoxynivalenol are metabolized rapidly in animal models.[37,43–47] Therefore, it is possible that the trichothecenes for which we were testing were broken down in a rapid fashion prior to our analyses. To our knowledge, metabolic studies have not been conducted concerning the macrocyclic trichothecenes produced by *Stachybotrys chartarum*. Because all trichothecene mycotoxins are structurally related compounds, it is reasonable to believe that the macrocyclics would be metabolized in a similar fashion.

LC/MS/MS analysis was performed only on select samples. This was based on the low trichothecene concentrations (as determined by ELISA) and the limits of detection for the LC/MS/MS system. For the 2 serum samples analyzed, results showed the presence of 2 trichothecene-like compounds, but neither could be positively identified on the basis of the 9 purified standards used. Because these samples demonstrated spectral qualities similar to the purified trichothecenes (related adduct patterns) and demonstrated high ELISA reactivity,

it is highly likely that trichothecenes were present. One of the isolated compounds had a molecular mass of 484, which is the mass of verrucarin J, a macrocyclic trichothecene produced by *Stachybotrys chartarum*. However, on the column it had a different retention time than verrucarin J, indicating that it may have been a positional isomer. There are several possible reasons why we could not make a positive identification. The simplest explanation is that we did not have the proper standards for comparison. On the basis of high ELISA reactivity, we analyzed the serum samples for the presence of intact macrocyclic trichothecene mycotoxins, because non-macrocyclic trichothecenes do not impart a high degree of reactivity in the assay.[27] It is possible that we detected an uncharacterized macrocyclic trichothecene or unknown metabolic breakdown product that still conferred positive ELISA reactivity. Because of the cleanup/extraction method used and the specificity of the ELISA, it is unlikely that we were detecting false positives. This is particularly true for samples 1 and 2 of group 1.

One of the negative controls (NC 2) was statistically different from NHS when tested. This particular control was retested several times, and similar results were seen for each evaluation. Serum samples used as negative controls were randomly selected from clinical samples and were categorized as being from individuals with unknown degrees of mold and/or mycotoxin exposure. It is possible, therefore, that the particular individual from whom NC 2 was collected had been or was currently being exposed (i.e., living or working in a contaminated environment) to *Stachybotrys* and its associated trichothecene mycotoxins.

Conclusions

We have shown that trichothecenes can be extracted and detected in human sera from individuals who have been exposed to *Stachybotrys* in contaminated indoor environments. Our results point to the need for further study in this area. Specifically, there is a great need to understand the distribution and metabolic processes that take place following inhalation of *S. chartarum* mycotoxins. To date, this has not been examined in vivo. Controlled animal studies could clarify these types of issues and offer a better understanding of the health risks associated with exposure to *Stachybotrys* and its associated trichothecene mycotoxins in contaminated buildings. Furthermore, the study described here could be expanded and more strictly controlled. This could consist of extensive documentation of control and test samples, including extent of *Stachybotrys* growth and airborne trichothecene concentrations in the buildings; time duration of occupant exposure when the serum was drawn; symptomatology; and physical examinations on a per-patient basis. Taken together, future studies could further clarify the relationship between the presence of mold and mycotoxins and adverse human health effects.

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