Comparison of Two Analytical Methods for Detecting (1-3)-β-D-Glucan in Pure Fungal Cultures and in Home Dust Samples

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Abstract: There are two methods available for the analysis of (1-3)-β-D-glucan: the *Limulus* Amebocyte Lysate assay (LAL) and the inhibition Enzyme Immunoassay (EIA). The aim of this study was to compare the accuracy and specificity of these two methods in detecting eight alpha and beta-glucan standards, and their sensitivity for the analysis of (1-3)-β-D-glucan content of common indoor fungal species and indoor dust samples. The results show that the LAL assay is more accurate, specific, and sensitive in measuring linear and branched β-D-glucans than the EIA. The greatest LAL-analyzed (1-3)-β-D-glucan content per spore (241 pg/spore) was found with *E. nigrum*, which also had the largest spore size (28 μm). The biomass-normalized (1-3)-β-D-glucan content of fungal spores from pure cultures was within similar range with the two assays but no correlation was found between the results from the two assays. In contrast, there was a significant correlation between the EIA and LAL-measured (1-3)-β-D-glucan concentrations (μg/m^2 of floor area) in field dust samples.

Keywords: (1-3)-β-D-glucan, fungi, indoor, LAL, EIA.

INTRODUCTION

Exposure to fungi in occupational and indoor environments is associated with respiratory (nose and throat irritation, cough) and general symptoms (tiredness and headache), allergic reactions and organic dust toxic syndrome [1-3]. In adults, similar general and respiratory symptoms and airways inflammation have been associated with occupational and indoor exposures to (1-3)-β-D-glucan, polyglucose component of the cell wall of fungi, pollen, and some bacteria [4,5]. In young children, however, increased dustborne (1–3)-β-D-glucan concentrations have been associated with a decreased risk for several respiratory health outcomes (asthma, persistent wheeze at age 1-4 [6], recurrent wheezing combined with allergen sensitization in infants [7]), and with a decreased risk of sensitization to inhalant allergens [8]. This may be explained with the Hygiene hypothesis, which postulates that microbial stimulation (such as endotoxin) in early childhood induces Th1 response, which counterbalances the allergen-induced Th2 responses [7,9,10].

The biological properties of (1-3)-β-D-glucan are not dependent on cellular viability and (1-3)-β-D-glucan from dead organisms may thus be equally relevant in causing potential health effects. Therefore, exposure assessment of (1-3)-β-D-glucan may be a better predictor for health risk than the commonly used determination of viable fungal spores. In addition, performing (1-3)-β-D-glucan analysis is less time consuming and labor intensive than cultivation or microscopic counting of fungal spores [5,11,12].

Currently there are two methods available for the analysis of (1-3)-β-D-glucan in environmental and occupational samples. One method is based upon the bioactivity of this molecule in the factor-G-mediated Limulus coagulation pathway - the *Limulus* Amebocyte Lysate assay (LAL) [13]. The other method is an Enzyme-Linked ImmunoSorbent Assay (ELISA) based on (1-3)-β-D-glucan antigen-antibody reaction; the traditional form of this assay is the inhibition Enzyme Immunoassay (EIA) [11], but also other ELISA modifications have been developed [14,15]. The EIA assay has been used mostly by European research groups [11,16,17], while the commercially available LAL assay has been used by both research and clinical groups in USA, Europe and Asia [18-22]. The LAL method is more sensitive with a Lower Limit of Detection (LOD) reported as low as 3.125 pg/ml [7] compared to EIA with a reported LOD = 40 ng/ml [11], which limits the traditional EIA assay to settled dust and air samples collected from high-exposure environments only. Due to its high sensitivity, the LAL assay has predominantly been used for the analysis of air samples [5,18,23].

Both linear and branched (1–3)-β-D-glucans have been shown to enhance allergen induced airway inflammation by increasing eosinophil infiltration and specific IgE in guinea pigs and mice sensitized to ovalbumin [24-27]. The EIA assay described by Douwes *et al.* [11] reacts with both linear and branched β-D-glucans. The LAL assay was suggested to recognize both linear and branched β-D-glucans [28,29], as well as yeast α-D-mannan, which in previous studies was viewed as a disadvantage indicating low specificity [28]. Therefore, information on the content and structure of (1-3)-β-D-glucan in common indoor fungal species will help in better understanding the health effects associated with these fungi. While there are some data on the content of (1-3)-β-D-glucans in spores of the indoor fungal species of *Penicillium*, *Aspergillus*, *Cladosporium* and *Stachybotrys* [23,30], analyzed by the LAL assay, very little is known on the EIA-analyzed (1-3)-β-D-glucan of spores from different species. LAL-analyzed (1-3)-β-D-glucan is a recognized indicator of fungal biomass based on health effects and correlation with

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total fungal count [18-21]. EIA-analyzed (1-3)-β-D-glucan in settled dust has also been used as an indicator of fungal biomass [11,16,17], but there are very little data on correlations between EIA-analyzed air samples and fungal counts due to the low sensitivity of EIA.

In order to better understand the health effects associated with (1-3)-β-D-glucan exposure, it is necessary to compare the commonly used assay methods. Thus, the aim of this study was to compare the specificity, sensitivity, and accuracy of LAL and EIA methods through the analysis of (1-3)-β-D-glucan concentration in purified glucan standards, common indoor fungal species, and field dust samples, in order to determine which assay is a more appropriate measure of (1-3)-β-D-glucan concentration.

MATERIALS AND METHODS

Laboratory Analysis of (1-3)-β-D-Glucan

The LAL test is a quantitative direct method for the detection of (1-3)-β-D-glucans that uses (1-3)-β-D-glucan-sensitive factor G [13]. The kinetic chromogenic *Limulus* Amebocyte lysate assay [Glucatell^{TM}, Associates of Cape Cod, East Falmouth, MA] was performed using laboratory materials certified as glucan-free by the manufacturer (Associates of Cape Cod). From each sample, 0.5 ml aliquot was extracted with 0.5 ml of 0.6 M NaOH by shaking for 1 hour at room temperature, to unwind the triple-helix structure of (1-3)-β-D-glucan and make it water-soluble. Fifty µl of Glucatell reagent was added to each well of serially diluted (from 1:1 to 1:10^{11}) extract and a control standard (1-3)-β-D-glucan (Pachyman as provided in the Glucatell kit), placed in a 96-well, flat-bottom microplate. The prepared (expected) concentrations of the glucan standards for the LAL assay were: 3.125, 12.50, 50 and 100 pg/ml. The optical density (OD) at 405 nm and the time of onset at OD = 0.03 was recorded. All samples were above the lower limit of detection (LOD) of the Glucatell assay protocol (3.125 pg/ml). The median coefficient of variation (CV) was 9% for the intra-plate variability and 27% for the inter-plate variability. Based on these values, the cumulative error was calculated to be 28%.

The EIA assay was performed as described by Douwes *et al*. [11], except that the source of the antibodies was different. The primary monoclonal antibody to (1-3)-β-D-glucan was mouse IgG, kappa light (Biosupplies Australia, Parkville Victoria, Australia) (31). The secondary antibody was peroxidase-conjugated affinipure sheep anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA). The sample extraction was accomplished by heat extraction in an autoclave at 120^{o}C for 1 hour. The standard against which the concentrations were measured was Laminarin (as described in the protocol by Douwes *et al*., 1996) [11]. The prepared (expected) concentrations of the glucan standards for the EIA assay were 250, 1000, 2500, and 5000 ng/ml. Values below the LOD (250 ng/ml) were replaced with LOD divided by the square root of two for the data analyses. For the EIA assay, the median CV was 13.6 % for the intra-plate variability and 24.2 % for the inter-plate variability.

Specificity, Sensitivity and Accuracy in Detecting Branched β-D-Glucans

The specificity was defined as the ability of each test to detect the (1-3)-beta-D-glucan structure, i.e. detect the beta but not the alpha structure, and detect the (1-3)-β-D-glucan structure in both linear and branched molecules. The sensitivity was defined as the ability of each test to detect the (1-3)-β-D-glucan molecule, i.e. the lowest the (1-3)-β-D-glucan concentration that a test can detect, the greater its sensitivity. The accuracy of each test was determined by comparing the results with a known standard (Pachyman for LAL, and Laminarin for EIA). The closer the result comes to the true value, the greater the accuracy.

The following glucan standards were used to test the specificity of the LAL and EIA tests in detecting linear (Pachyman, Curdlan, and Mannan) *vs* branched (1-3)-D-glucans [Laminarin, Schizophyllan, MG-glucan (Macro-Gard^{R}), Dextran, and Pullulan] (Table **2**). The glucan standards were purchased from Sigma Chemical Co. (St. Louis, MO), except for Pachyman and MG-glucan, which were obtained from Megazyme International Ireland Ltd (Bray, Ireland) and Nutritional Scientific Corporation (Liberty, TX), respectively. Each standard was measured following the above stated protocol for laboratory analysis of (1-3)-β-D-glucan.

Pure Fungal Cultures

Selection of fungal species. Thirteen fungal species were selected based on their prevalence in field samples, genus variability, and public health concerns: two *Cladosporium* species, five *Aspergillus* species, *Alternaria alternata, Aureobasidium pullulans, Epicoccum nigrum, Penicillium brevicompactum, Stachybotrys chartarum, and Wallemia sebi*. Results from an ongoing field study [Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS), see section Field Samples below] were used to identify species that are commonly found in homes. Based on Polymerase Chain Reaction (PCR) analysis of dust samples from 297 homes [32], eight fungal species that were most commonly found (>90% frequency) were selected for this study (Table **1**).

This list included three *Aspergillus* species. Two additional *Aspergillus* species were included in order to study the within species variability of (1-3)-β-D-glucan content. *P. brevicompactum* and *S. chartarum* were chosen to represent the species of medium frequency and concentration. In addition, *S. chartarum* was included due to reasons of being of health concern in indoor environments [33,34]. A non-toxic strain of *S. chartarum* was provided by the National Institute for Occupational Safety and Health (NIOSH), Morgantown, WV (research collection, No: 29-51-05; characterized as isolate JS5105) [35]. All the other species were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

Preparation of spore suspension from pure fungal cultures. The freeze-dried pure fungal cultures were re-hydrated and inoculated on media by following the ATCC instructions. Spores from one-week old pure cultures were harvested from the agar surface by using micro-beads [36], and transferred into 5 ml sterile tube, containing 0.02% Tween solution in pyrogen and glucan-free reagent water. Spore suspensions were placed in a bright-line hemocytometer (Model 3900, Hausser Scientific Company, Horsham, PA) and observed under a microscope to get an estimate of the spore concentration and to confirm the purity of spores. Serial dilutions of 10^{0} to 10^{-6} were prepared for each fungal

Table 1. Fungal Species Selected for this Study and their Indoor Frequency and Concentration (Geometric Mean), as Measured by PCR Analysis of 297 Dust Samples

Fungal Species Name	Isolate Designation*	Frequency**	Concentration***
Alternaria alternata	ATCC 44501	287 (97%)	401
A. chevalieri	ATCC 66451	291 (98%)	154
A. flavus	ATCC 11489	103 (35%)	3
A. penicillioides	ATCC 16910	277 (93%)	39
A. unguis	ATCC 10032	86 (29%)	2
A. versicolor	ATCC 52173	68 (23%)	4
Aureobasidium pullulans	ATCC 58926	297 (100%)	4365
C. cladosporioides	ATCC 6721	297 (100%)	1521
C. herbarum	ATCC 58927	293 (99%)	184
E. nigrum	ATCC 58875	293 (99%)	251
P. brevicompactum	ATCC 9056	212 (71%)	31
S. chartarum	29-51-05, NIOSH	153 (52%)	4
W. sebi	ATCC 42694	275 (93%)	62

*Isolate designation of the strain used for the (1-3)-β-D-glucan content analysis.
**Number and % of samples in which the species was detected, Cincinnati Childhood Allergy and Air Pollution Study [32].
***Cells/mg dust.
ATCC=American Type Culture Collection, Manassas, VA.

spore suspension, which was used for determining (1-3)-β-D-glucan concentration (as described above), spore concentration, and spore size. Spores were counted under a microscope as described by Adhikari *et al*. [37].

For determining the spore size, about 50 µl of the pure spore suspension was placed on a microscopic slide, covered by a cover slide and immediately observed at 1000X magnification oil immersion using an optical microscope. Digital images were taken by a color video camera (SPOT advanced software, version 3.4, Diagnostic Instruments Inc, Sterling Heights, MI, USA) and the spore size of 30 spores of each species was measured from the images. Based on the average spore size (diameter for spherical; width and length for ellipsoidal spores), the spore surface area and volume were calculated. Spores of *Cladosporium* species, *S. chartarum, A. alternata* and *A. pullulans* are ellipsoidal, and therefore, equations for a prolate ellipsoid were used. The surface area and volume for the spores of the other fungal species were calculated according to the formulas for a sphere.

The (1-3)-β-D-glucan results of both assays were converted from pg/ml to pg/spore for the spore suspensions. After measuring the spore size and calculating the spore surface area and volume, the results were also converted to pg/µm^2 and pg/µm^3.

Field Samples

Field samples were obtained through the CCAAPS project. The CCAAPS is a prospective birth cohort study aimed at investigating the role of aeroallergens and diesel exhaust particles in the development of atopy and atopic respiratory disorders [38]. When participating infants reached an average age of 8 months, families were visited at their homes and dust samples were vacuumed from the floor of baby's primary activity room [39]. The home dust sample was sieved (355 µm sieve), and the fine dust was divided into sub-samples and stored at -20°C before analyses.

Sub-samples of 50 mg and 40 mg were analyzed for (1-3)-β-D-glucan concentration by the LAL (n=297) and EIA assays (n=70), respectively. Results of the (1-3)-β-D-glucan concentration in dust samples were reported as µg/g of dust and µg/m^2 of floor area.

Data Analyses

The (1-3)-β-D-glucan content of fungal spores, as well as their respective spore sizes, surface areas and volumes, were not normally distributed even after log-transformation. The correlations with and between the LAL- and EIA-analyzed (1-3)-β-D-glucan contents of fungal spores were tested with the non-parametric Spearman correlation, and the difference of means with the Wilcoxon statistics.

(1-3)-β-D-glucan concentration in 297 dust samples collected from the CCAAPS homes followed the normal Gaussian distribution after log-transformation. The EIA-analyzed (1-3)-β-D-glucan concentration in indoor dust samples did not follow the Gaussian distribution even after log-transformation. Thus non-parametric analyses were used to test for correlation and difference between the LAL- and EIA-analyzed (1-3)-β-D-glucan concentrations in dust samples.

RESULTS

Specificity and Accuracy of LAL and EIA in Measuring Glucans of Different Linkage and Branching

The reactivity of LAL and EIA assays to α- and β-glucans of various degree of branching are shown in Fig. (**1**). As the LAL assay (Fig. **1A**) is a kinetic assay measuring the onset of time at OD = 0.03, the later the reaction occurs (mean onset time), the lower the specificity for the particular

purified glucan at that concentration in comparison to the other glucan standards. In the endpoint EIA assay, the best curve is curvilinear, with a rapid straight decrease in the absorbance units with the increase of the concentration until saturation of the curve is reached (Fig. **1B**). As seen in Fig. (**1**), both LAL and EIA assay were specific to linear (1-3)-β-D-glucans (Curdlan and Pachyman: change in concentration is reflected by change in mean onset time/absorbance units) and non-reactive to α-glucans (Mannan, Dextran, and Pullulan: presented by straight horizontal lines, i.e. no change in detection with increase in concentration). The reactivity of the LAL assay slightly decreased with the increase in the degree of branching (Fig. **1A**). Although the EIA assay was also specific in recognizing close to linear structures (Laminarin), its specificity to branched structures was negligible [(Schizophylan, branched (1-3)(1-6)-β-D-glucan)] (Fig. **1B**).

The measured concentrations for glucan standards were calculated based on a standard curve of Pachyman for the LAL assay and Laminarin for the EIA assay. These values, expressed as % of the expected concentrations, are presented in Table **2**. For each standard concentration the LAL was more accurate in measuring concentrations of (1-3)-β-D-glucan standards than the EIA as demonstrated by the narrower range and the smaller median value of the % expected concentration (Table **2**).

Sensitivity of LAL and EIA in Measuring (1-3)-β-D-Glucan in Pure Fungal Spores

The spore size, spore surface area, and spore volume of the thirteen fungal species analyzed are provided in Table **3**. Among the fungal species, *E. nigrum* had the largest spore size, and thus the biggest surface area and volume. The *Aspergillus* species were small in size, and *Aspergillus versicolor* was the smallest among them. The measured spore sizes and surface areas were within the range reported earlier [30,40,41].

Based on the spore characteristics reported in Table **3**, we compared the sensitivity of the two assays (i.e., ability to detect lower concentrations of (1-3)-β-D-glucan) by calculating the (1-3)-β-D-glucan content per spore, spore surface area, and spore volume (Tables **4A** and **4B**). Although *E. nigrum* was the species of greatest (1-3)-β-D-glucan content per spore (241 pg/spore), this was mainly due to having also the largest spore size (28 μm). The biomass-normalized (1-3)-β-D-glucan content (pg per spore surface area and pg per spore volume) measured by both assays was within similar range (LAL: 0.03 to $146.33*10^{-3}$ pg/μm^2, 0.22-$240.54*10^{-3}$ pg/μm^3; EIA: 0.04 – $197.00*10^{-3}$ pg/μm^2, 0.03-$300*10^{-3}$ pg/μm^3). The LAL assay determined *C. herbarum*, followed

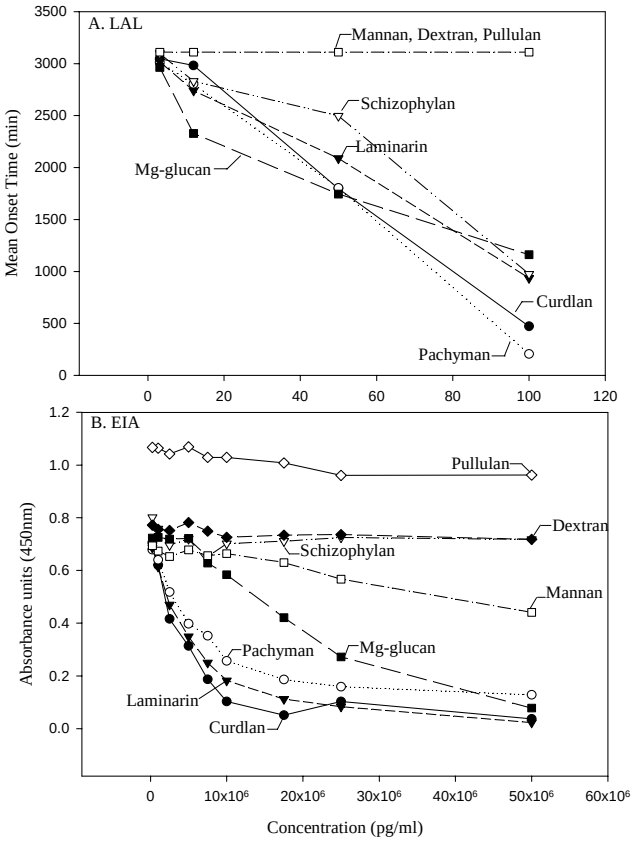

Fig. (1). Comparison of eight purified glucans and their reactivity as measured by (**A**) the kinetic LAL and (**B**) endpoint EIA assays.

Table 2. Comparison of Eight Purified Glucans and their Relative Reactivity as Measured by the LAL and EIA Assays*

Glucan	Linkages	% of the Expected Concentration**			
		LAL		EIA	
		Range	Median Value	Range	Median Value
Curdlan	Linear (1-3)-β-D-glucan	43-100	96.4	95-150	97.0
Pachyman	Linear (1-3)-β-D-glucan	97-118	104.8	56-94	73.7
Laminarin	(1-3)(1-6)-β-D-glucan (some branching)	78-118	99.3	71-120	98.2
Schizophylan	(1-3)(1-6)-β-D-glucan (33% branching)	47-118	84.7	No response	
MG-glucan	Branched (1-3)(1-6)-β-D-glucan extract from baker's yeast	74-240	96.0	No response	
Mannan	Linear (1-3)-α-D-Mannose	No response		No response	
Dextran	Branched (1-3)(1-6)-α-D-glucose	No response		No response	
Pullulan	Branched (1-3)(1-4)-α-D-glucose	No response		No response	

*The standard against which the concentrations are measured is Pachyman for the LAL assay, and Laminarin for the EIA assay.

**The prepared (expected) concentrations of the glucan standards for the LAL assay were: 3.125, 12.50, 50, and 100 pg/ml. The prepared (expected) concentrations of the glucan standards for the EIA assay were 250, 1000, 2500, and 5000 ng/ml. The measured values were expressed as % of the expected concentrations.

Table 3. Characteristics of Fungal Species Grown in Pure Fungal Cultures – Spore Size (µm), Spore Surface Area (µm^2) and Spore Volume (µm^3)[*]

Fungal Species	Spore Size (µm)	Spore Surface Area (µm^2)	Spore Volume (µm^3)
A. alternata	17[**]	643	1021
A. chevalieri	4.5	64	48
A. flavus	5	76	62
A. penicillioides	4.5	62	46
A. unguis	3	30	16
A. versicolor	3	28	14
A. pullulans	12[**]	628	786
C. cladosporioides	5.6[**]	83	60
C. herbarum	5[**]	59	36
E. nigrum	28	2463	11494
P. brevicompactum	3	29	15
S. chartarum	7.5[**]	148	172
W. sebi	4	49	32

[*]Mean of n=30 spores for each fungal species.
[**]Geometric mean of width and length for ellipsoidal spores.
Spores of *Aspergillus* species, *E. nigrum*, *P. brevicompactum* and *W. sebi* are spherical. Spores of *A. alternata*, *A. pullulans*, *Cladosporium* species, and *S. chartarum* are ellipsoidal.

by *E. nigrum* and *P. brevicompactum*, as the fungi of highest (1-3)-β-D-glucan content per surface area and volume. The EIA assay ranked *W. sebi* as having the highest (1-3)-β-D-glucan content, followed by *E. nigrum, P. brevicompactum* and *C. cladosporioides* (Table **4B**). Both assays recognized *Aspergillus* species, *A. alternata* and *S. chartarum* to have the lowest (1-3)-β-D-glucan content. Three fungal species, *C. herbarum, A. pullulans*, and *S. chartarum*, were below the LOD of the EIA assay. *A. alternata* (1-3)-β-D-glucan content was measured only with the LAL assay (0.03 pg/spore, $0.05*10^{-3}$ pg/µm^2, $0.03*10^{-3}$ pg/µm^3).

The data were used to analyze the variations between the 13 investigated fungal species and within 5 *Aspergillus* species. Both assays showed lower variation of (1-3)-β-D-glucan content within the 5 *Aspergillus* species than between the 13 fungal species (LAL: Coefficients of variation = 45% and 58%, respectively; EIA: Coefficients of variation = 22% and 42%, respectively).

No correlation was found between LAL- or EIA-analyzed (1-3)-β-D-glucan content (pg/spore) and spore size, spore surface area, or volume ($p>0.20$). Furthermore, no

Table 4A. Average† (1-3)-β-D-glucan Contents of Twelve Common Indoor Fungal Species as Measured by the LAL Assay

Fungal Species	pg/Spore	pg/Spore Surface Area*	pg/Spore Volume**
A. chevalieri	0.22	3.46	4.61
A. flavus	0.03	0.39	0.48
A. penicillioides	0.01	0.16	0.22
A. unguis	0.005	0.16	0.32
A. versicolor	0.03	0.91	1.84
A. pullulans	3.76	5.98	4.78
C. cladosporioides	0.25	3.00	4.19
C. herbarum	8.66	146.33	240.54
E. nigrum	241.57	98.08	21.02
P. brevicompactum	0.21	7.02	13.81
S. chartarum	0.004	0.03	0.33
W. sebi	0.12	2.40	3.72

* pg/µm^{2}x10^{-3}.
** pg/µm^{3}x10^{-3}.
†(1-3)-β-D-glucan content is an average of 4-6 experiments, CV=27%.

Table 4B. Average† (1-3)-β-D-Glucan Contents of Twelve Common Indoor Fungal Species as Measured by the EIA Assay

Fungal Species	pg/Spore	pg/Spore Surface Area*	pg/Spore Volume**
A. chevalieri	0.24	3.81	5.08
A. flavus	0.10	1.34	1.64
A. penicillioides	0.13	2.06	2.78
A. unguis	0.02	0.57	1.10
A. versicolor	0.08	3.01	6.10
A. pullulans[***]	(0.02)	(0.04)	(0.03)
C. cladosporioides	7.20	86.30	120.55
C. herbarum[***]	(0.06)	(1.01)	(1.72)
E. nigrum	379.97	153.25	32.72
P. brevicompactum	3.39	116.10	228.39
S. chartarum[***]	(0.58)	(3.94)	(3.40)
W. sebi	9.68	197.00	300.01

* $pg/\mu m^2 x10^{-3}$.
** $pg/\mu m^3 x10^{-3}$.
[***]The value for this fungal (1-3)-β-D-glucan concentration was below the lower detection limit of the EIA assay (250 ng/ml). These values were replaced with LOD divided by the square root of two.
†(1-3)-β-D-glucan content is an average of 4-6 experiments, CV=24%.

correlation was observed between LAL-analyzed and EIA-analyzed (1-3)-β-D-glucan content per spore.

Comparison of LAL *vs* EIA Analyzed (1-3)-β-D-Glucan in Dust Samples

The EIA-measured (1-3)-β-D-glucan levels [geometric mean (GM) = 15,998 μg/g and 3,775 $\mu g/m^2$] in dust samples were higher than the LAL-measured (GM = 40 μg/g and 9 $\mu g/m^2$). This difference was significant in both units (μg/g: z-value = -7.21, $p<0.001$; $\mu g/m^2$: z-value = -7.26, $p<0.001$). However, the variability in (1-3)-β-D-glucan levels was greater as measured by the LAL [Geometric standard deviation (GSD) = 1.70 and 2.00] than the EIA assay (GSD = 1.45 and 1.81). There was no correlation between LAL- and EIA-analyzed (1-3)-β-D-glucan in dust samples, when concentration was expressed per gram of dust (Fig. **2**), but moderate and statistically significant when expressed per square meter of floor area (Fig. **3**).

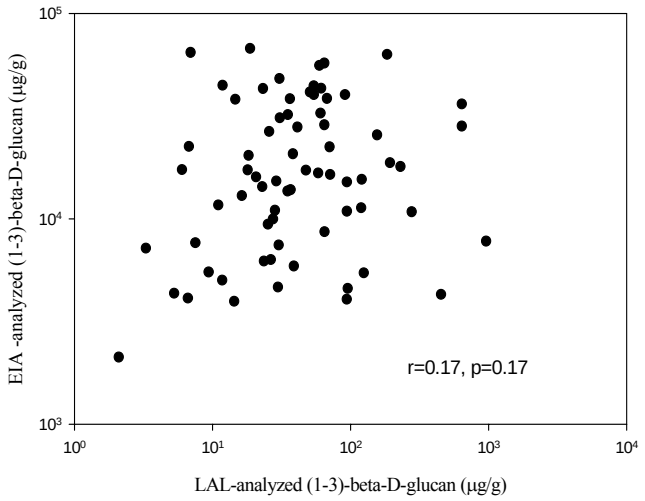

Fig. (2). LAL *vs* EIA-analyzed (1-3)-β-D-glucan in 70 dust samples in μg/g.

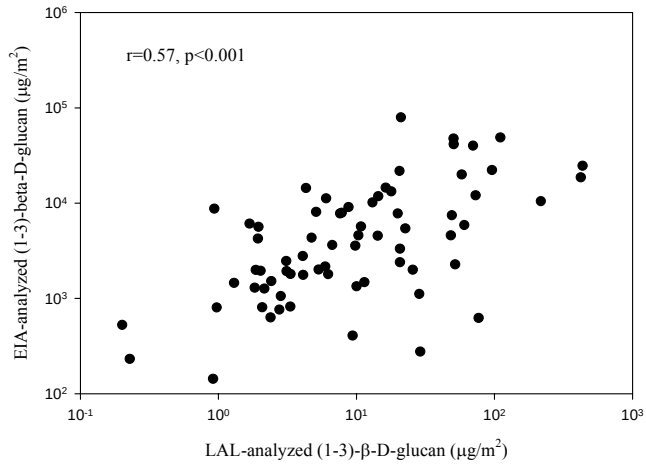

Fig. (3). LAL *vs* EIA-analyzed (1-3)-β-D-glucan in 70 dust samples in $\mu g/m^2$.

DISCUSSION

The utility of the LAL assay in measuring serum fungal (1-3)-β-D-glucans has been evaluated in numerous laboratory and clinical studies and the assay is currently routinely used in Japan and Europe for the detection of invasive fungal infections [42-44]. The EIA assay is not commercially available, and is not generally performed in most clinical laboratories. In addition, less data are available on the utility of LAL and EIA assays in the analysis of fungal (1-3)-β-D-glucan concentration in environmental samples [4,5,45,46]. Thus, the current study is the first one to directly compare the specificity, sensitivity, and accuracy of LAL versus EIA assays in detecting fungal (1-3)-β-D-glucans.

The current study confirmed that the LAL assay recognized both linear and branched (1-3)-β-D-glucans, as previously reported by Tanaka *et al*. [28] and Thorne *et al*. [29]. This study also confirmed that the EIA immunoassay reacts with linear (1-3)-β-D-glucans, as earlier reported by Douwes

et al. [11]. New modifications of the ELISA assay are highly specific to branched (1-3)(1-6)-β-D-glucans only [14,15]. It has been reported that both linear and branched type β-D-glucans are ubiquitous in the cell wall of fungi [47], and both types of β-D-glucans are important for *in vivo* priming of macrophages [48], as well as potentiate allergic reactions with the elevation of IgE in mice and guinea pigs [24-27]. Thus, the measurement of both linear and branched β-D-glucans appears to be important. From this perspective, the LAL and inhibition EIA assays seem to be more desirable to use for fungal exposure assessment than the new ELISA methods. However, the LAL assay showed greater sensitivity and specificity (LOD=3.125 pg/ml, detected both linear and branched β-D-glucans with comparable sensitivity) in comparison to the EIA assay (LOD=250 ng/ml, detected preferably the linear (1-3)-β-D-glucans). In addition, the LAL was more accurate in measuring concentrations of (1-3)-β-D-glucan standards than the EIA as demonstrated by the smaller difference between measured and expected concentrations.

A major disadvantage of the EIA assay is its low sensitivity. Thus, the low content of EIA-reactive (1-3)-β-D-glucan antigens in the pure fungal suspensions of *C. herbarum*, *A. pullulans* and *S. chartarum* is a limitation of the study. This difference in the detection sensitivity and specificity between assays may explain the difference of (1-3)-β-D-glucan content in the same fungus species when determined by LAL *vs* EIA. This may also explain the lack of correlation between LAL- *vs* EIA-analyzed (1-3)-β-D-glucan content of fungal spores.

Previous studies that have used the LAL assay on serum and culture supernatants of clinical fungal isolates have shown that the (1-3)-β-D-glucan content of different fungal species varies widely [42,43]. This study also showed a wide range of (1-3)-β-D-glucan content between 13 species from 7 genera and within *Aspergillus* genus, as demonstrated by the large coefficients of variation measured both with the LAL and EIA.

In this study, *Aspergillus* species, *A. alternata* and *S. chartarum* had the lowest (1-3)-β-D-glucan contents. Although no previous study reported the LAL-analyzed (1-3)-β-D-glucan content of *A. alternata*, studies have isolated elicitor-active components from *A. alternata* 102 that consisted almost solely of (1-3)(1-6)-β-D-glucan [49]. Fogelmark and Rylander have reported that the *Stachybotrys atra* (=*S. chartarum*) (median 3.9 pg/spore, range 0.9-39.3 pg/spore) has 1,000 times higher LAL-analyzed (1-3)-β-D-glucan than *Aspergillus fumigatus* (median: 0.11 pg/spore, range 0.008-0.7 pg/spore) [23]. The much lower (1-3)-β-D-glucan content in the *Stachybotrys* spores in the present study may be due to the fact that it was a non-toxic strain. Though, there is very little information on the association between (1-3)-β-D-glucan content and fungal toxicity, low (1-3)-β-D-glucan content is known to be associated with lower pathogenicity [50,51]. Also, growth conditions, and thus growth media, can affect the content of fungal (1-3)-β-D-glucans [30,52-54]. A study by Foto *et al*. showed that the LAL-analyzed (1-3)-β-D-glucan content in *S. chartarum* (mean 0.012 pg/μm^2) is lower than that of *A. versicolor* (mean 0.022 pg/μm^2) and *C. cladosporioides* (mean 0.060 pg/μm^2), all grown on 2% malt extract agar [30]. Although the (1-3)-β-D-glucan contents measured by the LAL in the present study are higher than those reported by Foto *et al*. [30], the same trend among species was observed.

The rank order of EIA analyzed fungal (1-3)-β-D-glucan content in the present study was different from what was reported for a mixture of fungal mycelia and spores assayed by the monoclonal IgM ELISA [15]. *Aspergillus flavus* isolated from stored urine and *Aspergillus ochraceus* isolated from outdoor air samples had higher (1-3)-β-D-glucan content (measured as ng per mg of cultured fungal isolates) than *Cladosporium* spp. isolated from bedroom air and *Wallemia* spp. isolated from outdoor air. This may be due to the different sources from which species were isolated and inclusion of both spores and mycelia, which may have different (1-3)-β-D-glucan content per cell. Furthermore, the IgM ELISA measures the content of branched (1-3)(1-6)-β-D-glucan only, and thus may underestimate the (1-3)-β-D-glucan content of *Cladosporium* spp., as they have been reported to contain predominantly linear glucans [55,56].

This appears to be the first report on LAL-analyzed (1-3)-β-D-glucan concentration in a large number of dust samples. The LAL-analyzed dust (1-3)-β-D-glucan levels measured in this study were lower than those measured in Canadian homes (n=28) of asthmatic children (GM=172 μg/g and GM=160 μg/m^2) [45] and homes perceived as having satisfactory indoor quality (n=20) (median=230 μg/m^2) [46]. These differences may be due to different geography and/or heating [16] (in colder climate) in the Canadian homes. The EIA assay used in this study measured much higher indoor dust (1-3)-β-D-glucan (GM=15,998 μg/g and 3,775 μg/m^2) than those reported in other studies. However, difference in sampling protocols (e.g., mesh size for sieving), seasonal and geographical variations can contribute to this difference. A wide range in the levels of EIA-analyzed dust (1-3)-β-D-glucan in European Union countries was reported – from 35.1 μg/g (GSD=1.80, n=20) to 2,959 μg/g (GSD=1.94, n=441) [15,17,57,58], and from 90 μg/m^2 (no GSD reported, n=508) to 1,197 μg/m^2 (GSD=2.5, n=395) [6,17,58,59]. (1-3)-β-D-glucan levels can vary between seasons; Chew *et al*. [16] observed GM=1,551μg/g (GSD=2.4, n=26) in spring and GM=2,219μg/g (GSD=2.7) in fall. In addition, the source of primary and secondary antibodies used is also different between the EIA assay used in this study *vs* in European studies, and thus data may not be directly comparable.

Although the (1–3)-β-D-glucan content of pure fungal cultures showed a similar rank of order whether measured by the LAL or EIA assay, there was about 1,000 times difference in the (1-3)-β-D-glucan concentration in dust samples when analyzed by the LAL *vs* the EIA assay. Previous studies show that (1–3)-β-D-glucan concentrations do not consistently correlate with total culturable fungal spore counts [17,21,60]. (1–3)-β-D-glucan concentrations in the environment more likely reflect exposure from multiple environmental sources of (1–3)-β-D-glucan, including fungi, pollen, plants and their fragments [30,61]. Since, these various sources may also contain other sugars, some interference of these (inhibition or enhancement) with the assay results may be expected [62,63]. In addition, these assays differ in measurement of β-D-glucans of different branching and conformation, which may lead to the orders of magnitude difference between the LAL and EIA assay measurements. It

seems that a combination of both assays can provide better perspective on the content and types of (1-3)-β-D-glucan exposure.

This and previous studies [30] have shown that (1–3)-β-D-glucan content varies between fungal species, and this may lead to variance of health outcomes by fungal genera [64]. For example, in the CCAAPS cohort, we have found an inverse association between the health outcomes and some fungal types [the concentration of airborne *Cladosporium* and SPT(+) to any allergen ($P < 0.05$), and *Cladosporium* and SPT(+) to aeroallergens ($P < 0.05$)], but positive associations with some other fungal types [*Penicillium/Aspergillus* and SPT(+) to any allergen ($P < 0.01$) and between *Alternaria* and SPT(+) to any allergen ($P < 0.01$)] [64]. Previous studies utilizing the EIA assay have shown stronger association between dust (1–3)-β-D-glucan exposures and the health outcome (peak flow variability) in per square meter units than per gram units [65], or no association at all (asthma and wheeze) per gram unit [6]. The current study showed that LAL and EIA (1–3)-β-D-glucan exposures per square meter correlate significantly, but not when expressed per gram of floor dust. Therefore, comparison of published LAL and EIA data per gram of dust is not plausible. It seems (1–3)-β-D-glucan may be an independent measure of biologically active exposure and measuring exposures per square meter can provide a better understanding of health outcomes, as well as a possibility for comparison of (1–3)-β-D-glucan exposures measured by these two different assays.

In conclusion, the LAL assay was shown to be more specific, sensitive, and accurate in detecting both linear and branched β-D-glucans. Although the (1-3)-β-D-glucan concentration in field samples measured by the LAL and EIA assay correlated, data should be analyzed with caution, as assays measure different aspects of the (1-3)-β-D-glucan structure and give different weight to different fungal species.

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