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Quantification of *C. globosum* spores in house dust samples

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Abstract

Chaetomium globosum is one of the most common fungi that grows in damp buildings and occurs in agricultural and forestry workplaces. Using sera from atopic patients, we characterized and purified an extracellular chitosanase (Chg47) from *C. globosum* that is antigenic to humans. The study reports the production of monoclonal antibodies to the protein. Three capture ELISAs were developed for Chg47 for detection of spores and spore and mycelial fragments in dust samples using different mono- and polyclonal antibody combinations. One method is based on an enhanced biotinylated polyclonal antibody as the secondary antibody and coating anti-IgM to capture one of two clones of IgM monoclonal antibodies as the capture antibody. The other method makes use of an enhanced rabbit polyclonal antibody as both the primary and capture antibody. The detection limit of the double PAb method for the Chg47 antigen was 7.6 pg/ml. When the anti-IgM+10B3 clone was used, the detection limit was 61 pg/ml and for anti-IgM+5F12, 122 pg/ml. The detection limit of double PAb method is comparable to methods for the allergen and spores of *Aspergillus versicolor* in house dust and is more sensitive than other immunoassays for allergens in house including for *Stachybotrys chartarum*, *Aspergillus fumigatus* and *Alternaria alternata*. All three methods had limited cross-reactivity to fungi common in house dust representing a diverse array of taxa.

Key words

Chaetomium globosum, Capture ELISA, allergen exposure, damp buildings, agricultural exposures

INTRODUCTION

The presented study concerned the identification of human fungal allergens and antigens from species common on water damaged or damp building materials. ELISA assays to these proteins could then be used to assess allergen exposure and for diagnostic tests for allergy. New allergens from a number of fungi are reported that reflect various types of moisture failures. This is a species growing on building materials with a high available moisture content (a_w 0.9) and those growing at low values [1]. We have reported *Stachybotrys chartarum sensu lato* [2, 3], *Penicillium chrysogenum* [4], *Aspergillus versicolor* [5, 6] and *Chaetomium globosum* [7]. Using a number of conventional and innovative ELISA methods, we have reported antibody-based assays for the allergens in settled dust from *S. chartarum sensu lato* [8], *P. chrysogenum* [9] and *A. versicolor* [10], with appropriate analytical performance data in different matrices. Commercial antibodies for the detection of allergens from *A. fumigatus* and *Alernaria alternata* have typically been used without such critical data [see 9].

C. globosum is common on wallboard, solid wood, textiles, manufactured wood, ceiling tiles, and frequently found on insulation [1, 11, 12]. Natural substrates of this species have been isolated from straw, wood chips, seeds, cereals and cardboard. Occupational exposure is possible from wood chips [e.g. 13] and in damp buildings [14]. Niedoszytko et al. [15] found that 7% of an atopic population in Poland had an IgE response to *C. globosum,* a similar proportion to that found in West Virginia, USA [16]. In a larger collection of human sera from throughout the USA, 2.5% had a strong IgE response to this fungus and 3.1 % had a weak response [7].

We reported the discovery of a 47 kDa chitosanase from *C. globosum* that was antigenic to humans, mice and rabbits [7]. The protein Chg47 was not glycosylated and had an acidic pI of 4.5 [7]. Monoclonal antibodies (mAb) were produced from the purified protein from liquid fungal cultures [17]. Many of the mAb, as well as the rabbit polyclonal antibodies (PAb) were found to have high affinity to Chg47. This prompted further investigations using various immunological techniques to develop ELISA methods for the detection of Chg47 antigens in house dust samples. The objective of this work was to characterize the sensitivity and specificity of various combinations of an enhanced polyclonal antibody and two monoclonal antibodies in three capture ELISA methods for *C. globosum* spores in house dust samples.

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MATERIALS AND METHOD

Monoclonal antibody preparation. The Chg47 protein was purified as described by Provost et al. [7]. Monoclonal antibodies (mAb) were prepared after Xu et al. [8] at Immunprecise Laboratories (Victoria, BC, Canada). The monoclonal antibodies that resulted were the IgM isotype. Those that were positive for the target protein by ELISA and by dot blot at Immuoprecise [4] were selected for further study. Each mAb was partially purified by buffer exchange with 50 mM pH=7.4 PBS containing 200 mM NaCl with 100kDa MWCO filters (Millipore, Etobicoke, Ontario, Canada). The concentration of IgMs in each sample was normalized by anti-IgM-HRP (Sigma), ensuring that the amount of IgM on each plate was similar. The affinities of the selected MAb to Chg47 were determined by western blotting, by indirect ELISA with different concentrations of Chg47 coated in the plate, and by capture ELISA with coated anti-IgM IgG to immobilize the different monoclonal IgM as capture antibodies in the plate and with biotinylated polyclonal antibody as the primary antibody.

Polyclonal antibody preparation. A rabbit anti-Chg47 polyclonal antibody (PAb) was produced according to the procedure described by Xu et al. [2] and Shi et al. [10]. Briefly, a rabbit was subjected to a pre-immune bleed and immunized with 0.5 mg of *C. globosum* spores. After 28, 47 and 66 days, respectively, the rabbit was boosted using 0.5 mg purified Chg47, this has the effect of increasing the titre of antibodies to the target protein and reducing reactions with the others [2, 5, 18]. The final bleed of the immunized rabbit was completed at 78 days. This was carried out by Cedarlane Laboratories, Ltd. (Hornby, Ontario, and meets the requirements of the Canadian Council on Animal Care).

The final antigen-specific PAb was purified and decontaminated following the protocol of Shi et al. [10]. Briefly, the details of the purification steps are as follows:

Step 1: 20 ml boosted rabbit serum was adjusted to pH 7.4, and then loaded to a 5 ml Protein G affinity column (GE Healthcare) for affinity purification. Afterwards, 100 ml of Buffer A (50 mM PBS buffer containing 200mM NaCl) was used to wash the column, and the antibody was eluted by Buffer B (20 ml 20mM pH=2.5 sodium acetate). The eluted solution was immediately neutralized by Buffer C (400 mM pH=9.0 PBS), to a pH of around 7. The antibody solution was then concentrated using a 15ml 30 kDa MWCO filter (Millipore) and the buffer was exchanged with Buffer A and concentrated with a 30kD MWCO filter for future usage.

Step 2: purified Chg47 was prepared as previously reported [7]. Two milligrams of biotinylated Chg47 was then applied to a 1 ml streptavidin affinity column (GE Healthcare). The column was washed with 20 ml of Buffer A, loaded with PAb from Step 1, incubated for 5–10 minutes at 4°C, and then washed with 2 X 20 ml Buffer A. The antigen-specific PAb was then eluted with Buffer B and immediately neutralized by Buffer C to a pH of around 7.0.

Step 3: The PAb from step 2 was then loaded to an empty 1 ml streptavidin column to eliminate any leached biotinylated Chg47 from the previous step. The resulting antibody solution from the above 3 steps of the purification process was concentrated and buffer exchanged with Buffer A. 50% glycerol was added for storage, for ELISA and for immune blot assays.

Biotinylation. Purified 1 ml of Chg47 (2 mg/ml) or 0.5 mg of purified antigen and decontaminated PAb were reacted with an excess of 80 mol NHS-LC-LC-Biotin (Pierce) for 2 hours at room temperature, according to the manufacturer's instructions. The reaction was stopped by adding 0.2 ml 1 M pH=7.0 Tris buffer and the buffer exchanged using a 5 ml G-25 column (GE Healthcare) to remove unbound biotin molecules. Biotinylated antigen was then loaded to streptavidin columns to prepare the antigen-biotin streptavidin columns for the antigen-specific PAb purification described above. The biotinylated PAb was used as the primary antibody in all antigen capture ELISA.

SDS-PAGE and Western blotting. SDS-PAGE and Western blots were performed following the methods of Xu *et al.* [2]. Briefly, purified Chg47 was loaded to 10% SDS-PAGE with SeeBlue® Plus2 Pre-Stained Standard (Invitrogen). The gel was transferred to a Hybond-PVDF membrane (Amersham Biosciences) with a Hoefer miniVE electrotransfer unit (Amersham Biosciences). The transfer was carried out at a constant current of 350 mA for 60 min. 1:1,000 dilutions of different monoclonal IgM sera (1mg/ml) were used as primary antibodies followed by corresponding 1:5,000 anti-Mouse-IgM conjugated with alkaline phosphatase (Jackson Immunoresearch). Detection was achieved by incubating membranes with BCIP/NBT (Sigma) for 5 to 10 min.

Indirect and Chg47 capture ELISA. Indirect ELISA was carried out by coating 100 µl aliquots of different concentrations of antigen/spore extracts in 50 mM pH 9.6 carbonate bicarbonate buffer (Sigma) to each well of microplates (NUNC Maxisorp, Nalgene). The plate was incubated overnight at 4°C, and then blocked with 200 µl PBST solution with 1% BSA for 4 h at room temperature. After washing twice with 200 µl PBS buffer with 0.1% Tween-20 (PBST), 100 µl of 1:4,000 partly purified monoclonal IgM (adjusted to 1mg/ml) in 1% BSA/PBST was added and the plate incubated at room temperature for 1 hour. After washing twice with 200 µl PBST, 100 µl anti-mouse IgM with horseradish peroxidase in 1% BSA/PBST (1mg/ml, 1:5,000) was then applied to each well and incubated at room temperature for 30 min. After 4 x final washes with 200 µl PBST, 100 µl TMB substrate (Sigma) was added per well and the plate incubated for 10 min at room temperature for the blue colour development. The enzyme reaction was stopped by adding 50 µl 0.5M H_2SO_4. The optical density at 450 nm was read using a Molecular Devices Spectra Max 340PC reader (Sunnyvale, CA, USA).

The Chg47 capture ELISA was performed by coating 20 ng of anti-IgM in 50 mM pH 9.6 of carbonate-bicarbonate buffer to each well in the microplate, which was incubated overnight at 4°C. Afterwards, the plate was blocked by 1% BSA/PBST at room temperature for 4 h. 100 µl of 1:500 dilutions of different partly purified IgM were added to each well and the plate incubated overnight at 4°C. After washing twice with 200 µl PBST, different concentrations of Chg47, spore extracts or dust sample extracts in 1% BSA/PBST were added for antigen capture and incubated at room temperature for 2 h. Titration curves were established with serial dilutions of Chg47 and *C. globosum* ascospore extracts, respectively, with the optimized capture antibodies as follows: 20 ng/well for anti-IgM and then 1:500 dilution of partly purified 10B3 and 5F12, 20 ng/well for partly purified 10B3 and 5F12, or 10 ng/well for purified PAb). The samples were then analyzed as above.

C. globosum UAMH 7142 ascospores were prepared as described previously [7]. Spores of other fungi, together with their culture collection numbers are found in Shi et al. [9]. Cross reactivity studies were conducted using spores from 18 species of taxonomically divergent fungi common in house in damp buildings. Based on the detection limits of the various methods obtained, this was carried out with capture ELISAs comprised of the double PAb-PAb, Anti-IgM+10B3 and Anti-IgM+5F12.

Analysis of *C. globosum* ascspores in house dust. House dust sieved to < 300µm was obtained from samples from a research study [19]. An amount of fine dust (0.1 g) was spiked with different concentrations of *C. globosum* spores. The spores were fragmented using a Spex-Certiprep mixer mill (model 5100, Metuchen, NJ, USA) [9]. Ascospores (50 mg) were treated in a similar fashion. The spore fragments and spiked dust samples were dissolved in PBST containing 5 µl of a fungal protease inhibitor cocktail (Sigma) and sonicated for 2 h at 4°C. The solution was then centrifuged at 10,000 × g.

The supernatants were then loaded to ELISA microplates for capture ELISA detection.

RESULTS

Monoclonal antibodies. A single fusion was performed and the resulting hybridomas were screened for MAb production. Nine cell lines produced MAbs that reacted strongly in western blot with Chg47 (Figure 1A). All 9 cell lines (5C11, 7C4, 5G5, 3D7, 10E12, 5F7, 5F9, 5F12 & 10B3) obtained were of the IgM isotype. To detect their ability to bind Chg47, MAbs were further tested by indirect ELISA with different concentrations of Chg47 (Fig. 1B). With the exception of MAbs 5F12 and 10B3, which reacted strongly with the purified antigen, the remaining MAbs had lower affinities. Additionally, the Chg47 capture ELISA with anti-IgM coated in the plate to immobilize the nine respective MAbs as capture antibodies and with the biotinylated PAb as the primary antibody, indicated that MAbs 5F12 and 10B3 had the strongest interactions with the antigen and had the most sensitive limit of quantification compared to the other 7 MAbs tested (Figure 1C).

Purification and characterization. More than 99% purity of the PAb was achieved with a Protein G affinity column, as determined by SDS-PAGE with CBB staining (data not shown). The PAb was further purified by Chg47 affinity column in a manner similar to the methods reported by Shi et al. [9, 10]. As expected, most of the PAbs after the Protein G purification were not specific to the Chg47 antigen. From 50 mg total IgG, a yield of ca. 2 mg was purified PAb after the Chg47 affinity column purification. The final PAb was characterized by Western blot and capture ELISA. Both indirect ELISA and western blot indicated that the final purified PAb was strongly bound to Chg47 (data not shown).

Capture ELISA. Five different capture antibodies were screened (Tab. 1). The optimized primary and secondary antibody concentrations were a 1:8,000 and 1:16,000 dilution, respectively, were developed (Fig. 2A, 2B). The limits of detection (LOD) of the different capture ELISA protocols tested were as follows:
- for the double PAb-PAb assay, the LOD was 7.6 pg/ml;
- for the combination of PAb + monoclonal 10B3, the value was 31 ng/ml;
- for PAb-5F12, 62 ng/ml;
- for Anti-IgM + 10B3, 61 pg/ml;
- for Anti-IgM + 5F12, 122 pg/ml Chg47.

Table 1. Analytical performance of the capture ELISA methods

capture antibody	primary antibody	LOD (Chg47 ng/ml)	Linear Range (ng/ml antigen (R^2))	LOD (spores, µg/ml (# of spores))	Linear Range (µg/ml spores (R^2))
Pab	Pab	0.0076	0.0076–0.122 (0.993)	0.076 (20)	0.15–1.22 (0.991)
10B3	Pab	31	31–500 (0.985)	–	–
5F12	Pab	62	62–500 (0.971)	–	–
Anti-IgM +10B3	Pab	0.061	0.061–0.977 (0.989)	9.8 (2.8×10^3)	1.22–39.1 (0.998)
Anti-IgM +5F12	Pab	0.122	0.061–0.977 (0.989)	19.5 (5.7×10^3)	2.44–156 (0.981)

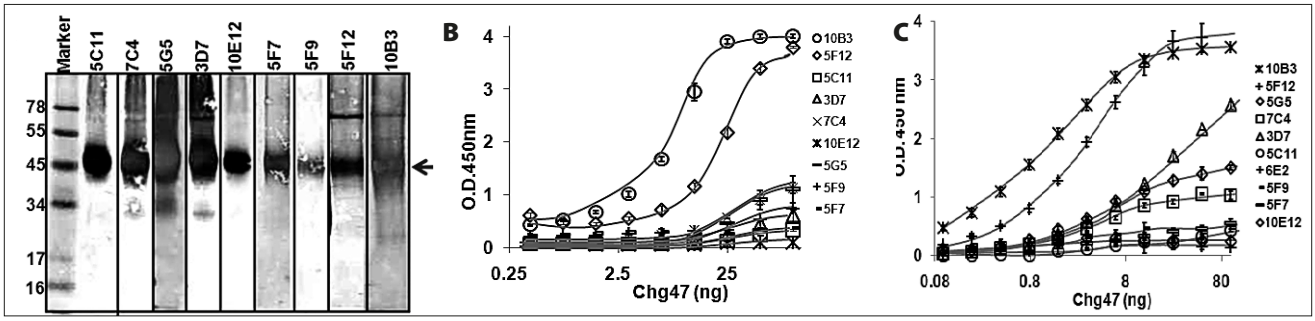

Figure 1. Performance of different IgM mAb against Chg47
A – Western blots of different Chg47 monoclonal antibodies; 1 µg Chg47 was loaded to each lane. Chg47 mAb by indirect ELISA with different concentrations of coated Chg47.
B – indirect ELISA with various IgM antibodies as the primary antibodies.
C – capture ELISA with anti-IgM immobilizing various IgM antibodies as the capture antibody and 1:8,000 biotinylated PAb as the primary antibody.

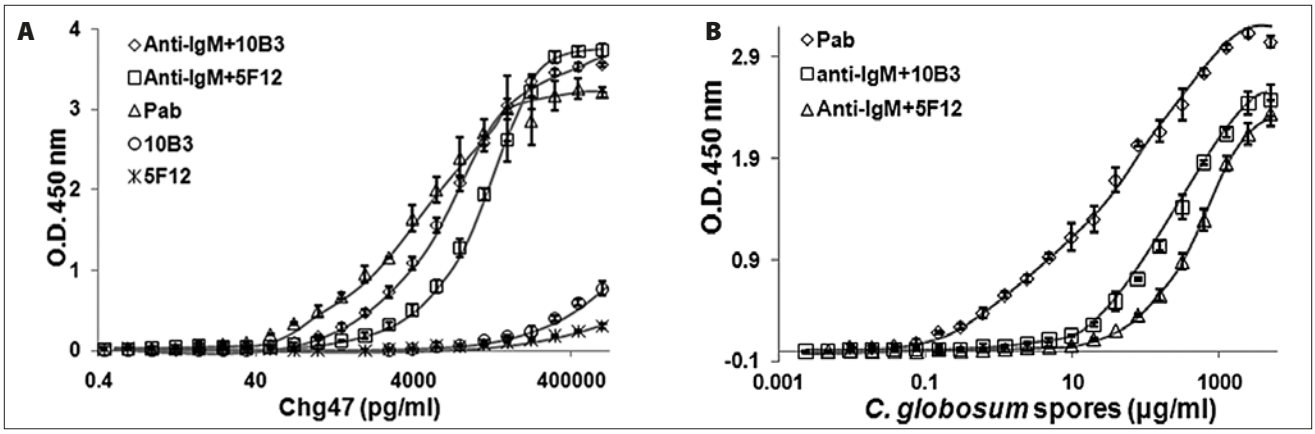

Figure 2. Chg47 capture ELISA for antigen and *C. globosum* ascospores
A – Chg47 capture ELISA with different concentrations of purified Chg47; B – Chg47 capture ELISA with *C. globosum* ascospore fragments detected

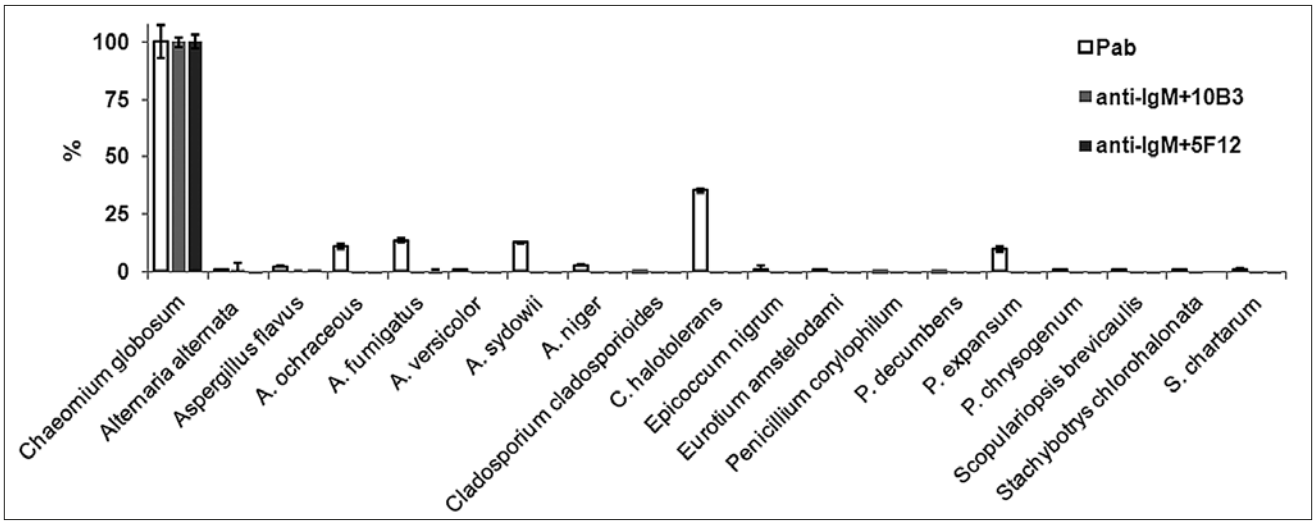

Figure 3. Cross-reactivity of various capture ELISA to spore fragments of other fungi
1,000 ug/ml of *C. globosum* ascospore fragments or the equivalent amount (weight/volume) of spore fragments from the other species. The capture antibodies were PAb (PAb), anti-IgM + 5F12 and anti-IgM + 10B3 respectively. 1:8,000 biotinylated PAb was used as the primary antibody and 1:16,000 Strep-HRP used as the secondary antibody

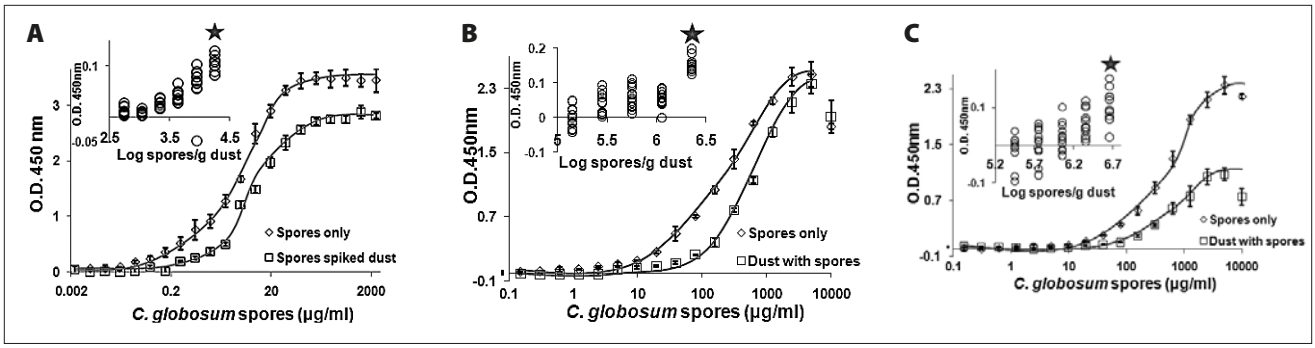

Figure 4. Analysis of *C. globosum* spore-spiked dust by various Chg47 capture ELISA
Panel A-C – Capture ELISA with PAb (A), anti-IgM + 10B3 (B) and anti-IgM + 5F12 (C) as the respective capture antibody. The limitation of quantification was 0.31 µg/ml (A), 39 µg/ml (B) and 78 µg/ml (C), respectively, in 50 mg/ml dust samples or 1.8×10^4 (A), 2.3×10^6 (B), 4.5×10^6 (C) spores per gram dust (upper left in each panel, $p<0.05$; Fisher's LSD)

These LODs were significantly different ($p<0.05$; Fisher's LSD). The LOD for the most sensitive assay for *C. globosum* ascospores was the double PAb-Pab assay (table 1). Table 1 includes the linear ranges for the various assays shown. For the double PAb-PAb combination, the linear range was 7.6–122 pg/ml ($R^2=0.993$).

For the double PAb-PAb ELISA, cross-reactivity to the other species was low on a wt/wt basis for all fungi except *C. halotolerans*. The methods based on the two monoclonal antibodies had no detectible response to the other fungi tested (Fig. 3).

Analysis of house dust samples spiked with *C. globosum* spores. This analysis was carried out with the 3 most sensitive capture ELISA assays, PAb-PAb, Anti-IgM + 10B3 and Anti-IgM + 5F12. The detection limit of the Chg47 protein in house dust using the PAb-PAb assay was 6.2 µg/g house dust (~10^4 spore fragment equivalents). For the 2 IgM-based assay the values were 780 µg/g house dust (Anti-IgM + 10B3) and 1,560 µg/g house dust (Anti-IgM + 5F12) (Fig. 4). The linear range were between 6.2–98 µg per gram dust ($R^2=0.970$) for the most sensitive method, and between 98 – 626 µg/g dust ($R^2=0.998$) for the Anti-IgM + 10B3 assay, and 400 – 1250 µg/g dust ($R^2=0.994$) for the Anti-IgM + 5F12 combination.

DISCUSSION

As noted, *C. globosum* is one of the most common fungi that grows in damp buildings as well as in agricultural and forestry workplaces. Using sera from atopic patients, we characterized and purified an extracellular chitosanase (Chg47) from *C. globosum* that is antigenic to humans, and collected preliminary data on a polyclonal antibody [7]. The World Health Organization (WHO) [20], among other cognizant authorities, has examined the relationship of exposure to fungi growing in damp buildings to respiratory disease. Mould growth indoors is associated with exacerbation of asthma in mould-sensitized individuals and increased upper respiratory disease. Reliable tools for exposure assessment are critical to resolving causality and attributable risk of fungal contamination [21, 22]. The NAS panel stated that the best way to assess the causality of allergic disease associated with fungi would be to develop 'standardized methods for assessing exposure to fungal allergens ranges for each analysis preferably based on measurements of allergens rather than culturable or countable fungi' [22]. We have focused on proteins that are excreted on spores and mycelia and produced on building materials [e.g. 18].

Nine MAbs were developed from purified Chg47 and all these MAbs were of the IgM isotype. This was also the

outcome with the allergen from *Aspergillus versicolor*, Asp v 13, a subtilisin-like serine protease. In contrast, the allergen from *Stachybotry chartarum*, was an alkaline, Mg-dependent exodeoxyribonuclease [10] and an acidic glucoamylase in *Penicillium chysogenum* [4]. Both of the allergens produced monoclonals primarily of the IgG isotype. Monoclonals of the IgM isotype are typically less useful for a two-site capture ELISA due to the high molecular weight [23, 24]. In addition, purification of biologically-active IgM remains a challenge due to the instability of IgM at suboptimal pH and salt concentrations, and when binding to matrixes such as cation/anion/hydrophobic affinity resins [25, 26]. However, some successful assays have been reported using IgM-based capture ELISA [23, 27, 28].

Among the 9 MAbs cell lines, MAbs 10B3 and 5F12 had the strongest interaction with Chg47, in both quantitative assays with indirect or capture ELISA (Fig. 1B, 1C). Direct coating of MAb 10B3 and 5F12 reduced the interaction with the target antigen, Chg47. The LOD for either monoclonal with the capture antibody was reduced 500-fold. This reduction may be due to the anion affinity purification process or covalent/hydrophobic binding to the ELISA plate surface, which may critically alter the immunological activity of IgM. [25, 26]. Nevertheless, the 2 IgM antibodies had exceptional specificity to *C. globosum* ascospore fragments in relation to a range of taxonomically diverse fungi.

In studies of antibody methods for the analysis of the allergens from *P. chrysogenum* and *A. versicolor*, novel methods for increasing the sensitivity of the polyclonal antibody were developed. This allowed the development of useful assays for the analysis of the respective allergens in house dust. This method involved improving the quality of the PAb preparation using Protein G columns, followed by antigen-affinity column chromatography [9, 10]. By applying this technique, different capture ELISA methods for the Chg47 antigen were developed by either coating partly purified MAb IgM, by coating anti-IgM to immobilize partly purified IgM, or by using enhanced PAb as the capture antibody, and using the enhanced biotinylated PAb as the primary development antibody.

The LOD of Chg47 using PAb-PAb pair was ~0.7 ng Chg47 antigen per gram dust. This represented ~ 10^4 spore fragments/g house dust. In this method, cross-reactivity was observed with *C. halotolerans* (Figure 3). The spores of *C. halotolerans* have a surface area approximately 25 times less than the ascosopores of *C. globosum*. Chg47 is an exoallergen found on the spore surface, at a high concentration [7]. The amount of allergen per spore is a function of the surface area of the spore [see 5, 29]. On a weight/weight basis, approximately 35 times more spores were present for *C. globosum* (3×10^6 ascospores/mg) than *C. halotorerans* (7×10^7 spores/mg). In a practical sense, the PAb-PAb assay is suitable for house dust analysis. A false positive could only occur in the event that the amount of *C. halotoerans* present exceed >10–100 times that of *C. globosum*. While both species require high water contents, *C. halotolerans* is mainly associated with wood products and insulation [1]. The sensitivity of this assay comparable to the capture ELISA for *A. versicolor* [3], but is somewhat more sensitive than the assay for *S. chartarum* [8], *A. fumigatus* Asp f1 [9, 30, 39], and is much more sensitive than the assay for the *A. alternata* allergen Alt a1 [9, 32].

The limit of detection for the target antigen Chg47 using the anti-IgM + 10B3 or anti-IgM + 5F12 pairs were ~ 20 ng and ~ 40 ng Chg47 antigen per gram dust, respectively. Unlike the modest cross-reactivity seen in the double PAb method for Chg47, essentially no cross reactivity was observed (Figure 3). Little or no cross-reactivity has been seen in the assays for the allergens from *S. chartarum*, *A. versicolor* or *P. chrysogenum* [8, 9, 10]. This is not always the case with other assays for fungal allergens [33, 34].

CONCLUSIONS

Two useful ELISA methods emerged for the detection of *C. globosum* spores in dust samples. One involves using enhanced biotinylated PAb as the primary antibody and coating anti-IgM to immobilize 10B3/5F12. An alternative method is based on the PAb as the capture and detection antibody for capture ELISA. The methods have little or no cross-reactivity to the common fungi that occur in damp housing or agriculture environments. Some cross-reactivity was observed to *C. halotolerans* with the double PAb method. This fungus is uncommon in homes or in agricultural environments. The detection limit of the double PAb method was comparable to ELISA methods for *A. versicolor* in house dust, and is more sensitive than assays for allergens of *S. chartarum*, *A. fumigatus* and *A. alternata*. All 3 methods are suitable for the measurement of the dominant *C. globosum* antigen Chg47 in dust and each presents particular advantages.

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