

Identification of Immunoglobulin E-Binding Proteins of the Xerophilic Fungus *Aspergillus penicilliioides* Crude Mycelial Mat Extract and Serological Reactivity Assessment in Subjects with Different Allergen Reactivity Profiles

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Keywords

Xerophilic fungi · *Aspergillus penicilliioides* · Allergy · Immunoglobulin E-binding proteins · Serologic reactivity

Abstract

Background: *Aspergillus penicilliioides* is a very common indoor xerophilic fungus and potential causative agent of respiratory conditions. Although people are constantly exposed to *A. penicilliioides*, no proteins with allergenic potential have been described. Therefore, we aim to confirm allergic sensitization to *A. penicilliioides* through reactivity in serological assays and detect immunoglobulin E (IgE)-binding proteins. **Methods:** In an indirect ELISA, we compared the serological reactivity to *A. penicilliioides* between subjects with specific IgE (sIgE) (group 1, $n = 54$) and no sIgE reactivity (group 2, $n = 15$) against commercial allergens. Correlations and principal component analysis were performed to identify associations between reactivity to commercial allergens and *A. penicilliioides*. IgE-binding proteins in *A. penicilliioides* were visualized using Western blotting

(WB) in group 1. The IgE-binding proteins with the highest reactivity were analyzed by mass spectrometry and confirmed by transcript matching. **Results:** There was no statistical significance ($p = 0.1656$) between the study groups in serological reactivity. Correlations between reactivity to *A. penicilliioides*, dog epithelia, *Aspergillus fumigatus*, and *Penicillium chrysogenum* were observed. WB experiments showed 6 IgE-binding proteins with molecular weights ranging from 45 to 145 kDa. Proteins of 108, 83, and 56 kDa showed higher reactivity. Mass spectrometry analysis of these 3 proteins led to the putative identification of NADP-specific glutamate dehydrogenase and catalase B. This was confirmed with transcriptome analysis. **Conclusions:** These results provide evidence of the presence of potential allergenic components in *A. penicilliioides*. Further analysis of the putatively identified proteins should reveal their allergenic potential.

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Introduction

Spores from xerophilic fungi (molds capable of growing at low levels of water activity, a_w), such as *Aspergillus penicillioides*, are prevalent in the indoor environments in many areas of the world and tropics, including Puerto Rico, where *A. penicillioides* has been reported [1–4]. Contrary to various fungi such as *Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp., and *Alternaria* spp., which can be recovered in standard culture media such as malt extract agar, the allergenic potential of xerophilic fungi has been underestimated. Isolation of xerophilic fungi from environmental settings requires special culture media such as glycerol nitrate agar (G25N), which is not routinely used in commercial production of fungal extracts. For this reason, only a few studies have documented the allergenic potential of xerophilic fungi, such as *Aspergillus restrictus*, *Eurotium* spp., and *Wallemia sebi* [5, 6]. As a result, proteins originating from xerophilic fungi that could be included in allergy testing are limited [7]. Considering that xerophilic fungi contribute significantly to the proportion of total fungal spores in indoor air samples, allergen panels may not be representative of the composition of the most common Puerto Rican indoor airborne fungal allergens [1, 7].

The current pilot study investigated the serological reactivity of Puerto Rican subjects to *A. penicillioides* crude mycelial mat extract (MME) using in vitro serological tests. An indirect enzyme-linked immunosorbent assay (ELISA) was employed to detect immunoglobulin E (IgE) antibodies against *A. penicillioides*, and the serological reactivity of individuals with different allergic profiles was compared. Furthermore, Western blot (WB) analysis was performed to identify IgE-binding proteins using the serum samples from allergic individuals previously tested in the indirect ELISA. The protein bands with the highest proportion of reactivity were analyzed and putatively identified by mass spectrometry (MS). Based on previous findings and the high prevalence of respiratory diseases present among Puerto Ricans, we expected to find a high serological reactivity to *A. penicillioides* MME among the serum samples tested [1, 8–12]. We supplemented our findings with a transcript analysis from *A. penicillioides* (PRJNA344885) [unpubl. data] by matching the proteins identified by MS with high count transcript presence that had a similar predicted sequence and molecular weights (MWs). We report evidence of the serological reactivity and IgE-binding components of *A. penicillioides*.

Materials and Methods

Identification and Culture Conditions

A. penicillioides colonies were recovered from air samples collected in a room that stores student medical records in the Students' Health Services office of the University of Puerto Rico (UPR) Medical Sciences Campus (MSC) (San Juan, PR, USA). Air samples were collected with the MicroBio2 air sampler (Cantium Scientific Limited, UK) in 25% glycerol nitrate agar (G25N, 0.75 g dipotassium phosphate, 250 mL glycerol, 7.5 mL Czapek concentrate, 3.5 g yeast extract, 12 g agar, and 750 mL of ultrapure water) and incubated at 25°C. The identification of *A. penicillioides* was confirmed by Dr. S. Vesper at the US Environmental Protection Agency using the MSqPCR assay [13]. Conidia were harvested from the purified mycelia by adding sterilized, pyrogen-free water. The suspension was aseptically transferred to a 2-L Erlenmeyer flask with 250 mL of G25N broth and cultured at 25°C with constant shaking for 2–3 weeks. This culture was used for continuous subculturing to obtain the necessary mycelial mat material for the experiments. The mycelial mats were separated from the culture media by centrifugation in a 5810-R centrifuge (Eppendorf International, Hamburg, Germany) at 3,000 rpm for 10 min, washed once with ultrapure water, and stored at –80°C until further processing.

Crude MME Preparation

A. penicillioides MME was prepared as described by Fratamico and Buckley [14] with slight variations. Briefly, mycelial mat previously collected was thawed on ice and washed with lysis buffer (PBS containing 1 mM phenylmethylsulfonyl fluoride; G-Biosciences, MO, USA). Previously sterilized glass beads (Biospec Products, OK, USA) were used to disrupt mycelia during a 60-min cycle (1 min agitation and 1 min rest) using a BeadBeater (Biospec Products) connected to a GraLab electronic timer/interval meter (GraLab Co., OH, USA). The homogenate was centrifuged at 417 g and 4°C for 30 min, and then at 216,179.7 g at 4°C for 90 min in an Optima L-100 XP centrifuge (Beckman Coulter, CA, USA). The supernatant was collected and filter sterilized with a Nalgene 115-mL filter unit (0.45- μ m pore size; Nalge Nunc International, NY, USA); 5-mL aliquots were transferred into sterile 15-mL Falcon conical tubes (Becton Dickinson, NJ, USA) and frozen at –80°C for 24 h or until further processing. The frozen tubes were placed in a 600-mL Virtis drying flask (SP Scientific, PA, USA), and lyophilization was performed for 1 day in an Edwards Modulyo freeze dryer (Edwards High Vacuum International, Crawley, UK). The resulting solid was stored at –80°C until further processing.

Determination of Extract Protein Concentration

The protein concentration of the *A. penicillioides* MMEs was determined with the DC Protein Assay Kit II (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instructions. The protein concentrations of the MME used in the serological assays ranged from 1.5 to 5.0 mg/mL.

Human Sera

With the approval of the UPR-MSC Institutional Review Board (A9830113), human serum remnants were donated by the Toledo Laboratory (Arecibo, PR). The donated serum remnants came from individuals who had been tested for specific IgE (sIgE) with

Table 1. Commercial allergen panels tested on sera donated by the reference clinical laboratory (Toledo Clinical Laboratory, Arecibo, PR, USA)

Respiratory allergen panel ¹	Respiratory/food allergy panel ¹	
<i>Alternaria alternata</i> ²	Acacia	Egg
American cockroach	<i>Alternaria alternata</i> ²	English plantain
<i>Aspergillus fumigatus</i> ²	<i>Aspergillus fumigatus</i> ²	<i>Fusarium moniliforme</i>
Cat dander/epithelium ²	Australian pine	Lamb's quarter
Chicken feathers	Bermuda grass	Mango
<i>Cladosporium herbarum</i> ²	Cat dander/epithelium ²	<i>Mucor racemosus</i>
<i>Dermatophagoides farinae</i> ²	<i>Cladosporium herbarum</i> ²	<i>Penicillium chrysogenum</i> ²
Dog epithelia ²	Cockroach	<i>Phoma betae</i>
Horse dander	Corn	Queen palm
Mouse epithelium	Cow milk	Rough pigweed
<i>Penicillium chrysogenum</i> ²	<i>Dermatophagoides farinae</i> ²	Western ragweed
Rat epithelium	Dog epithelia ²	Wheat

¹ Serum samples were tested to only one of the panels. Some serum samples did not get tested for all the allergens comprised in the panels.

² The allergens marked were the ones taken into consideration for principal component analysis.

a radioallergosorbent test (RAST) (Immulite 2000 3gAllergy) against various commercial allergens included in either of 2 panels. These commercial allergens consisted of respiratory allergens alone or respiratory and food allergens combined (Table 1). The clinical history of the subjects was not available, and the serum samples were not tested with the same battery of commercial allergens. Donated sera were divided into 2 groups according to their allergen reactivity profiles determined by RAST with commercial extracts: (a) group 1 consisted of 54 subjects with sIgE to at least 1 commercial allergen, and (b) group 2 comprised 15 subjects with no sIgE against any allergen (Table 2). The negative control was a serum sample from 1 volunteer with no previous history of allergies and no skin reactivity to commercial allergen extracts (Greer Laboratories, NC, USA), which included fungal extracts from *Penicillium* spp., *Aureobasidium* spp., *Alternaria* spp., *Cladosporium* spp., *Acremonium* spp., *Fusarium* spp., *Aspergillus* spp., *Chaetomium* spp., animal extracts from dog, cat, mite, and cockroach, and pollen extracts from Bermuda, Johnson, and Bahia, among others, and total IgE of 4.20 IU/L.

Indirect ELISA

Indirect ELISAs against *A. penicilliioides* MME were performed on human serum samples as described by Figueroa-Santiago et al. [15] with minor modifications. The assay was optimized by checkerboard titration. High-binding flat-bottom disposable polystyrene plates (Corning, NY, USA), were coated overnight at 4°C with 10 µg of MME from *A. penicilliioides* prepared in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6). The next day, the plates were blocked with 300 µL of 3% bovine serum albumin (BSA) per well (AMRESCO, OH, USA) in PBS containing 0.05% Tween 20 (PBST) and incubated for 1 h at 37°C in a wet chamber. The blocking solution was discarded, and the plates incubated for 1 h in a humid chamber at 37°C with 100 µL of human sera (1:100) per well. Subsequently, the plates were incubated for 1 h at 37°C in a humid chamber with 100 µL (1:2,500) of affinity-

Table 2. Sample groups for the indirect ELISA against *Aspergillus penicilliioides* crude mycelial mat extract

Serum sample groups	Origin	Samples, <i>n</i>
<i>Group 1:</i> Individuals with sIgE reactivity to ≥1 allergen from the commercial panels tested	Toledo Clinical Laboratory, Arecibo, PR, USA	54
<i>Group 2:</i> Individuals with no sIgE reactivity to the allergens from the commercial panels tested	Toledo Clinical Laboratory, Arecibo, PR, USA	15
Total		69
The clinical history of the individuals is unknown.		

purified peroxidase-labeled goat anti-human IgE per well (KPL, Gaithersburg, MD, USA). Incubation with 100 µL of the substrate solution per well, containing 20 mg o-phenylenediamine and 30% H₂O₂, pH 5.0 (Sigma-Aldrich Co.), was performed for 30 min at room temperature in the dark. The reaction was stopped with 50 µL of 10% HCl per well. Before any antibody and substrate incubation, the plates were washed 3 times with PBST and read at 490 nm to obtain the absorbance values (A₄₉₀) using a Bio-Rad microplate reader.

SDS-PAGE of MME and WB Analysis

MMEs were separated under denaturing conditions using 4–15% Mini-PROTEAN[®] TGX[™] Precast Protein Gels (Bio-Rad Laboratories). One-dimensional SDS-PAGE was carried out following the method of Laemmli [16]. The gels were run in a Mini-

PROTEAN Tetra cell electrophoresis system (Bio-Rad Laboratories) with Tris-glycine-SDS buffer (pH 8.3) for 50 min at 100 V in a cold chamber and subsequently stained with the Silver Stain Plus Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Thirty micrograms of protein were applied to each well. The proteins were transferred to a 0.45- μ m nitrocellulose membrane (Bio-Rad Laboratories), at 100 V and a constant current of 300 mA for 50 min in a cold chamber, using the Mini Trans-Blot Cell System (Bio-Rad Laboratories) in Tris-glycine buffer (pH 8.0) containing 20% methanol. The membranes were blocked with constant shaking and 5% BSA in PBST for 3–5 h at 4°C. After blocking, patient serum was diluted 1:400 and incubated in 3% BSA in PBST at 4°C overnight. The next day, the membranes were washed 6 times for 5 min with PBST and then incubated for 2 h with ReserveAP™ (affinity-purified) phosphatase-labeled goat anti-human IgE (KPL, Gaithersburg, MD, USA) diluted 1:20,000 in 5% BSA in PBST at room temperature. Chromogenic detection was carried out by adding 6 mL of BCIP/NBT phosphatase substrate (KPL) for 25 min and stopped by washing with 5 mL of de-ionized water twice for 5 min. Immunoblots were obtained from 26 subjects of group 1. Previously, all subjects from group 1 were separated into serum pools to perform WB analysis and determine which were the most reactive (data not shown). Sera from individuals comprising the most reactive pools were individually analyzed for detection and identification of the IgE-binding proteins. The immunoblotting analysis was complemented with image analysis using ImageJ® (National Institutes of Health) as previously described by Vila-Hereter et al. [17].

MS for Protein Identification

A lyophilized *A. penicilliioides* MME sample was sent to the Proteomics and Mass Spectrometry Facility at Cornell University Institute of Biotechnology (Ithaca, NY, USA) for MS analysis and database search. The sample was reconstituted in PBS (pH 7.4) containing 0.05% SDS, run on a 10% Bis-Tris gel in a MOPS buffer system, and stained with SYPRO Ruby (Invitrogen). Extraction and in-gel digestion with trypsin of the proteins of interest were performed, followed by a nanoscale liquid chromatography coupled to MS/MS (nano-LC-MS/MS) in an Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer. The data search was carried out using PD 1.4 (Proteome Discoverer v. 1.4) with the SEQUEST HT search engine against the UniProt database for the fungal kingdom, which contains approximately 30,000 entries. The following settings were used: 2 missed cleavage sites by trypsin allowed with fixed carboxamidomethyl modification of cysteine and variable modifications of methionine oxidation, and deamidation of asparagine and glutamine residues. The PD 1.4 reports for each band were then interpreted for protein identification.

Transcriptome Sequence Retrieval

Database searches for matching sequence retrieval between the results obtained from the MS analysis and the transcripts obtained from *A. penicilliioides* (PRJNA344885) [unpubl. data] were performed with the Basic Local Alignment Search Tool (BLAST) using blastx with default parameters to confirm the presence of the proteins [18–20]. An E-value $<10^{-05}$ was considered a significant match. This was performed on the Bridges supercomputing system at the Pittsburgh Supercomputing Center using the GALAXY interface [21–23].

Statistical Analysis

Statistical analysis was performed with XLSTAT software (Addinsoft SARL) and R version 3.3 [24]. A Shapiro-Wilk test was performed to test for normality in the A_{490} data from the indirect ELISA in groups 1 and 2 [25]. A Mann-Whitney U test [26] was performed to determine if there were statistically significant differences between group 1 and group 2 A_{490} values. To compare the proportion of group 1-reactive sera to each of the 6 proteins detected in WB, the Fisher exact test with 95% confidence intervals was applied.

Exploratory Data Analysis

To identify associations between group 1 and group 2 reactivity to *A. penicilliioides* and 7 commercial allergens, among all those tested (Table 2), a Spearman correlation matrix was calculated. Principal component analysis (PCA) using the Spearman correlation matrix was carried out. For the PCA, the allergen extracts included were *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Penicillium chrysogenum*, *Dermatophagoides farinae*, dog epithelia, and cat dander epithelium. These allergens were chosen because they were common to both allergen panels (respiratory and food/respiratory panels). From the total serum samples (69), only the 59 samples that were tested for the chosen allergens were used for the analysis.

Results

Groups 1 and 2 Showed Serological Reactivity to *A. penicilliioides* MME

To detect IgE antibodies and compare serological reactivities to *A. penicilliioides* MME between subjects with different allergen reactivity profiles (Fig. 1), an indirect ELISA was performed. The median A_{490} values of groups 1 and 2 were 0.6243 ± 0.3509 and 0.403 ± 0.3308 , respectively. The differences between groups 1 and 2, although A_{490} was 54% higher in group 1, were not statistically significant ($p = 0.1656$; Mann-Whitney U test). The A_{490} of the negative control was 0.2120. These findings suggest that sensitized and nonsensitized subjects recognized allergenic components in the *A. penicilliioides* MME.

Spearman Correlation and PCA Reveal an Association between Fungal Allergens and *A. penicilliioides* MME

To further explore the relationship between the reactivity to *A. penicilliioides* MME and sIgE to commercial allergens, Spearman correlation and PCA were performed. Spearman correlation was employed for both correlation analysis and PCA because, except for A_{490} ($p = 0.130$), most variables pertaining to reactivity to commercial extracts were sparse and did not follow a normal distribution as determined by a Shapiro-Wilk test. This statistical approach (i.e., PCA) reduces the dimen-

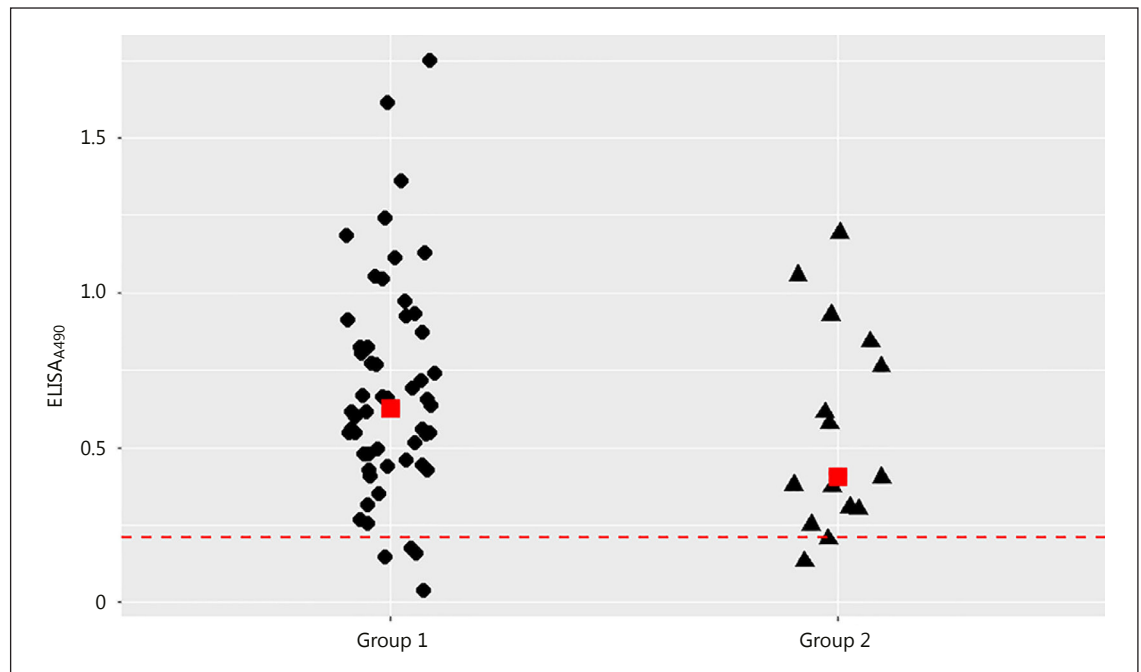


Fig. 1. Indirect ELISA reactivity results (A_{490}) of human sera against the crude mycelial mat extract of *Aspergillus penicillioides*. The x-axis represents the serum sample groups (group 1, $n = 54$; group 2, $n = 15$). The y-axis represents the absorbance values for each individual. The red square within each group represents the median value for each group. The dashed red line represents the A_{490} for the negative control.

Table 3. Spearman correlation matrix

Variables	A_{490}	Aalt	Afum	Cat	Cher	Dfar	Dog	Pchr
A_{490}	<i>1</i>	0.239	0.305	0.078	0.256	0.048	0.373	0.258
Aalt		<i>1</i>	0.620	0.379	0.780	0.284	0.349	0.664
Afum			<i>1</i>	0.231	0.816	0.209	0.257	0.522
Cat				<i>1</i>	0.214	0.482	0.647	0.262
Cher					<i>1</i>	0.310	0.288	0.648
Dfar						<i>1</i>	0.306	0.147
Dog							<i>1</i>	0.278
Pchr								<i>1</i>

A_{490} , indirect ELISA values; Aalt, *Alternaria alternata*; Afum, *Aspergillus fumigatus*; Cat, cat dander epithelium; Cher, *Cladosporium herbarum*; Dfar, *Dermatophagoides farinae*; Dog, dog epithelia; Pchr, *Penicillium chrysogenum*. Values in italic are significantly different from 0 (α level = 0.05; ρ coefficients with $p < 0.05$).

sionality of datasets with a larger number of variables and aids in identifying patterns between variables [27].

Spearman correlation analysis identified statistically significant positive correlations between A_{490} and *A. fumigatus*, dog epithelia, and *P. chrysogenum* allergens ($p = 0.019$, 0.004 , and 0.048 , respectively). Other correlations between the reactivity to commercial extracts were

also detected, including between fungal allergens (*A. alternata* with *A. fumigatus*, *C. herbarum*, *P. chrysogenum*; *A. fumigatus* with *C. herbarum* and *P. chrysogenum*; and *C. herbarum* with *P. chrysogenum*), fungal and non-fungal allergens (*A. alternata* with cat dander epithelium, *D. farinae*, and dog epithelia; *A. fumigatus* and dog epithelia; *P. chrysogenum* and cat dander epithelium; *C.*

Fig. 2. Correlation circle from factors 1 and 2 illustrating the correlation between allergens. Variability (x-axis) is 46.38% for factor 1 and 18.08% for factor 2 (y-axis), with a cumulative variability of 64.46%. The x-axis represents the correlations between the variables and the allergen contribution to factor 1, and the y-axis represents the correlations between the variables and the allergen contribution to factor 2. Red lines represent the different allergens.

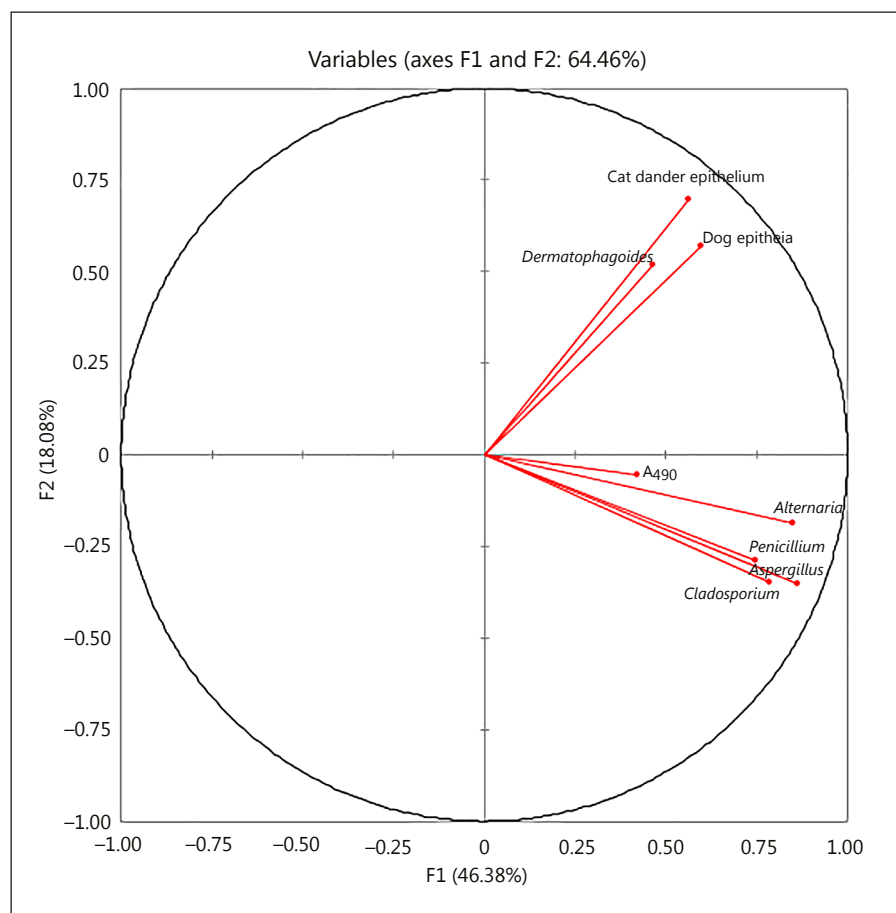


Table 4. Immunoblotting reactivity among serum samples ($n = 26$) from group 1

Reactivity	Detected bands in the Western blots	Serum samples recognizing the bands	Total
Reactive	1	3	18
	2	4	
	3	6	
	4	3	
	5	2	
Not reactive	0	8	8

herbarum with *D. farinae* and dog epithelia; and *P. chrysogenum* and dog epithelia), and between nonfungal allergens (cat dander epithelium with *D. farinae* and dog epithelia, and *D. farinae* and dog epithelia), as described in detail in Table 3. PCA revealed that, of the 8 compo-

nents identified, components 1 and 2 had higher eigenvalues (3.710 and 1.447, respectively), with a cumulative variability of 64.7%. These data indicate that the best quality projection for the variables will be visualized in an F1/F2 correlation circle, as shown in Figure 2. The correlation circle showed 2 groups of positively correlated allergens: one comprised *D. farinae*, cat dander epithelium, and dog epithelia, and a second corresponding to fungal allergens, including A₄₉₀ against *A. penicillioi-des* MME. Together, these results suggest an association between reactivity to *A. penicillioi-des* MME and fungal allergens.

Six IgE-Reactive Bands Were Recognized from *A. penicillioi-des* MME by Immunoblotting

Reactive IgE-binding protein bands were detected via WB of 26 sera because of the higher sample size corresponding to individuals from group 1. Sera from group 1 were used for WB analysis due to more availability (54 in group 1 vs. 15 in group 2). Six IgE-binding proteins were

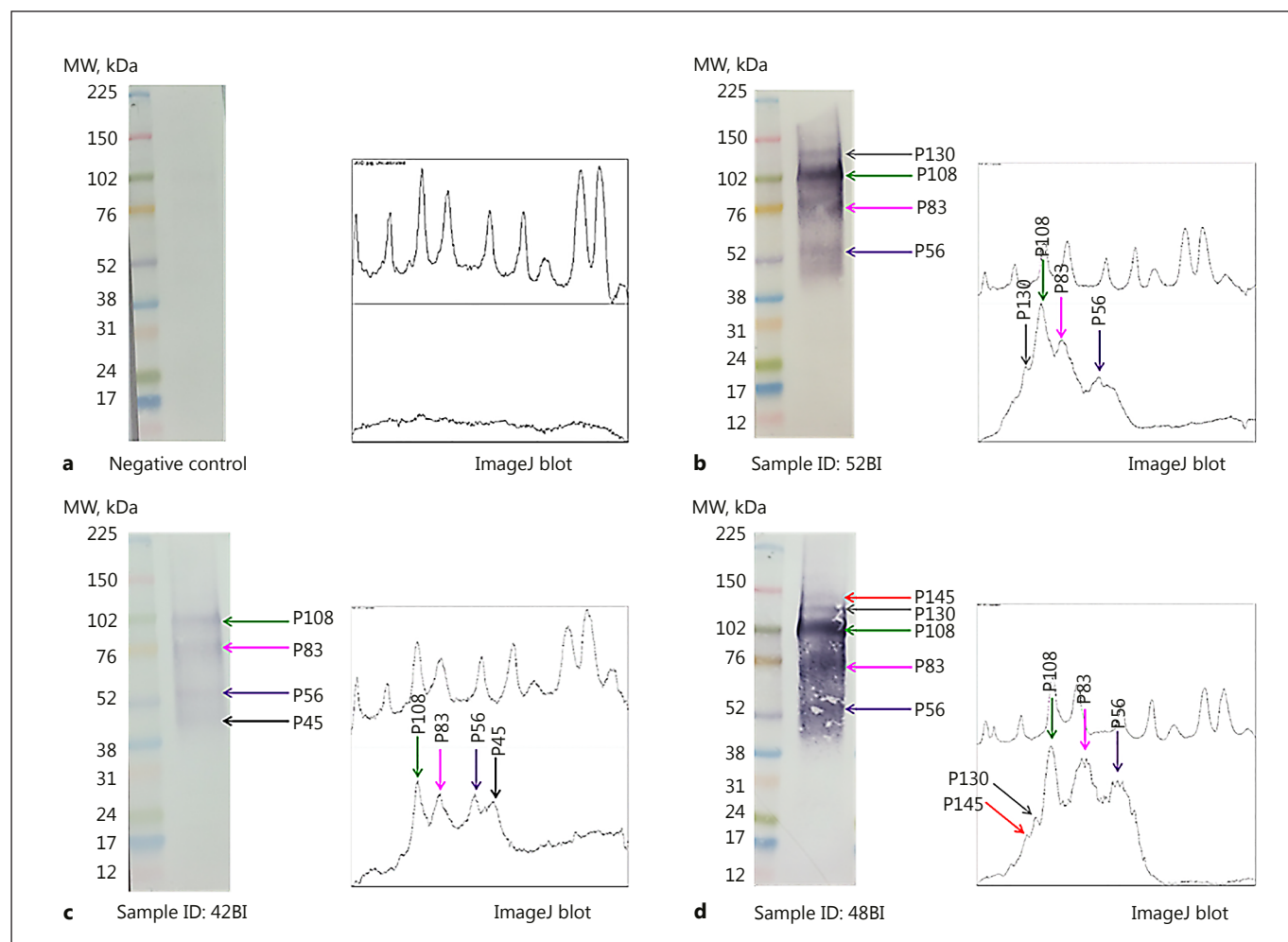


Fig. 3. Immunoblotting results illustrated by plots of the serum samples showing the 6 reactive IgE-binding bands. The reactive bands are represented by arrows and their estimated molecular weight (MW). **a** Negative serum sample. **b** Serum sample reactive to P130, P108, P83, and P56 IgE-binding protein bands. **c** Serum sample reactive to the P108, P83, P56, and P45. **d** Serum sample reactive to P145, P130, P108, P83, and P56. On the left side of the panels, we have blotted membranes, and on the right side, we have

the blots from ImageJ software. The y-axis in the plot represents the intensity of the reactive bands from the Western blot, illustrated as peaks, while the x-axis represents the distance in the membrane. The peaks in the plot match the bands in the membrane. Proteins (30 µg crude mycelial mat extract) were separated in 4–15% gradient polyacrylamide gels. Serum dilution, 1:400; conjugate dilution, 1:20,000.

detected with MWs ranging from 145 to 45 kDa (Fig. 3). Eighteen of the 26 sera (69.2%) were reactive to at least 1 IgE-binding protein: 15 (57.7%) sera were reactive to ≥ 2 IgE-binding proteins (Table 4). Three of the 6 IgE-binding proteins (MW: 108, 83, and 56 kDa) showed $>40\%$ reactivity (Table 5). The negative control (Fig. 3a) showed a slight reaction to the 108-kDa band, which was not evident in the ImageJ plot. These results suggest that proteins with MWs of 108, 83, and 56 kDa could be relevant allergens in *A. penicilliioides* MME and justify molecular identification.

Table 5. Summary of Western blot band detection

Reactive bands	Estimated molecular weight	Reactive serum samples		
		<i>n</i>	%	95% CI, %
P45	45	1	3.8	0.00–19.6
P56	56	15	57.7	36.9–76.6
P83	83	12	46.2	26.6–66.6
P108	108	17	65.4	44.3–82.8
P130	130	4	15.4	4.43–34.8
P145	145	2	7.7	0.00–25.1

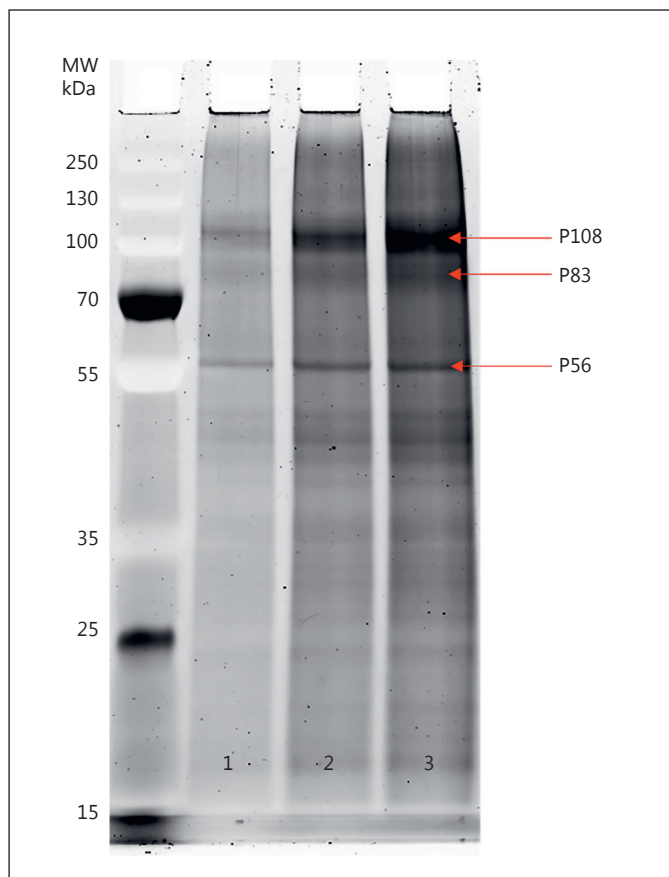


Fig. 4. SDS-PAGE gel image analysis of the *A. penicillioide* crude mycelial mat extract. Different protein concentrations (1, 3, and 5 µg for lanes 1, 2, and 3, respectively) were separated on a 10% polyacrylamide gel and stained with SYPRO Ruby (Invitrogen) for protein band pattern visualization and detection of the bands of interest (red arrows). MW, molecular weight. Gel image created by the Proteomics and Mass Spectrometry Facility at Cornell University Institute of Biotechnology (Ithaca, NY, USA).

MS Identification of 3 IgE-Binding Proteins

The 3 IgE-binding proteins showing increased reactivity, P108 (108 kDa), P83 (83 kDa), and P56 (56 kDa), were analyzed via MS for identification (Fig. 4). Results from the LC-MS/MS along with the transcripts matching the identified proteins can be found in Tables 6–8. The interpretation was based on the protein score, MW, number of unique peptides, the number of peptides, and confirmation of their presence by matching the protein hit with an *A. penicillioide* transcript. The match was considered significant if it had an E-value $<10^{-5}$. IgE-binding proteins P108 and P56, which demonstrated reactivity in WB, were identified as NADP-specific glutamate dehydrogenases, while P83 was identified as catalase B.

Discussion

A. penicillioide is a xerophilic fungus commonly found in indoor environments, including house dust [1–3, 28]. This fungus was suggested as a probable cause of allergies; however, its allergenic potential has not been studied [3]. In this pilot study, we evaluated the allergenic potential of the crude *A. penicillioide* MME in individuals with different allergen reactivity profiles. Serum from subjects with sIgE had higher reactivity to *A. penicillioide* MME than those with no sIgE to the commercial allergens tested. Six IgE-binding proteins were identified via WB, and in 3 of them, reactivity was $>40\%$ in sera from subjects with sIgE to commercial allergens. LC-MS/MS identification revealed that these 3 proteins have dehydrogenase and catalase activity. This study is the first to report IgE-binding proteins from *A. penicillioide* mycelia and provides evidence of *A. penicillioide* as an allergen source.

One-dimension SDS-PAGE immunoblotting revealed the presence of 6 reactive bands with MWs ranging from 145 to 45 kDa. Three of these bands, P108, P56, and P83, were recognized at higher frequencies (65.4, 57.7, and 46.2%, respectively). MS analysis of P108 and P56 putatively identified these 2 IgE binding proteins as NADP-specific glutamate dehydrogenases (Tables 6, 7). These enzymes are essential for the carbon and nitrogen metabolism [29]. The NADP⁺-linked protein structure in fungal species has 6 identical subunits, each with an MW around 48 kDa [29–32]. These findings suggest that P108 may be a subunit dimer of NADP⁺-specific glutamate dehydrogenase. MS analysis for the 3rd reactive band, P83, identified the IgE binding protein as an NADP⁺-specific glutamate dehydrogenase, too. However, the MW of this protein hit does not match the estimated MW determined by WB analysis. The protein pattern (Fig. 4) from our crude MME shows P108 as a very thick band, close to P83, which might indicate the presence of residues from P108 that might have altered the protein identification results. Analysis of the next set of protein matches obtained for P83 putatively identified the IgE-binding protein as catalase B, an enzyme essential for the conversion of hydrogen peroxide to water and molecular oxygen [33]. It is noteworthy that catalase B of *Aspergillus nidulans* has been shown to have an MW of 360 kDa and is composed of 4 identical glycosylated subunits [34]. The Allergome database (allergome.org) has identified this enzyme as an allergen, and each subunit has an MW of approximately 80 kDa. This would be a reasonable match for our protein identification.

Table 6. Protein identification report of IgE-binding protein P56 by mass spectrometry from PD 1.4 with the SEQUEST HT search engine against the UniProt database for the fungal kingdom

Protein identification data								Transcriptome data		
ID	PD	MW	S	#P	#UP	#Pe	C	MT	%ID	eV
P18819	NADP-specific glutamate dehydrogenase OS = <i>Emericella nidulans</i>	49.578	511.5150616	6	2	12	27.67	TRINITY_DN14200_c0_g1_i1 TRINITY_DN14200_c0_g1_i2	90 70.83	6.00E-05 0.008
Q9URS1 ¹	NADP-specific glutamate dehydrogenase OS = <i>Penicillium chrysogenum</i>	49.801	494.65	7	2	12	21.91	TRINITY_DN15766_c1_g1_i1 TRINITY_DN15766_c1_g1_i1 TRINITY_DN15766_c1_g4_i1 TRINITY_DN15766_c1_g5_i1 TRINITY_DN15766_c1_g7_i1 TRINITY_DN15766_c1_g10_i8 TRINITY_DN15766_c1_g11_i1 TRINITY_DN15766_c1_g12_i1	83.33 54.05 82.95 82.95 82.95 82.57 77.46 85.34	1.00E-54 5.00E-06 6.00E-67 5.00E-67 9.00E-67 2.00E-53 1.00E-71 0
O93934	NADP-specific glutamate dehydrogenase OS = <i>Botryotinia fuckeliana</i>	49.013	341.85	5	1	6	12.67	–	–	–

ID, accession number; PD, protein description; OS, organism-species; MW, molecular weight; S, score; #P, number of proteins; #UP, number of unique peptides; #Pe, number of peptides; C, coverage; MT, matching transcripts; %ID, percentage of identity; eV, E-value; – means no transcript match found for the identified protein.

¹ Identifies the protein hit chosen for the identification of the reactive band of interest.

Table 7. Protein identification report of IgE-binding protein P108 by mass spectrometry from PD 1.4 with the SEQUEST HT search engine against the UniProt Database for the fungal kingdom

Protein identification data								Transcriptome data		
ID	PD	MW	S	#P	#UP	#Pe	C	MT	%ID	eV
Q9URS1 ¹	NADP-specific glutamate dehydrogenase OS = <i>Penicillium chrysogenum</i>	49.8	172.90984	3	1	6	15.62	TRINITY_DN15766_c1_g1_i1 TRINITY_DN15766_c1_g1_i1 TRINITY_DN15766_c1_g4_i1 TRINITY_DN15766_c1_g5_i1 TRINITY_DN15766_c1_g7_i1 TRINITY_DN15766_c1_g10_i8 TRINITY_DN15766_c1_g11_i1 TRINITY_DN15766_c1_g12_i1	83.33 54.05 82.95 82.95 82.95 82.57 77.46 85.34	1.00E-54 5.00E-06 6.00E-67 5.00E-67 9.00E-67 2.00E-53 1.00E-71 0
P18819	NADP-specific glutamate dehydrogenase OS = <i>Emericella nidulans</i>	49.58	167.14528	2	2	7	21.57	TRINITY_DN14200_c0_g1_i1 TRINITY_DN14200_c0_g1_i2	90 70.83	6.00E-05 0.008
Q877A8	Catalase B OS = <i>Aspergillus oryzae</i>	79.8	156.83108	1	3	8	12.97	TRINITY_DN22135_c0_g1_i1 TRINITY_DN16004_c0_g2_i1 TRINITY_DN16004_c0_g2_i2 TRINITY_DN16004_c0_g2_i3	68.57 71.8 73.68 67.09	4.00E-23 8.00E-105 4.00E-89 2.00E-18

ID, accession number; PD, protein description; OS, organism-species; MW, molecular weight; S, score; #P, number of proteins; #UP, number of unique peptides; #Pe, number of peptides; C, coverage; MT, matching transcripts; %ID, percentage of identity; eV, E-value.

¹ Identifies the protein hit chosen for the identification of the reactive band of interest.

Table 8. Protein identification report of IgE-binding protein P83 by mass spectrometry from PD 1.4 with the SEQUEST HT search engine against the UniProt Database for the fungal kingdom

Protein identification data								Transcriptome data		
ID	PD	MW	S	#P	#UP	#Pe	C	MT	%ID	eV
P18819	NADP-specific glutamate dehydrogenase OS = <i>Emericella nidulans</i>	49.5781	188.0285	5	2	7	16.78	TRINITY_DN14200_c0_g1_i1 TRINITY_DN14200_c0_g1_i2	90 70.83	6.00E-05 0.008
Q9URS1	NADP-specific glutamate dehydrogenase OS = <i>Penicillium chrysogenum</i>	49.8	186.121071	6	1	6	16.27	TRINITY_DN15766_c1_g1_i1 TRINITY_DN15766_c1_g1_i1 TRINITY_DN15766_c1_g4_i1 TRINITY_DN15766_c1_g5_i1 TRINITY_DN15766_c1_g7_i1 TRINITY_DN15766_c1_g10_i8 TRINITY_DN15766_c1_g11_i1 TRINITY_DN15766_c1_g12_i1	83.33 54.05 82.95 82.95 82.95 82.57 77.46 85.34	1.00E-54 5.00E-06 6.00E-67 5.00E-67 9.00E-67 2.00E-53 1.00E-71 0
Q877A8 ¹	Catalase B OS = <i>Aspergillus oryzae</i>	79.81	91.58744	1	2	7	12.28	TRINITY_DN22135_c0_g1_i1 TRINITY_DN16004_c0_g2_i1 TRINITY_DN16004_c0_g2_i2 TRINITY_DN16004_c0_g2_i3	68.57 71.8 73.68 67.09	4.00E-23 8.00E-105 4.00E-89 2.00E-18
Q92405	Catalase B OS = <i>Neosartorya fumigata</i>	79.86	64.04767	1	1	6	10.3	TRINITY_DN25055_c0_g1_i1 TRINITY_DN16039_c0_g1_i1 TRINITY_DN16039_c0_g1_i3 TRINITY_DN16039_c0_g1_i4 TRINITY_DN16004_c0_g1_i1 TRINITY_DN16004_c0_g1_i1	92.5 80.87 87.57 70.56 86.67 47.37	6.00E-48 0 0 2.00E-77 0.18 0.18

ID, accession number; PD, protein description; OS, organism-species; MW, molecular weight; S, score; #P, number of proteins; #UP, number of unique peptides; #Pe, number of peptides; C, coverage; MT, matching transcripts; %ID, percentage of identity; eV, E-value.

¹ Identifies the protein hit chosen for the identification of the reactive band of interest.

Catalases are part of the proteins responsible for the oxidative stress responses in the organisms, and they have already been identified as allergens in fungal species like *Neosartorya fumigata* (*A. fumigatus*), *Penicillium citrinum*, and *Aspergillus versicolor* [35–37]. On the other hand, dehydrogenases are involved in cellular metabolic pathways. Some of these include malate dehydrogenases, NADP-dependent mannitol dehydrogenases, aldehyde dehydrogenases, and alcohol dehydrogenases [38–41]. The previous enzymes have already been described as allergens in species like *Malassezia furfur*, *A. alternata*, *C. herbarum*, and *Candida albicans* [38–41]. Moreover, NADP-specific glutamate dehydrogenases are involved in the cellular amino-acid metabolic processes, and yet no reports describing NADP-specific glutamate dehydrogenase as an allergen was obtained after a search in the Allergome database (<http://www.uniprot.org/uniprot/?query=Allergome&sort=score>) [42].

Indirect ELISA results suggest that both groups were more reactive than the control serum. Also, the highest reactivity belonged to sera from group 1 (i.e., individuals with sIgE reactivity to ≥ 1 allergen from the panel) although there was no statistically significant difference to group 2 reactivity. These results were expected since it has been described that individuals with fungal sensitization may present sIgE to other inhalant allergens, and both groups were suspected to have some allergy due to the medical referral for sIgE testing [17, 43]. The Mann-Whitney U test showed no significant difference between both groups.

Taking into consideration the different allergen profiles from the individuals of groups 1 and 2, we performed exploratory data analysis with 7 allergens (*D. farinae*, cat dander epithelium, dog epithelia, *A. alternata*, *A. fumigatus*, *P. chrysogenum*, and *C. herbarum*) and the crude MME from *A. penicillioides* (A₄₉₀, Abs₄₉₀). The results showed strong significant correlations (Table 3) between

all the fungal allergens (*A. alternata*, *A. fumigatus*, *P. chrysogenum*, and *C. herbarum*), between animal dander allergens (dog epithelia and cat dander epithelium), with an association between mostly all allergens. Additionally, *Aspergillus* and *Penicillium* genera are considered major indoor fungi [44]. Thus, we unsurprisingly found a correlation between *A. penicilliioides* MME and other indoor allergens like dog epithelia, *A. fumigatus*, and *P. chrysogenum*. PCA was performed as an exploratory analysis to identify relevant information from the data, like allergen correlation patterns. The analysis of factors 1 and 2 revealed 2 clusters of uncorrelated allergens. The first is represented by cat dander epithelium, dog epithelia, and mite (*D. farinae*) allergens, while the second is defined by the fungal allergens (*A. alternata*, *A. fumigatus*, *C. herbarum*, and *P. chrysogenum*) along with A₄₉₀ (Fig. 2). The results are consistent with the correlations determined by the Spearman correlation matrix (Table 3). However, in the factor 1 and 2 map, a strong correlation for A₄₉₀ and dog epithelia was not observed. The map suggests a more robust association of *A. penicilliioides* to the fungal allergens. The results obtained are consistent with data indicating that fungal allergens often co-occur with other indoor allergens, suggesting *A. penicilliioides* as a potential indoor allergen source [43].

The use of 1-dimensional immunoblotting to identify IgE-binding proteins, the lack of serum samples with the associated clinical history, and the use of mycelia from a 2- to 3-week fungal culture can be considered limiting factors in this study. The use of 2-dimensional immunoblotting has proven to be an outstanding strategy to identify allergenic proteins [45]. This technique, which could not be performed due to limited resources, could aid in expanding the identification of *A. penicilliioides* MME IgE-binding proteins that might have migrated together because of similar MWs [46]. The small number of serum samples and the absence of their clinical history prevented us from evaluating relationships between reactivity and risk factors to understand the reactivity of the subjects better and complement the allergen correlation analysis. Also, having only 1 individual diagnosed as a negative control is very limiting factor, as it prevents meaningful statistical comparisons to the negative control. Furthermore, the use of mycelia as the allergen source might affect the extracts, and medium culture of slow-growing fungi has proven to be an excellent source of antigens [44]. In a subsequent investigation, *A. penicilliioides* culture filtrate extracts will be used to assess the presence of IgE-binding proteins and determine serological reactivity in order to compare them with MME to

obtain a more comprehensive view of the full allergenic potential of proteins from this fungus.

In conclusion, this is the first study to provide evidence of the serological reactivity of individuals with different allergen reactivity profiles to the xerophilic fungus *A. penicilliioides* crude MME. However, confirmation of the IgE-binding protein identity with further purification and characterization needs to be performed. Whether the identified proteins could activate cells in a strong manner and elicit an allergic response remains to be determined. Nevertheless, this study provides insights into the respiratory allergenic potential of *A. penicilliioides* and is a major step towards the development of high-quality *A. penicilliioides* allergen sources in the diagnosis of allergy and for allergen-specific immunotherapy against these xerophilic fungi.

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Disclosure Statement

The authors have not conflict of interest to disclose.

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