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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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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. Correlation and principal component analysis (PCA) were performed to identify relations between reactivity to commercial allergens and *A. penicilliioides*. IgE-binding proteins in *A. penicilliioides* were visualized with Western blots in group 1. The IgE-binding proteins with the highest reactivity were analyzed by mass spectrometry and confirmed by transcript match.

Results—There was no statistical significance ($p = 0.1656$) between the study groups in serological reactivity. A correlation between reactivity to *A. penicilliioides*, dog epithelia, *A. fumigatus* and *P. chrysogenum* was observed. WB experiments showed six 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 three proteins led to the putative

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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. penicillioides*. Further analysis of the putatively identified proteins should reveal their allergenic potential.

Keywords

Xerophilic fungi; *Aspergillus penicillioides*; Immunoglobulin E-binding proteins; serologic reactivity; allergy

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 in Puerto Rico, where *A. penicillioides* has been reported [7, 11, 12, 36]. Contrary to various fungi such as *Penicillium spp.*, *Aspergillus spp.*, *Cladosporium spp.*, *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 of the allergenic potential of xerophilic fungi, such as *A. restrictus*, *Eurotium spp.*, and *Wallemia sepii*, have been documented [8, 9]. As a result, proteins originating from xerophilic fungi that could be included in allergy testing are limited [35]. Considering that xerophilic fungi contribute a significant proportion of the 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 [7, 35].

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 IgE antibodies against *A. penicillioides*. This serological reactivity was compared in serum from individuals with different allergic profiles. 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 rate to *A. penicillioides* MME among the serum samples tested [7, 14 – 18]. We supplemented our findings with a transcript analysis from *A. penicillioides* (PRJNA344885, Unpublished data) by matching the proteins identified by MS with high count transcript presence that had a similar predicted sequence and molecular weights. We report evidence of the serological reactivity and IgE-binding components of *A. penicillioides*.

MATERIALS AND METHODS

Identification and Culture Conditions

Aspergillus 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 Medical Sciences Campus (San Juan, PR). Air samples were collected with the MicroBio2 air sampler (Cantium Scientific Limited, UK) in 25 % Glycerol Nitrate Agar (G25N, 0.75g dipotassium phosphate, 250 mL glycerol, 7.5 mL Czapek concentrate, 3.5 g yeast extract, 12g agar and 750mL 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 [19]. Conidia were harvested from the purified mycelia by adding sterilized, pyrogen-free water. The suspension was aseptically transferred to a 2L Erlenmeyer flask with 250mL of G25N broth, and left in constant shaking at 25°C 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, DE) at 3,000rpm for 10 minutes, washed once with ultrapure water, and stored in –80°C until further processing.

Crude Mycelial Mat Extract (MME) Preparation

A. penicillioides mycelial mat extract (MME) was prepared as described by Fratamico et al. [20] with slight variations. Briefly, mycelial mat previously collected was thawed on ice and washed with lysis buffer (PBS containing 1mM Phenylmethylsulfonyl Fluoride, PMSF, G-Biosciences Missouri, USA). Previously sterilized glass beads (Biospec Products, Oklahoma, USA), were used to disrupt mycelia during a 60-minute cycle (1 minute agitation and 1 minute rest) using a Bead Beater (Biospec Products, Oklahoma, USA) connected to a GraLab electric timer/interval meter (GraLab Co., Ohio, USA). The homogenate was centrifuged at 417 *g* at 4°C for 30 minutes, and afterward was centrifuged at 216,179.7 *g* at 4°C for 90 minutes in an Optima L-100 XP Centrifuge (Beckman Coulter, California, USA). The supernatant was collected and filter-sterilized with a Nalgene 115mL filter unit (0.45µm pore size; Nalge Nunc International, New York, USA) and 5mL aliquots were transferred into sterile 15 ml Falcon conical tubes (Becton Dickinson New Jersey, USA), and frozen at –80 C for 24 hours or until further handling. The frozen tubes were placed in a 600 ml Virtis drying flask (SP Scientific, Pennsylvania, USA) and lyophilization performed for one day in an Edwards Modulyo Freeze-Dryer (Edwards High Vacuum International, Crawley, England). 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, Hercules, CA) 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 University of Puerto Rico, Medical Sciences Campus (UPR-MS) 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 immunoglobulin-E (sIgE) with radioallergosorbent assay (RAST) (Immulite 2000 3gAllergy) against various commercial allergens included in either one of two 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 two groups according to their allergen reactivity profiles as determined by the RAST with commercial extracts: a) group 1 consisted of 54 subjects with sIgE to at least one commercial allergen, and b) group 2 consisting of 15 subjects with no sIgE against any allergen (Table 2). The negative control was a serum sample from one volunteer with no previous history of allergies and no skin reactivity to commercial allergen extracts (Greer Laboratories, North Carolina, USA), which included fungal extracts from *Penicillium spp.*, *Aerobasidium 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, Bahia, among others, and total IgE of 4.20 IU/L.

Indirect Enzyme Linked Immunosorbent Assay (ELISA)

Indirect ELISA assays against *A. penicilloides* MME were performed on human serum samples as described by Figueroa-Santiago et. al [21] with minor modifications. The assay was optimized by checkerboard titration. Highbinding flat bottom disposable polystyrene plates (Corning Incorporated, NY), were coated overnight at 4°C with 10µg of MME from *A. penicilloides* prepared in coating buffer (0.05M carbonate-bicarbonate buffer pH 9.6). The next day, the plates were blocked with 300µL/well of 3% bovine serum albumin (3% BSA) (AMRESCO, OH, USA) in PBS containing 0.05% Tween 20 (PBST), and incubated for 1 hour at 37°C in a wet chamber. The blocking solution was discarded and the plates incubated for 1 hour in a humid chamber at 37°C with 100µL/well (1:100) of human sera. Afterward, the plates were incubated for 1 hour at 37°C in a humid chamber with 100µL/well (1:2,500) of affinity purified peroxidase labeled goat anti-human IgE (KPL, Gaithersburg, MD). Incubation with 100µL/well of the substrate solution, containing 20mg o-phenylenediamine and 30% H₂O₂ pH 5.0, (Sigma-Aldrich Co.) proceeded for 30 minutes at room temperature in the dark. The reaction was stopped with 50µL/well of 10% HCl. Before any antibody and substrate incubation, the plates were washed three times with PBST. The plates were read at 490nm to obtain the absorbance values (A₄₉₀) using a BioRad microplate reader (Hercules, CA).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of MME and Western Blot Analysis

The MMEs were separated under denaturing conditions using 4 – 15% Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad Laboratories, California, USA). The one-dimensional SDS-PAGE was carried out following the method of Laemmli [22]. The gels were run in a

Mini-Protean Tetra Cell electrophoresis system (Bio-Rad Laboratories Inc. Hercules, CA) with Tris – Glycine – SDS buffer pH 8.3 for 50 minutes at 100V in a cold chamber, and subsequently stained with Silver Stain Plus Kit (BioRad Laboratories, California, USA) according to manufacturer's instructions. Thirty micrograms (30µg) of protein were applied to each well. The proteins were transferred to a 0.45µm nitrocellulose membrane (Bio-Rad Laboratories Inc. Hercules, CA), at 100 V and a constant current of 300mA for 50 minutes in a cold chamber, using the Mini Trans-blot cell system (Bio-Rad Laboratories Inc. Hercules, CA) in Tris – glycine buffer (pH 8.0) containing 20% methanol. The membranes were blocked in constant shaking with 5% BSA in PBST for 3 to 5 hours at 4 C. After blocking, incubation with patient's serum proceeded, diluted at 1:400 in 3% BSA in PBST at 4 C overnight. The next day, the membranes were washed six times for five minutes with PBST and then incubated for 2 hours with ReserveAP™ (affinity purified) phosphatase labeled goat anti-human IgE (KPL, Gaithersburg, MD) diluted to 1:20000 in 5% BSA in PBST at room temperature. Chromogenic detection was carried out by adding 6mL BCIP/NBT Phosphatase Substrate System (KPL, Gaithersburg, MD), for twenty-five minutes and stopped by washing with 5mL of deionized water twice for 5 minutes. Immunoblots were performed with 26 subjects from group 1. Previously, all subjects from group 1 were separated into sera 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 with ImageJ® (National Institutes of Health) as previously performed by Vila-Hereter *et al.* [43].

Mass Spectrometry (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) 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 SyproRuby (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) Instrument. 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 kingdom Fungi, which contains approximately 30,000 entries. The following settings were used: two missed cleavage site by trypsin allowed with fixed carboxamidomethyl modification of cysteine and variable modifications of methionine oxidation, 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, Unpublished data) were performed with the Basic Local Alignment Search Tool (BLAST) using blastx with default parameters to confirm the presence of the proteins [44 – 46]. An E-value lower than 10^{-05} was considered a significant match. This was performed on the Bridges

supercomputing system at the Pittsburgh Supercomputing Center using the GALAXY interface [47 – 49].

Statistical Analysis

The statistical analysis was performed with XLSTAT software (Addinsoft SARL) and R version 3.3 [50]. A Shapiro-Wilk test was performed to test for normality in the A₄₉₀ data from the indirect ELISA in group 1 and group 2 [51]. A Mann-Whitney U test [52] was performed to determine if there were statistically significant differences between group 1 and group 2 A₄₉₀ values. To compare the proportion of group 1 reactive sera to each of the six proteins detected in the western blot, a Fisher's exact test, with 95% confidence intervals was performed.

Exploratory Data Analysis

To identify associations between group 1 and group 2 reactivity to *A. penicilliioides* and seven 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 (Figure 1), an indirect ELISA was performed. The median A₄₉₀ from group 1 was 0.6243 ± 0.3509 and for group 2 was 0.403 ± 0.3308 . The differences between group 1 and group 2, although 54% higher A₄₉₀ in group 1, were not statistically significant ($p = 0.1656$) as determined by the Mann-Whitney U test. The A₄₉₀ of the negative control was 0.2120. These findings suggest that sensitized and not-sensitized subjects recognized allergenic components in the *A. penicilliioides* MME.

Spearman correlation and principal component analysis reveals 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 principal component analysis (PCA) were performed. The Spearman correlation was employed for both correlation analysis and PCA because, except for A₄₉₀ ($p = 0.130$), 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 dimensionality of datasets with a larger number of variables and aids in identifying patterns between variables [37].

The Spearman correlation analysis identified statistically significant positive correlations between A₄₉₀ and *A. fumigatus*, dog epithelia, and *P. chrysogenum* allergens ($p = 0.019$, 0.004 , 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*; *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. herbarum* with *D. farinae* and dog epithelia; *P. chrysogenum* and dog epithelia), and between non-fungal allergens (cat dander epithelium with *D. farinae* and dog epithelia; *D. farinae* and dog epithelia), as described in detail in Table 3. The PCA revealed that, of the 8 components identified, components 1 and 2 had the higher eigenvalues, 3.710 and 1.447 respectively, with a cumulative variability of 64.7%. This data indicates 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 two groups of positively correlated allergens: one was comprised by *D. farinae*, cat dander epithelium, and dog epithelia, and a second corresponding to the fungal allergens, including A₄₉₀ against *A. penicillioide*s MME. Together, these results suggest an association between reactivity to *A. penicillioide*s mycelial mat extract with the fungal allergens.

Six IgE reactive bands were recognized from the *A. penicillioide*s MME in the immunoblotting analysis

Reactive IgE-binding protein bands were detected via WBs with 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 ($n = 54$ in group 1 vs. $n = 15$ in group 2). Six IgE-binding proteins were detected with molecular weights ranging from 145 kDa to 45 kDa (Figure 3). Eighteen out of the 26 sera (69.2%) were reactive to at least one IgE-binding protein: fifteen (57.7%) sera were reactive to two or more IgE-binding proteins (Table 6). Three of the six IgE-binding proteins (MWs of 108, 83, and 56 kDa) showed higher than 40% proportion of reactivity (Table 7). The negative control (Figure 3A) showed a slight reaction to the 108 kDa band, which was not evident in the Image J plot. These results suggest that the proteins with MWs of 108, 83, and 56 kDa could be relevant allergens in *A. penicillioide*s mycelial mat extract, and justify molecular identification.

Mass spectrometry analysis identification of three IgE-binding proteins

The three reactive IgE binding proteins found reactive with a higher proportion of reactivity, P108 (108 kDa), P83 (83 kDa), and P56 (56 kDa) were analyzed via MS for identification (Figure 4). Results from the LC – MS/MS identification along with the transcripts matching the identified proteins can be found in Tables 8, 9, and 10. The interpretation was based on the protein score, molecular weight, number of unique peptides, the number of peptides, and confirmation of their presence by matching the protein hit with an *A. penicillioide*s transcript. The match was considered significant if it had an E-value lower than 10^{-5} . IgE binding proteins P108 and P56, which had reactivity in the WBs, were identified as NADP – specific glutamate dehydrogenases, while P83 was identified as Catalase B.

DISCUSSION

A. penicillioides is a xerophilic fungus commonly found in indoor environments, including house dust [7, 11, 12, 23]. This fungus was suggested as a probable cause of allergies; however, its allergenic potential has not been studied [12]. In this pilot study, we evaluated the allergenic potential of the crude *A. penicillioides* mycelial mat extract (MME) among individuals with different allergen reactivity profiles. Serum from subjects with sIgE had higher reactivity to *A. penicillioides* MME than those with no sIgE to the commercial allergens tested. Six IgE-binding proteins were identified via Western Blot, three of which had reactivity higher than 40% among sera from subjects with sIgE to commercial allergens. LC – MS/MS identification revealed that these three proteins have dehydrogenase and catalase activity. This study is the first to report IgE-binding proteins from *A. penicillioides* mycelia and provides evidence of *A. penicillioides* as an allergen source.

One dimension SDS – PAGE immunoblotting analysis showed the presence of six reactive bands with MWs ranging from 145 kDa to 45 kDa. Three of these bands, P108, P56, and P83, were recognized in higher frequencies among the others, 65.4%, 57.7%, and 46.2% respectively. MS analysis of P108 and P56 putatively identified these two IgE binding proteins as NADP – specific glutamate dehydrogenases (Table 6 – 7). These enzymes are essential for the carbon and nitrogen metabolism [24]. The NADP⁺ - linked protein structure in fungal species has six identical subunits, each with a mass around 48 kDa [24 – 27]. These findings suggest that P108 maybe a subunit dimer of NADP⁺ – specific glutamate dehydrogenase. MS analysis for the third reactive band, P83, identifies the IgE binding protein as an NADP⁺ – specific glutamate dehydrogenase as well. However, the MW of this protein hit does not match the estimated molecular weight determined by WB analysis. The protein pattern (Figure 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 identifies the IgE binding protein as a Catalase B, which are enzymes essential for the conversion of hydrogen peroxide to water and molecular oxygen [28]. It is noteworthy that the Catalase B of *A. nidulans* has been shown to have a molecular weight of 360kDa and is composed of four identical glycosylated subunits [53]. The Allergome Database (allergome.org) has identified this enzyme as an allergen, and each subunit has a molecular weight of approximately 80kDa. This would be a reasonable match for our protein identification.

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*), *P. citrinum*, and *A. versicolor* [29 – 31]. 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*, *Alternaria alternata*, *Cladosporium 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>) [54].

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 one or more allergens 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 [32, 43]. The Mann-Whitney U test, showed no significant difference between both groups.

Taking into consideration the different allergen profiles from the individuals of group 1 and 2, we performed exploratory data analysis with seven 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* genus are considered major indoor fungi [4]. Thus, we unsurprisingly found a correlation between *A. penicillioides* mycelial mat extract and other indoor allergens like dog epithelia, *A. fumigatus* and *P. chrysogenum*. The principal component analysis (PCA) was carried as an exploratory analysis to identify relevant information from the data, like allergen correlation patterns. The analysis of factors 1 and 2 revealed two 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 (Figure 2). The results are consistent with the correlations determined by the Spearman correlation matrix (Table 3). However, in the factors 1 and 2 map, a strong correlation for A₄₉₀ and dog epithelia, was not observed. The map suggests a more robust association of *A. penicillioides* to the fungal allergens. The results obtained are consistent with data indicating that fungal allergens often co-occur with other indoor allergens, suggesting *A. penicillioides* as a potential indoor allergen source [32].

The use of one-dimensional immunoblotting to identify IgE-binding proteins, the lack of serum samples with the associated clinical history, and the use of mycelia from a two to three-week fungal culture can be considered limiting factors in this study. The use of two-dimensional immunoblotting has proved to be an outstanding strategy to identify allergenic proteins [33]. This technique, which couldn't be performed due to limited resources, could aid in expanding the identification of *A. penicillioides* MME IgE-binding proteins that might have migrated together because of similar MWs [42]. 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 one individual was diagnosed as a negative control is very limiting, as it prevents meaningful statistical comparisons to the negative control. Furthermore, the use of mycelia as the allergen source might affect the

extracts, and media culture from slow growing fungi have proven to be an excellent source of antigens [4]. As a follow-up to this investigation, *A. penicillioides* culture filtrate extracts will be used to assess the presence of IgE-binding proteins and determine serological reactivity in order to compare them with the mycelial mat extracts to obtain a more comprehensive view of the full possible allergenic 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. penicillioides* mycelial mat crude extract. However, confirmation of the IgE binding proteins identity with further purification and characterization needs to be performed. Whether the identified proteins could activate cells strongly and elicit an allergic response remains to be determined. Nevertheless, this study provides insights into the respiratory allergenic potential of *A. penicillioides* and is a major step towards the development of high-quality *A. penicillioides* allergen sources for use in allergy diagnoses and allergen-specific immunotherapy to this xerophilic fungi.

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Figure 1.

Indirect ELISA reactivity results (A_{490}) of human sera against the crude mycelial mat extract (MME) of *A. penicilliioides*. 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 squares within each group represents the median value for each group. The red line represents the A_{490} for the negative control.

Figure 2.

Correlation circle from Factors 1 and 2 illustrating the correlation between allergens. Variability percentage for factor 1 (x-axis) is 46.48%, for factor 2 (y-axis) is 18.08%, for 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.

Figure 3.

Immunoblotting results illustrated by plots of the serum samples showing the six reactive IgE-binding reactive bands. The reactive bands are represented by arrows and their estimated molecular weight. Panel A illustrates the immunoblot for the negative serum sample. Panel B shows a serum sample reactive to the P130 (gray arrow), P108 (green arrow), P83 (pink arrow), and P56 (purple arrow) IgE-binding protein bands; panel C illustrates a serum sample reactive to the P108 (green arrow), P83 (pink arrow), P56 (purple arrow), and P45 (black arrow); panel D demonstrates a serum sample reactive to the P145 (red arrow), P130 (gray arrow), P108 (green arrow), P83 (pink arrow), and P56 (purple arrow). MW (kDa) = molecular markers; on the left side of the panels we have blotted membranes, and on the right side, we have the blots from Image J Software. The y-axis in the plot represents the intensity of the reactive bands from the WB, 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 (30µg crude MME) were separated in 4 – 15% gradient polyacrylamide gels. Sera dilution 1:400, Conjugate dilution 1:20000.

Figure 4. SDS-PAGE gel image analysis of the *A. penicilliioides* crude mycelial mat extract. Different protein concentrations (lane 1: 1 µg, lane 2: 3 µg, and lane 3: 5 µg) were separated on a 10% polyacrylamide gel and stained with SyproRuby (Invitrogen) for protein band pattern visualization and detection of the bands of interest (red arrows). MW (kDa) = molecular markers. Gel image created by the Proteomics and Mass Spectrometry Facility at Cornell University Institute of Biotechnology (Ithaca, NY).

Table 1

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

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

[†] Serum samples were tested to only one of the panels. Some serum samples did not get tested for all the allergens comprising the panels.

^{*} The allergens marked were the ones taken into consideration for the PCA.

Table 2

Samples groups for the indirect ELISA against *A. penicilliioides* crude mycelial mat extract (MME).

Serum Sample Groups	Origin	Number of Samples
Group 1: Individuals with sIgE reactivity to one or more allergens from the commercial panels tested	Toledo Clinical Laboratory, Arecibo, PR	54
Group 2: Individuals with no sIgE reactivity to the allergens from the commercial panels tested	Toledo Clinical Laboratory, Arecibo, PR	15
Total		69

Note: The clinical history of the individuals is unknown.

Table 3

Spearman Correlation Matrix.

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

Note: Values in bold are different from 0 with a significance level alpha = 0.05. A₄₉₀ = indirect ELISA values, Aalt = *A. alternata*, Afum = *A. fumigatus*, cat = cat dander epithelium, Cher = *C. herbarum*, Dfar = *D. farinae*, dog = dog epithelia, Pchr = *P. chrysogenum*. Bold numbers represent rho coefficients with *p*-values < 0.05.

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	
Non – Reactive	0	8	8

Table 5

Western Blot band detection summary results.

Reactive Bands	Estimated Molecular Weight	Reactive Serum Samples	Proportion of Reactive Samples Percentage (%)	95% CI of Reactive Samples
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%

Protein identification report of IgE-binding protein P56 by mass spectrometry from PD 1.4 with Sequest HT search engine against UniProt Database for the kingdom Fungi.

Table 6

ID	Protein Identification Data				Transcriptome Data			
	PD	MW	S	#P	#UP	#Pe	C	MT
P18819	NADP-specific glutamate dehydrogenase OS=Emeritella nidulans	49.578	511.5150616	6	2	12	27.67	TRINITY_DN14200_c0_g1_i1
								TRINITY_DN14200_c0_g1_i2
*Q9URSI	NADP-specific glutamate dehydrogenase OS=Penicillium chrysogenum	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
O93934	NADP-specific glutamate dehydrogenase OS=Boryotinia fuckeliana	49.013	341.85	5	1	6	12.67	-

Note: ID refers to accession number, PD to protein description, MW to molecular weight, S to score, #P is the number of proteins, #UP is the number of unique peptides, #Pe is the number of peptides, C refers to coverage. MT is matching transcripts, %ID refers to percentage of identity, and eV is eValue. – means no transcript match the identified protein.

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

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

Table 7

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

Note: ID refers to accession number, PD to protein description, MW to molecular weight, S to score, #P is the number of proteins, #UP is the number of unique peptides, #Pe is the number of peptides, C refers to coverage, MT is matching transcripts, %ID refers to percentage of identity, and eV is eValue. – means no transcript match the identified protein.

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

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

Table 8

Protein Identification Data										Transcriptome Data			
ID	PD	MW	S	#P	#UP	#Pe	C	MT	% ID	eV			
P18819	NADP-specific glutamate dehydrogenase OS=Emericella nidulans	49.5781	188.0285	5	2	7	16.78	TRINITY_DN14200_c0_g1_i1	90	6.00E-05			
								TRINITY_DN14200_c0_g1_i2	70.83	0.008			
Q9URSI	NADP-specific glutamate dehydrogenase OS=Penicillium chrysogenum	49.8	186.121071	6	1	6	16.27	TRINITY_DN15766_c1_g1_i1	83.33	1.00E-54			
								TRINITY_DN15766_c1_g1_i1	54.05	5.00E-06			
								TRINITY_DN15766_c1_g4_i1	82.95	6.00E-67			
								TRINITY_DN15766_c1_g5_i1	82.95	5.00E-67			
								TRINITY_DN15766_c1_g7_i1	82.95	9.00E-67			
								TRINITY_DN15766_c1_g10_i8	82.57	2.00E-53			
								TRINITY_DN15766_c1_g11_i1	77.46	1.00E-71			
TRINITY_DN15766_c1_g12_i1	85.34	0											
*Q877A8	Catalase B OS=Aspergillus oryzae	79.81	91.58744	1	2	7	12.28	TRINITY_DN22135_c0_g1_i1	68.57	4.00E-23			
								TRINITY_DN16004_c0_g2_i1	71.8	8.00E-105			
								TRINITY_DN16004_c0_g2_i2	73.68	4.00E-89			
								TRINITY_DN16004_c0_g2_i3	67.09	2.00E-18			
Q92405	Catalase B OS=Neosartorya fumigata	79.86	64.04767	1	1	6	10.3	TRINITY_DN25055_c0_g1_i1	92.5	6.00E-48			
								TRINITY_DN16039_c0_g1_i1	80.87	0			
								TRINITY_DN16039_c0_g1_i3	87.57	0			
								TRINITY_DN16039_c0_g1_i4	70.56	2.00E-77			
								TRINITY_DN16004_c0_g1_i1	86.67	0.18			
TRINITY_DN16004_c0_g1_i1	47.37	0.18											

Note: ID refers to accession number, PD to protein description, MW to molecular weight, S to score, #P is the number of proteins, #UP is the number of unique peptides, #Pe is the number of peptides, C refers to coverage, MT is matching transcripts, %ID refers to percentage of identity, and eV is eValue. – means no transcript match the identified protein.

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