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ALLERGENIC POTENTIAL AND CROSS-REACTIVITY OF FUNGAL SPECIES ISOLATED FROM THE INDOOR ENVIRONMENT

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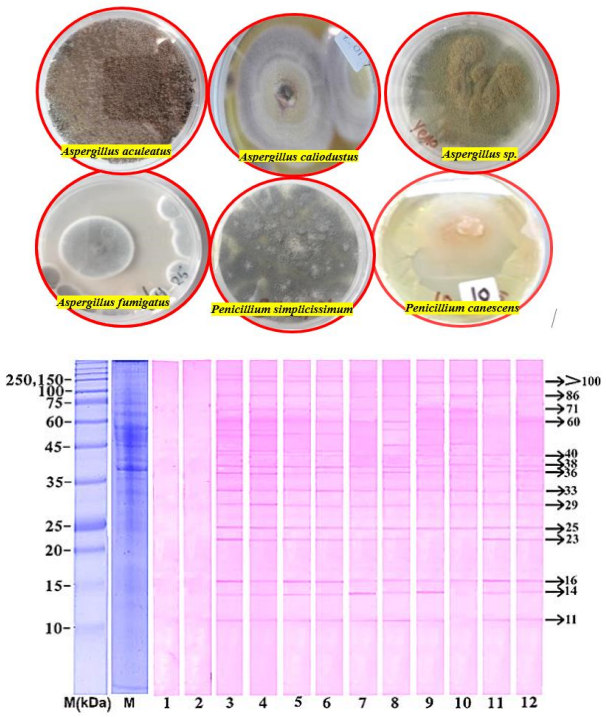
Graphical abstract



Abstract

Indoor fungi are potential sensitizing agents. Their detection and quantification in indoor environments are important in the diagnosis and environmental management of fungal allergies. This study aims to analyse the allergenic potential of ten fungal species and the cross-reactivity of the two most common fungi isolated from the indoor environment samples from Sultan Idris Education University buildings. Employing *in vivo* (skin prick test) and *in vitro* (immunoblotting), the major and minor allergenic proteins of ten fungi sensitized subjects were identified. *Aspergillus fumigatus* and *Penicillium canescens* were the most common fungi with the highest potential to trigger allergies. The cross-reactivity between them was detected by immunoblotting inhibition experiments using three selected sera from subjects sensitized to each of the aforementioned species. The immunoblotting test revealed multiple major and minor allergens. Among them were 11, 25, 33, 36 > 100 kDa and were also listed as causative agent triggering allergy by IUIS Allergen Nomenclature Subcommittee. Cross-reactivity of *Aspergillus fumigatus* against *Penicillium canescens* revealed that 9(64.29%) allergenic bands and 13(76.47%) allergenic bands were inhibited, respectively. *Aspergillus* spp. and *Penicillium* spp. with high cross-reactivity are most prevalent in the indoor environment of identified contaminated buildings at UPSI. *Aspergillus fumigatus* and *Penicillium canescens* can elicit sensitization among the atopic population and implicates worsening the condition of the symptomatic subjects with prolonged these fungal exposures.

Keywords: Fungi, sensitisation, potential allergenic agent, cross-reactivity, indoor environment

Abstrak

Fungi dalaman berpotensi sebagai agen sensitisasi. Pengesanan dan pengkuantitatifan dalam persekitaran dalaman penting dalam mengdiagnosis dan pengurusan persekitaran alergi fungi. Kajian ini bertujuan untuk menganalisis potensi alergenik sepuluh spesies fungi dan reaktiviti-silang dua fungi yang kerap diisolasi daripada sampel persekitaran bangunan di Universiti Pendidikan Sultan Idris. Pengujian *in vivo* (Ujian cucuk kulit) dan *in vitro* (imunopemblotan) digunakan untuk mengenal pasti protein alergenik major dan minor sepuluh subjek yang tersensitisasi fungi. *Aspergillus fumigatus* dan *Penicillium canescens* adalah fungi yang paling kerap ditemui dengan potensi tertinggi bagi mencetus alergi. Ujian reaktiviti-silang antara kedua-duanya telah dikesan menggunakan tiga sera terpilih daripada subjek yang alergik kepada dua fungi tersebut untuk diuji dalam eksperimen imunopemblotan inhibisi. Ujian imunopemblot mempamerkan beberapa alergen major dan minor. Antaranya ialah 11, 25, 33, 36 > 100 kDa yang turut tersenarai sebagi agen yang mencetuskan alergi oleh *IUIS Allergen Nomenclature Subcommittee*. Reaktiviti-silang *Aspergillus fumigatus* terhadap *Penicillium canescens* menunjukkan jalur alergenik yang terinhibisi masing-masing pada 9(64.29%) dan 13(76.47%). *Aspergillus* spp. dan *Penicillium* spp. dengan reaktiviti-silang yang paling tinggi ini turut menunjukkan prevalens yang tertinggi dalam persekitaran dalaman bangunan yang telah dikenal pasti sebagai tercemar di UPSI. *Aspergillus fumigatus* dan *Penicillium canescens* boleh mencetuskan pensensitifan dalam kalangan populasi atopik dan menyebabkan keadaan yang lebih terok terhadap subjek yang bersimptom dengan pendedahan yang berpanjangan terhadap fungi ini.

Kata kunci: Fungi, sensitisasi, agen potensi alergenik, reaktiviti-silang, persekitaran dalam

1.0 INTRODUCTION

The detection and quantification of potential sensitizing of indoor fungi are important in the diagnosis and environmental management of fungal allergies. Airborne fungi allergens have been implicated as one of the main causes of allergic respiratory problems in temperate countries [1]. Exposure and sensitisation to fungal allergens can promote the development and worsen allergic diseases [2].

The allergic manifestations induced by fungi are more widely recognized and include asthma, rhinitis, allergic sinusitis, allergic bronchopulmonary mycoses and hypersensitivity pneumonitis [3]. The study of the allergenic protein components of mold may provide valuable clues for understanding that particular fungi species activate the innate immune system and enhance lung inflammation induced by unrelated allergens such as grass pollen [4]. Allergens are molecular markers contributing to improving diagnostic decisions by prediction of cross-reactivity and developing more robust detection tools to assess exposure allergen levels. Correctly assessing the exposure to aeroallergens in the environment is of major importance for predicting the risk of respiratory symptoms in an atopic population and informing the implementation of appropriate public health measures [5].

At least 590,214 of the Malaysian population (1.93%) is affected by a severe fungal infection annually, and this problem is severe enough to warrant further epidemiological studies to estimate the burden of human fungal infections in Malaysia [6]. According to the Occupational Health and Safety Act, the health and safety of workers must be ensured, and protecting them from mould in workplace buildings is necessary [7]. At the Sultan Idris Education University (UPSI), it was observed that fungal growth loaded the ducts of the central cooling system. Besides, fungal growth was also detected mainly in the corners and on furniture, this situation may likely cause allergies amongst the occupants of the building. The occupants with allergies could be suffering due to exposure to fungal spores in their office, lecturer's rooms, tutorial room, or laboratories, where they stayed for at least 40 hours weekly.

As a consequence of colonization, the fungal allergy plays a vital role in the exacerbation of asthma and allergic rhinitis, particularly among the employers in the specific study area, which eventually also contributes to the reduction of productivity by increasing sickness. Based on

enormous reports on severe health effects caused by prolonged exposure to indoor pollution, therefore, this study focuses on examining different types of indoor dust samples as surrogates of airborne inhalation exposure to assess the health effects of indoor fungi.

2.0 METHODOLOGY

Sample Collection

In the previous study, 10 most prevalent fungi species were isolated from 51 dust samples collected from the indoor environments i.e., offices, laboratories, and lecturers' rooms of the three academic blocks at UPSI's Sultan Azlan Shah Campus. Isolates were then identified by subculturing. Each isolated fungus was examined microscopically and sent for identification by using molecular method 18S rRNA via PCR at the Malaysian Agricultural Research and Development Institute (MARDI-UPM) [8].

Fungi Cultivation and Incubation Condition

Potato dextrose agar served as a medium for the isolates to grow. The isolates were incubated at room temperature (25°C) for 48-72 hours and were evaluated based on the morphological colony and microscopic study following the growth of fungi (Figure 1).

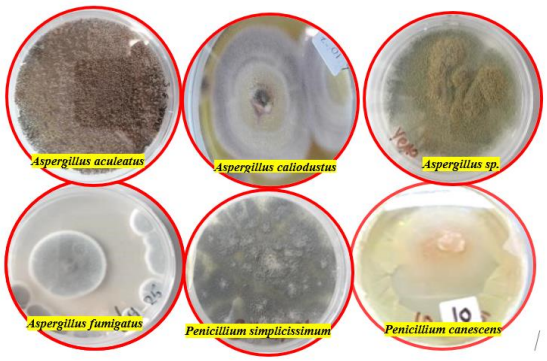


Figure 1 Samples of cultured fungi

Several hyphae were transferred to an Erlenmeyer flask containing 250 mL of Sabouraud glucose infused with chloramphenicol to obtain protein. The culture was mass-produced under the sterile condition and shaking (150 rpm) at 25°C for 12-14 days to get the maximum growth fungal biomass.

Fungus Colonies Separation and Cell Disruption

By using Wattman paper, number 1, and Buchner funnels in a fully sterile state, fungus colonies were separated from the medium and washed with three stages of sterile PBS [9]. Cells disruptions were performed using all below three methods: a freeze-tow, Bead beating and Sonication.

Fungi Crude Extracts Preparation

The crude extracts were extracted from the intact cells after cell disruption, and the remaining cell walls were centrifuged for 30 minutes at 4°C at 14,000 rpm. A sterile syringe filter (0.2) µm for solution sterilization bacteria removal were used for protein filtration and separation. The extracts were then lyophilized for one night using a freezer dryer. The lyophilized extracts were stored at -20°C until they were used [10]. The protein concentration of each extract was determined using the Bradford Reagent Kit (Biorad, CA, USA), following the manufacturer's instructions

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out with a gel containing 11% polyacrylamide separating gel and stacking gel of 4%. The samples were prepared by getting 5 mg of extracted fungi samples resolved in 50 µL of deionized water and vortexes. The samples were then treated with 5% 2-mercaptoethanol in a Laemmli sample buffer (BioRad, USA) in various ratios such as 1:1 and 1:2 (volume/volume) and heated to 97°C for 5 minutes before electrophoresis. Along with the samples, the Precision plus protein standards (BioRad, USA) were used as a reference.

Skin Prick Test and Sera Collection

To diagnose an atopy population, a skin prick test was done to obtain a positive SPT reaction to at least one of the ten allergens (diameter >3 mm more than negative control) and the subject sensitized to fungi was determined using six fungal allergens following the recommendations of the European Academy of Allergy and Clinical Immunology [11]. There was a total of 225 subjects made up of students, lecturers, and supporting staff undergoing SPT to common aeroallergens for evaluation of allergy by two allergists. The study was approved by the Ethical Committee of Sultan Idris Education University (Code: 2019-005-01). The emergency kit and transportation were provided by UPSI Medical Centre. Venous blood was collected from subjects showing markedly positive skin reactions (≥2+) to different antigenic extracts as recommended by Twaroah *et al*. [12]. Blood was allowed to clot for 1 hour at room temperature. Sera were separated and centrifuged at 2000 g for 5 minutes for sedimentation of erythrocytes. The sera obtained were stored at -20°C for immunoassay. Besides ten sera samples from the fungi sensitized respondents, non-allergenic sample sera were also collected from healthy volunteers and treated as control.

Detection of IgE-Binding Proteins

Immunoblotting protocol adapted from protocol by [13] was carried out by using fungi extracts to identify the major and minor allergenic proteins of sera from 10 fungi sensitized subjects identified from the SPT

procedure. The separated proteins of fungi extracts were electrophoretically transferred from unstained SDS-PAGE gel to a 0.45 mm pore size nitrocellulose membrane using a Mini Trans-Blot System (BioRad, USA). The unstained protein bands in the SDS-PAGE gel were electrophoretically transferred to a 0.45µm nitrocellulose at 100 V for 70 minutes. After that, Ponceau S (Sigma, USA) was used to stain the membrane for a minute. Next, the membrane was cut to strips with approximately 3mm width. Tris-buffered saline (TBS) containing 0.05% Tween 20 (TTBS) was used to wash the membrane and blocked in a blocking solution of 10% non-fat milk in TBS for 2 hours. With the patient's sera (diluted 1:5 in the block buffer) as the primary antibody under constant mixing at 50 rpm, the strips were incubated overnight at 4°C. The strips were then probed with diluted biotinylated goat-antihuman IgE (KPL, USA) for 30 minutes at ambient temperature after being washed in TTBS, as the secondary antibody in the blocking buffer with the ratio of 1:1000. The strips were incubated in diluted streptavidin-conjugated alkaline phosphatase (BioRad, USA) in a ratio of 1:5000 in TBS for half an hour at room temperature, to detect the IgE-binding protein. The procedure was followed by incubating the strips in the alkaline phosphatase conjugate substrate kit (BioRad, USA) for 15 minutes. The reactions were terminated by washing the strips in distilled water for 10 minutes. In this experiment, the blank being used was a strip without a serum sample, and the negative control of this experiment was the sera from non-allergic individuals.

3.0 RESULTS AND DISCUSSION

Immunoblotting of *Penicillium* spp.

In this study, the allergenic potential of the crude *P. simplicissimum* in individuals with serum from ten subjects with the positive fungal allergen in SPT was evaluated. Eight multiple allergenic bands were identified, and in three of them, reactivity was ≤40% (minor allergenic bands 17, 19, 36 kDa) in sera from subjects, whereas five of them reactivity was ≤50% (major allergenic bands 11, 25, 46, 85, >100 kDa) (Table 1, Figure 2).

Table 1 The Frequency of Specific-IgE Binding Proteins in Immunoblotting of *Penicillium simplicissimum* Using Sera from 10 Fungal Allergic Subjects

Molecular weights (kDa)	Sera Identification Number 1	2	3	4	5	6	7	8	9	10	Frequency of presence (%)
11 kDa						√	√	√	√	√	50*
17 kDa	√	√	√	√							40
19 kDa	√	√	√		√						40
25 kDa	√	√	√	√	√	√	√	√			80*
36 kDa					√	√					20
46 kDa	√	√	√	√	√	√	√	√			80*
85 kDa	√	√	√	√	√	√	√	√			80*
>100 kDa	√	√	√	√	√		√	√		√	80*

* Major allergens

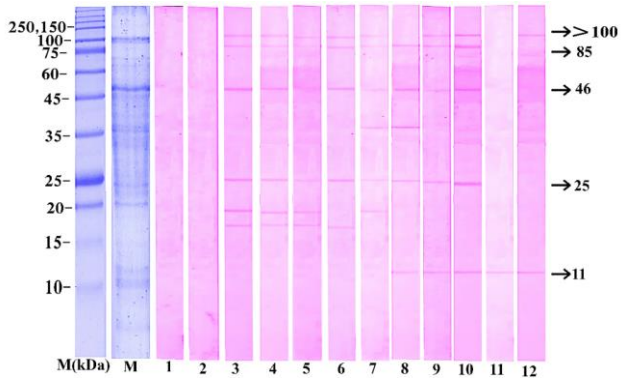


Figure 2 Immunoblotting result of *Penicillium simplicissimum* Using Sera from 10 fungal allergic subjects (Lanes 3 to 12). Lane M (kDa): Molecular Mass Marker in Kilo Dalton (kDa); Lane M Protein Profile of *Penicillium simplicissimum*; Lane 1: blank; Lane 2: immunoblot using a negative control serum. Arrows indicate the major allergens of *Penicillium simplicissimum*

In this study *Penicillium canescens* showed several major allergenic bands (11, 14, 16, 23, 25, 29, 33, 36, 38, 40, 60, 71, 86, >100 kDa) and some minor allergenic protein (41, 50, 55 kDa) (Table 2) (Figure 3).

Table 2 The Frequency of Specific-IgE Binding Proteins In Immunoblotting of *Penicillium canescens* Using Sera from 10 Fungal Allergic Subjects

Molecular weights (kDa)	Sera Identification Number 1	2	3	4	5	6	7	8	9	10	Frequency of presence (%)
11 kDa							√	√		√	30
14 kDa					√		√	√		√	40
15 kDa	√	√	√	√	√	√	√				70*
20 kDa	√	√	√	√	√	√	√				70*
25 kDa		√	√			√	√				40
28 kDa	√	√	√		√	√					50*
30 kDa	√		√		√	√					40
33 kDa			√			√					20
38 kDa	√		√								20
44 kDa		√	√		√	√	√	√		√	70*
55 kDa	√	√	√		√	√					50*
67 kDa							√		√		20
73 kDa	√	√	√		√	√	√				60*

*Major allergens

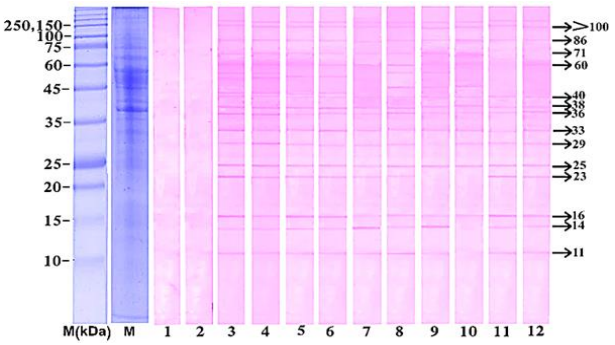


Figure 3 Immunoblotting result of *Penicillium canescens* using sera from 10 fungal allergic subjects (Lanes 3 to 12). Lane M (kDa): Molecular mass marker in Kilo Dalton (kDa); Lane M Protein Profile of *Penicillium canescens*; Lane 1: blank; Lane 2: immunoblot using a negative control serum. Arrows indicate the major allergens of *Penicillium canescens*

According to WHO/IUIS Allergen Nomenclature Sub-Committee 11, 16, 33, 36, 40 kDa allergenic protein band was identified as a major allergen for *P.crustosum*, *P. brevicompactum*, *P. citrinum, P. chrysogenum* respectively [14], [15]. 86 kDa was also reported and listed as a major allergen from the yeast *Malassezia sympodialis* [16]. Allergens similarity between *Penicillium* spp. in this study and other listed allergens by IUIS lead to the understanding of the hazards of exposure to this particular type of fungal species especially in an indoor environment at UPSI.

Immunoblotting of *Aspergillus* spp.

Immunoblotting test to *Asp. aculeatus* revealed six major allergens (25, 26, 34, 36, 48, >100) and two minor allergens (33, 40 kDa) (Table 3, Figure 4).

Table 3 The Frequency of Specific-IgE Binding Proteins In Immunoblotting of *Aspergillus aculeatus* Using Sera from 10 Fungal Allergic Subjects

Molecular weight (kDa)	Sera Identification Number 1	2	3	4	5	6	7	8	9	10	Frequency of presence (%)
11 kDa	√	√	√	√	√	√	√		√	√	90*
14 kDa	√	√	√	√	√	√	√		√	√	90*
16 kDa	√	√	√	√	√	√	√	√	√	√	100*
23 kDa	√	√	√	√	√	√			√	√	**80***
25 kDa	√	√	√	√	√	√	√	√	√	√	100*
29 kDa	√	√	√	√	√	√		√	√	√	**90***
33 kDa	√	√	√	√	√	√	√	√	√	√	100*
36 kDa	√	√	√	√	√	√	√	√	√	√	100*
38 kDa	√	√	√	√	√	√	√	√	√	√	100*
40 kDa	√	√	√	√	√	√	√	√	√	√	100*
41 kDa	√	√	√	√							**40**
50 kDa	√	√	√	√							**40**
55 kDa	√	√			√	√					**40**
60 kDa	√	√	√	√		√				√	60*
71 kDa	√	√	√	√	√	√	√	√			**80***
86 kDa	√	√	√	√	√	√	√	√	√	√	100*
>100 kDa	√	√	√	√	√	√	√	√	√	√	100*

* Major allergens

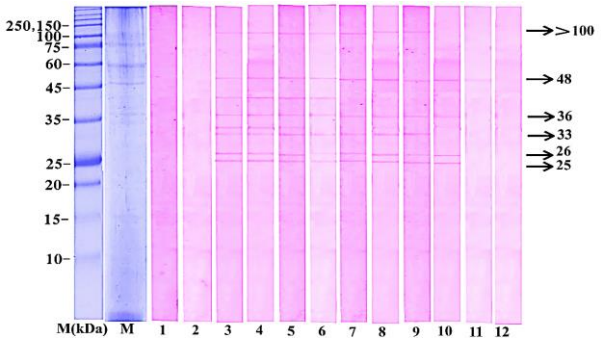


Figure 4 Immunoblotting result of *Aspergillus aculeatus* using sera from 10 fungal allergic subjects (Lanes 3 to 12). Lane M (kDa): is a molecular mass marker in kilodalton (kDa); Lane M is a protein profile of *Aspergillus aculeatus*; Lane 1 Is Blank; Lane 2 is an immunoblot using a negative control serum. Arrows indicate the major allergens of *Aspergillus aculeatus*

To this date, there is no specific study on the allergenicity of *Aspergillus aculeatus*. The 34 kDa allergenic protein in *Asp. aculeatus* compared to other *Aspergillus* species studied by other researchers were identified as Asp f I 13 in *Aspergillus flavus*, in *Asp. fumigatus* and other *Aspergillus species* (Asp f 9, Asp f10 Aspartate protease in *Asp. fumigatus*), (Asp n 18 –Vacuolar serine protease) in *Asp. niger* and *Asp. fumigatus* (Table 4).

Table 4 List of *Asp. fumigatus* allergenic proteins listed by IUIS Allergen Nomenclature Sub-Committee

Species	MW (kDa)	Allergens	Biochemical name	Reference
Asp. fumigatus	11	Asp f 8	Ribosomal protein P2	[17]
	12	Asp f 7	–	[16]
	13	Asp f 13	Alkaline serine protease	[18]
	19	Asp f 3	Peroxysomal protein	[19]
	30	Asp f 4	–	[20]
	34	Asp f 9 Asp f10	Aspartate protease	[16]
	37	Asp f 2	–	[21]
	40	Asp f 5	Metalloprotease	[16]

– = no Biochemical name listed

The same band also is identified as Asp o 13-Alkaline serine protease in *Aspergillus oryzae* [17], [18]. 40 kDa allergen is also listed as Asp f 5-Metalloprotease in *Asp. fumigatus*. The allergenic protein of >100 kDa was listed as Asp n 14 Beta-xylosidase in *Asp. niger and Asp. aculeatus.* Therefore, all these identified allergenic proteins could raise the possibility of sensitisation among the UPSI community.

Immunoblotting study on *Aspergillus caliodustus* with 60% prevalence in indoor dust revealed major allergens (15, 20, 28, 44, 55, 73 kDa) and minor allergens (11, 14, 25, 30, 33, 38, 67 kDa) (Table 5) (Figure 5).

Table 5 The Frequency of Specific-IgE Binding Proteins in Immunoblotting of Aspergillus caliodustus Using Sera From 10 Fungal Allergic Subjects

Molecular weight (kDa)	Sera Identification Number 1	2	3	4	5	6	7	8	9	10	Frequency of presence (%)
12 kDa	√	√	√	√	√	√				√	70*
13 kDa	√	√	√								**30**
15 kDa	√	√	√	√	√	√				√	70*
17 kDa	√							√			**20**
19 kDa	√							√			**20**
28 kDa							√	√	√	√	**40**
34 kDa	√	√	√	√	√	√	√			√	80*
36 kDa							√	√	√	√	40
39 kDa							√	√	√	√	**40**
42 kDa	√	√	√	√	√	√					**60***
55 kDa	√	√	√	√	√	√					**60***
59 kDa							√		√	√	30
73 kDa							√	√	√		30

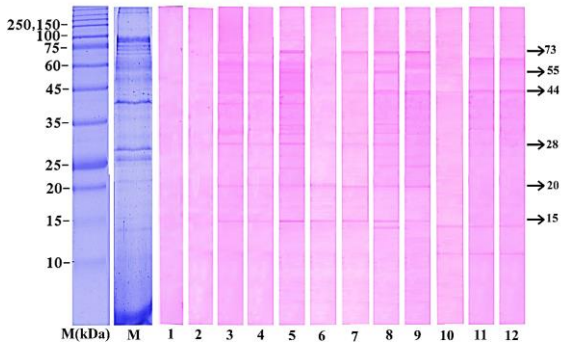


Figure 5 Immunoblotting result of *Aspergillus caliodustus*. Using sera from 10 fungal allergic subjects (Lanes 3 to 12). Lane M (kDa): is a molecular mass marker in kilo Dalton (kDa); Lane M is a protein profile of *Aspergillus caliodustus*; Lane 1 is blank; Lane 2 is an immunoblot using a negative control serum. Arrows indicate the major allergens of *Aspergillus caliodustus*

11 kDa allergenic protein was reported as Asp f 8 Ribosomal protein P2 in *Asp. fumigatus* [19]. Both 20 and 44 kDa allergenic proteins from *Asp. caliodustus* and unidentified *Aspergillus* sp. in this study were also listed as Asp f 34-PhiA cell wall protein and Asp f 23 L 3 ribosomal protein respectively in *Asp. fumigatus*. While 30 kDa allergenic protein was identified as Asp f 4 in *Asp. fumigatus*. Allergen Nomenclature Sub-Committee also listed 67 kDa which was detected within the range of allergens 66-100 kDa in *Asp. fumigatus* as Asp n 25- 3-phytase B [19].

Hence, the similarity between identified airborne dust fungal allergenic proteins in this study and other allergens from *Aspergillus* spp. which are listed by Allergen Nomenclature Sub-Committee. Other major and minor allergenic proteins detected in this study that are not listed by Allergen Nomenclature Sub-Committee should not be taken lightly as they may be hazardous as well.

In this study immunoblotting of unidentified *Aspergillus* which was prevalent at 76% in indoor dust revealed major allergenic protein bands (12, 15, 34, 42, and 55 kDa); and minor allergenic protein (13, 17, 19, 28, 36, 39, 59, and 73 kDa) (Table 6) (Figure 6).

Table 6 The frequency of specific-ige binding proteins in immunoblotting of *Aspergillus* sp. Using sera from 10 fungal allergic subjects

Molecular weight (kDa)	Sera Identification Number										Frequency of presence (%)
	1	2	3	4	5	6	7	8	9	10	
11kDa	√	√	√	√	√	√					**60***
12kDa	√	√	√	√	√	√					**60***
13kDa	√	√	√								**30**
17kDa			√	√	√						**30**
19 kDa						√	√	√	√	√	**50***
23kDa	√	√	√	√	√	√		√			**70***
25kDa	√	√	√	√	√	√		√			**70***
26kDa	√	√	√								**30**
30 kDa	√	√	√	√	√	√		√			**70***
34kDa	√	√	√	√							**40**
37 kDa	√	√	√	√	√	√					**60***
41 kDa	√	√	√	√	√	√					**60***
45kDa	√	√	√	√	√	√					**60***
68kDa							√	√	√	√	**40**

*Major allergens

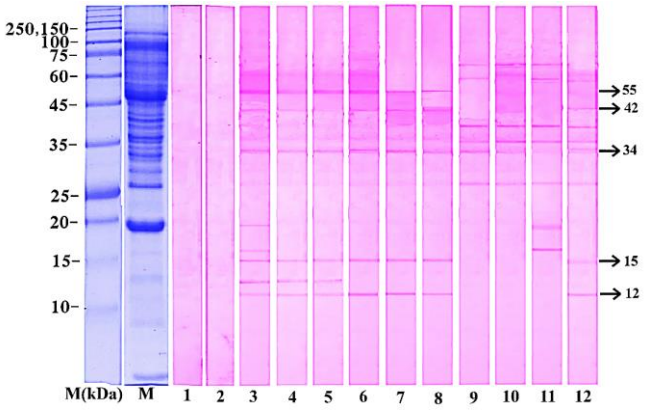


Figure 6 Immunoblotting result of *Aspergillus* sp.Using sera from 10 fungal allergic subjects (Lanes 3 to 12). Lane M (kDa): is a molecular mass marker in kilodalton (kDa); Lane M is a protein profile of *Aspergillus* sp.; Lane 1 is blank; Lane 2 is an immunoblot using a negative control serum. Arrows indicate the major allergens of *Aspergillus* sp.

12 kDa is also listed as a major allergen from *Asp. fumigatus* (Asp f 7) [20]. 13 kDa is also listed as Asp f 28 Thioredoxin, Asp f 29 Thioredoxin respectively in *Asp. fumigatus* and 19 kDa are also listed as Asp f 3 Peroxysomal proteins in *Asp. fumigatus* [21].

Immunoblotting study of *Asp. fumigatus* with 82% prevalence from indoor dust revealed major allergenic proteins (11, 12, 19, 23, 25, 30, 37, 41, and 45 kDa) and minor allergenic proteins (13, 17, 26, 34, and 68) (Table 7) (Figure 7).

Table 7 The Frequency of Specific-IgE Binding Proteins in Immunoblotting of *Aspergillus fumigatus* Using Sera from 10 Fungal Allergic Subjects

Molecular weight (kDa)	Sera Identification Number										Frequency of presence (%)
	1	2	3	4	5	6	7	8	9	10	
12 kDa	√	√	√	√	√	√				√	70*
13 kDa	√	√	√								**30**
15 kDa	√	√	√	√	√	√				√	70*
17 kDa	√								√		**20**
19 kDa	√								√		**20**
28 kDa							√	√	√	√	**40**
34 kDa	√	√	√	√	√	√	√			√	80*
36 kDa							√	√	√	√	40
39 kDa							√	√	√	√	**40**
42 kDa	√	√	√	√	√	√					**60***
55 kDa	√	√	√	√	√	√					**60***
59 kDa							√		√	√	30
73 kDa							√	√	√		30

*Major allergens

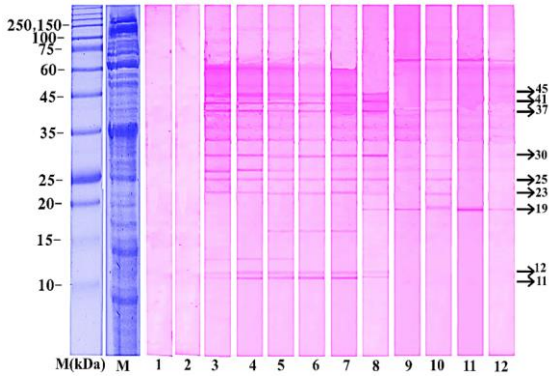


Figure 7 Immunoblotting Results of *Aspergillus fumigatus.*Using Sera from 10 fungal allergic subjects (Lanes 3 to 12). Lane M (kDa): Is a molecular mass marker in kilo Dalton (kDa); Lane M is a protein profile of *Aspergillus fumigatus*; Lane 1 Is Blank; Lane 2 is an immunoblot using a negative control serum. Arrows indicate the major allergens of *Aspergillus fumigatus*

Allergenic proteins with molecular weights of 11, 12, 19, 13, and 34 kDa were also listed by Allergen Nomenclature Sub-Committee as fungal allergens (Table 4). The allergenic protein of 17 kDa in this study was also reported as a major allergenic protein in *Aspergillus fumigatus* [22].

Immunoblotting of *Syncephalastrum sp.*

In this study, *Syncephalastrum sp* was isolated from indoor dust with a 66% prevalence. Immunoblotting of this species revealed the major allergenic proteins (20, 25, 30, 33, 35, 41, 44, 49, and 73 kDa) and minor allergens (28, 55, 60, and 83 kDa) (Table 8, Figure 8).

Table 8 The Frequency of Specific-IgE Binding Proteins in Immunoblotting of *Syncephalastrum* sp. Using Sera from 10 Fungal Allergic Subjects

Molecular weights (kDa)	Sera Identification Number 1	2	3	4	5	6	7	8	9	10	Frequency of presence (%)
20 kDa	√	√	√	√	√	√	√	√	√	√	**100***
25 kDa	√	√	√	√	√	√	√			√	**80***
28 kDa	√	√	√								**30**
30 kDa	√	√	√	√	√	√	√				**70***
33 kDa	√	√	√	√	√	√	√	√		√	**90***
35 kDa	√	√	√	√	√	√	√	√		√	**90***
41 kDa	√	√	√	√			√	√		√	**70***
44 kDa	√	√	√	√			√	√		√	**70***
49 kDa	√	√	√	√				√			**50***
55 kDa	√	√	√	√							**40**
60 kDa	√	√	√	√							**40**
73 kDa	√	√	√	√				√		√	**60***
83 kDa	√	√	√	√							**40**

*Major allergens

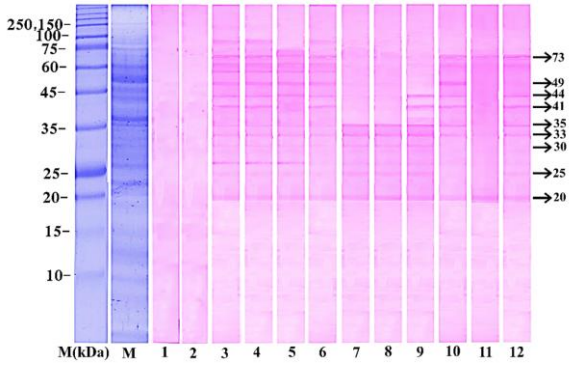


Figure 8 Immunoblotting result of *Syncephalastrum* sp. using sera from 10 fungal allergic subjects (lanes 3 to 12). Lane M (kDa): is a molecular mass marker in kilodalton (kDa); Lane M is a protein profile of *Syncephalastrum* sp.; Lane 1 is Blank; Lane 2 is an immunoblot using a negative control serum. Arrows indicate the major allergens of *Syncephalastrum* sp.

Most commonly, *Syncephalastrum* is identified as a contaminant. But it is very rarely associated with human disease. Only a single species is included in the genus *Syncephalastrum* i.e. *Syncephalastrum racemosum*. In terms of sensitisation, allergenicity or pathogenicity, it is considered to be a very rare causative agent of human zygomycosis. So far, it has also been isolated in cases of otomycosis as well as a cutaneous infection [23].

Immunoblotting of *Fusarium sp.*

Species of *Fusarium* causes superficial, locally harmful and diffuse disease in humans. Although *Fusarium verticillioides*, including *F. moniliforme* and *F. fujikuroi* species complex [24] are opportunistic pathogens, the species in the *F. solani* complex including the pathogenic species *F. solani*, *F. oxysporum*, *F. verticillioides*, and *F. proliferatum* infect the immune-compromised subjects.

In this study, immunoblotting of this species revealed the 11 and 58kDa as major and the 13, 25, 33, 36, 45, 71, and >100) kDa as the minor allergenic proteins (Table 9, Figure 9).

Table 9 The Frequency of Specific-IgE Binding Proteins in Immunoblotting of Fusarium sp. Using Sera from 10 Fungal Allergic Subjects

Molecular weight (kDa)	Sera Identification Number 1	2	3	4	5	6	7	8	9	10	Frequency of presence (%)
11 kDa	√	√	√	√		√					**50***
13 kDa	√	√	√				√				**40**
25 kDa					√						**10**
33 kDa									√	√	**20**
36 kDa	√	√	√								**30**
45 kDa					√	√	√	√			**40**
58 kDa	√	√			√	√	√	√			**60***
71 kDa	√	√	√								**30**
>100 kDa				√	√	√	√				**40**

*Major allergens

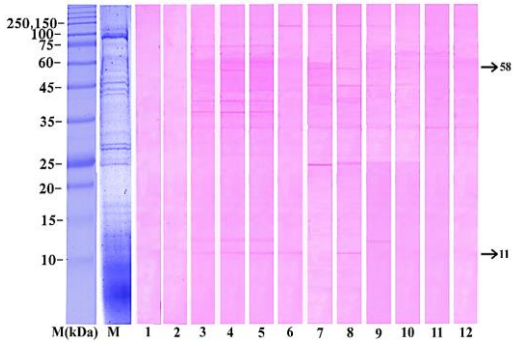


Figure 9 Immunoblotting Result of *Fusarium* sp.using sera from 10 fungal-allergic subjects (Lanes 3 to 12). Lane M (kDa): Is a molecular mass marker in kilo Dalton (kDa); Lane M is a protein profile of *Fusarium* sp.; Lane 1 is blank; Lane 2 is an immunoblot using a negative control serum. Arrows indicate the major allergens of *Fusarium* sp.

11 kDa (a major allergenic protein) and 13 kDa (a minor allergenic protein) were listed as Fus c 1 Ribosomal protein P2 and Fus c 2 Thioredoxin respectively *in Fusarium culmorum*. While 36.5 kDa allergenic protein was also listed as Fus p 9 Vacuolar serine proteases in *Fusarium proliferatum* [25].

The specific allergens from many different mould species have been cloned as well as produced as recombinant IgE-reactive proteins. But, for some of these, the skin prick tests, as well as basophile histamine release assays, help to demonstrate the ability to induce certain immediate-type reactions. Generally, the recombinant allergens with certain similar characteristics as their natural counterparts are well suited for both *in vivo* as well as *in vitro* diagnosis.

So, the proper identification of the genuine sensitisation to a single mould species is mainly allowed by the application of species-specific allergens such as Mala s 1, Cop c 1, Asp f 1 or Alt a 1. Additionally, the recombinant mould allergens with known cross-reactivity are considered to be a much useful tool as marker allergens to identify the cross-sensitisation. In this way, it is possible to improve the diagnosis of both specificities as well as the sensitivity of diagnosis [26], [27].

The frequency of major allergenic proteins 11 kDa was 50 %, while 33, 36, >100 kDa were 60%, and 25 kDa was 80% in all the ten sera from fungi sensitised subjects (Table 10).

Table 10 Summary of All Major Allergens for the Ten Fungal Species and Their Frequency

M.W (kDa)	Fungi species										
	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10	*%
11	√				√			√	√	√	50*
25	√	√	√		√	√		√	√	√	80*
33		√		√	√	√			√	√	60*
36	√	√	√	√			√		√	√	60*
>100	√	√	√	√					√	√	60*

Fusarium sp. and *Penicillium canescens* showed the highest frequency for all major allergenic proteins 100%. *Fusarium* sp. and *Penicillium canescens* prevalence is 82% and 88% respectively within the three buildings that led to an increase in the hazards of potent allergenicity of these species.

In summary, all the major fungal allergenic proteins revealed in this study are also documented by IUIS in its official website as major allergens. 11 kDa reported as major allergenic protein from *Alternaria, Cladosporium, Penicillium, and Aspergillus* species. 25 and 33 kDa, both are reported as major allergenic proteins from various *Penicillium* spp. Meanwhile, both 36 and **>**100 is reported as major allergenic proteins for *Cladosporium* and *Aspergillus* respectively (Table 11).

Table 11 Summary of All Major Allergens for the Ten Fungal Species and Their Frequency

MW kDa	Route of allergen exposure	Biochemical name	Allergen	Species
11 kDa	Airway	Acidic ribosomal prot. P1	Asp f 8	*Asp. fumigatus*
			Cla h 5	*Cladosporium herbarum*
			Fus c 1	*Fusarium culmorum*
			Pen b 26	*Penicillium brevicompactum*
			Alt a 5	*Alternaria alternata*
			Pen cr 26	*Penicillium crustosum*
25 kDa	Airway	Elongation factor 1 beta	Pen c 24	*Penicillium citrinum*
33 kDa	Airway	Alkaline serine protease	Pen b 13	*Penicillium brevicompactum*
			Pen c 13	*Penicillium citrinum*
36 kDa	Airway	Vacuolar serine protease	Cla c 9	*Cladosporium cladosporioides*
>100 kDa	Airway	Beta-xylosidase	Asp n 14	*Aspergillus niger*

Evaluation of Cross-Reactivity among Fungi Allergens

The cross-reactivity was done in between two species most commonly prevalent, i.e. *Penicillium canescens* and *Aspergillus fumigatus* using three selected sera and following specific methods. Using rabbit polyclonal antibodies against *Aspergillus fumigatus*, cross-reactivity with *Penicillium canescens* was analyzed by enzyme immune assay (EIA).

Due to the cross-reactivity, some of the allergenic bands were partial or completely disappeared to demonstrate inhibition. In this study, most of the IgE-binding protein of *Aspergillus fumigatus* cross-reacted with inhibitor extracts of *Penicillium canescens* at various molecular including the major allergenic

proteins 12, 19, 23, 25, and 30 kDa with completely inhibited IgE reactivity of the patient's sera. Only some IgE-binding major proteins at 11, 37, 41, 45, and 68 kDa (a minor allergen) were not cross-reacted with the certain inhibitor extracts in some subjects. The inhibition percentage of *Aspergillus fumigatus* against *Penicillium canescens* is 85.7% (Figure 9, Table 12). These results suggested that both fungi shared some antigenic components.

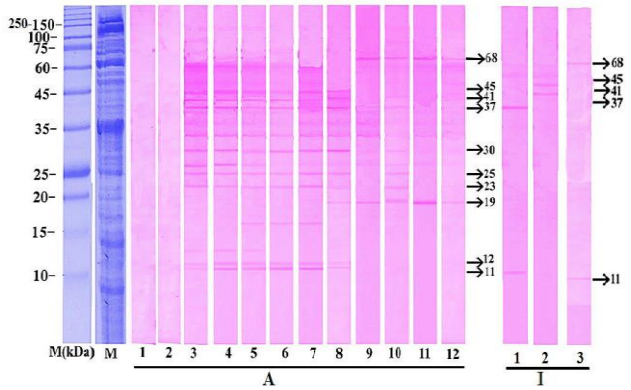


Figure 9 *Aspergillus fumigatus* allergenic bands after inhibition by using three reabsorbed sera from patient allergic to *Penicillium canescens*, Line M (kDa) is the molecular weight marker in kilo Dalton (kDa). Line M is the protein profile bands. Lines A, is immunoblotting results. Line I, Is *Aspergillus fumigatus* immunoblotting inhibition strips

Table 12 Immunoblotting inhibition analysis of *Aspergillus fumigatus*

Inhibitors	*Penicillium canescens* Subjects						
MW (kDa)	1	2	3	C (%)	P (%)	N (%)	Inhibition%
11*	N	C	N	33.3	-	66.3	33.3
12*	C	C	C	100	-	-	100
13	C	C	C	100	-	-	100
17	C	C	C	100	-	-	100
19*	C	C	C	100	-	-	100
23*	C	C	C	100	-	-	100
25*	C	C	C	100	-	-	100
26	C	C	C	100	-	-	100
30*	C	C	C	100	-	-	100
34	C	C	C	100	-	-	100
37*	N	C	C	66.6	-	33.3	66.6
41*	C	N	C	66.6	-	33.3	66.6
45*	C	N	C	66.6	-	33.3	66.6
68	C	C	N	66.6	-	33.3	66.6

C = Complete inhibition, P = Partial inhibition, N = No inhibition, % = frequency of inhibition *= Major allergen

In comparison, most of IgE-binding protein of *Penicillium canescens* cross-reacted with inhibitor extracts of *Aspergillus fumigatus* at various molecular including the major allergenic proteins 14, 16, 23, 25, 36, 38, 40, 60, 86, <100 kDa, with totally inhibited IgE reactivity of the patient's sera. Only some IgE-binding proteins at 11, 29, 33, 71 kDa were not cross-reacted with the certain inhibitor extracts in some subjects. The inhibition percentage of *Penicillium canescens* is 92.1% (Figure 10, Table 13). These results confirmed that both fungi highly shared common antigenic components.

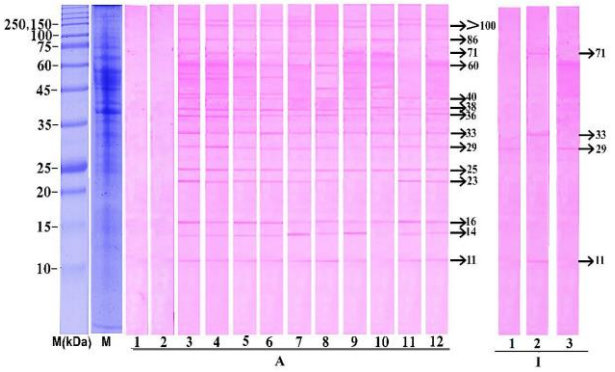


Figure 10 *Penicillium canescens* allergenic bands after inhibition by using three reabsorbed sera from patients allergic to *Aspergillus fumigatus*, line M (kDa) is the molecular weight marker in kilo Dalton (kDa), line M is the protein profile bands, lines A, is immunoblotting results, the line I, is *Penicillium canescens* immunoblotting inhibition strips

In this study, 86 kDa major allergenic protein from *Penicillium canescens* was cross-reacted by *A. fumigatus* extracts, and 86 kDa was reported and listed by IUIS as a major allergen from the yeast *Malassezia sympodialis* (skin fungus). The discovery could lead to new information in diagnosis and treatment as it is well known that the existence of cross-reactivity, particularly among different moulds, may have implications for the diagnosis and treatment [28]. In this study 68 kDa, the minor allergenic protein in A. *fumigatus* was not cross-reacted against *Penicillium canescens* extracts but from *P. notatum,* 68 kDa demonstrated a 54% sequence homology with b-N-acetylglucosaminase from *C. albicans.* Likewise, 33kDa in *Penicillium canescens* was not cross-reacted in this study was demonstrated IgE cross-reactivity in *P. citrinum,* against extracts of *P. notatum,* and *P. brevicompactum* [22]. They also reported and listed 19 kDa and 30 kDa as fungal allergenic proteins with cross-reactivity in *Aspergillus fumigatus*. 19 kDa (Asp f 3) from *A. fumigatus* was reported to share common IgE epitopes with two peroxisomal membrane proteins (PMPA and PMPB) of *C. boidinii* (yeast species). Recent investigations using highly pure recombinant allergens demonstrate that cross-reactivity between homologous fungal proteins can play a decisive role in the exacerbation of allergic complications related to fungal sensitisation [28] and risk assessment [29].

Table 13 Immunoblotting inhibition analysis of *Penicillium canescens*

Inhibitor	*Aspergillus fumigatus* Subject			Inhibition (%)			
MW (kDa)	1	2	3	C (%)	P (%)	N (%)	Total (%)
11*	C	N	C	33	-	33	66
14*	C	C	C	100	-	-	100
16*	C	C	C	100	-	-	100
23*	C	C	C	100	-	-	100
25*	C	C	C	100	-	-	100
29*	P	C	N	33	33	33	66
33*	P	N	C	33	33	33	66
36*	C	C	C	100	-	-	100
38*	C	C	C	100	-	-	100
40*	C	C	C	100	-	-	100
41	C	C	C	100	-	-	100
50	C	C	C	100	-	-	100
55	C	C	C	100	-	-	100
60*	C	C	C	100	-	-	100
71*	C	N	C	66	-	33	66
86*	C	C	C	100	-	-	100
>100*	C	C	C	100	-	-	100
Inhibition %	100	82	94				92

C = Complete inhibition, P = Partial inhibition, N = No inhibition, % = frequency of inhibition *= Major allergen

4.0 CONCLUSION

In summary, both most communal prevalent fungi i.e. *Penicillium canescens* and *Aspergillus fumigatus* are highly shared mutual antigenic components but with different allergenic components. In allergy management, both allergens should be included in the panel to ensure individuals are precisely diagnosed as both are hazardous and evident to elicit allergy among communities at UPSI.

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