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Evaluation of the sensitization of asthmatic patients to *A.alternata, P.citrinum* and *A.fumigatus* by IgE-immunoblotting

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Abstract

Background: There is evidence to demonstrate an association between fungal sensitization and asthma. Several studies have shown that saprophytic fungi such as *Alternaria, Penicillium* and *Aspergillus* species are the most prevalent fungal allergens worldwide. The main purpose of this study was to compare *Alternaria alternata*, *Penicillium citrinum* and *Aspergillus fumigatus* allergen bands by using the same patients and controls' sera in clinical investigations.

Methods: Forty-eight patients with asthma (23 males, 25 females) and Forty-eight healthy controls (23 males, 25 females) were collected in 2017. Glass beads and liquid nitrogen were used to disrupt the cell wall of cultured fungi. SDS-PAGE was used to isolate protein fractions. IgE immunoblotting against the patients and controls sera were performed to isolate protein bands after electrotransfering into the nitrocellulose membrane.

Results: Our findings demonstrated the most allergenic bands consist to *A. alternata* with 17 bands (44.7%) relative to *P. citrinum* and *A. fumigatus*, and we found that asthmatic patients in the age range of 41 to 70 years were more sensitive when compared to other age groups.

Conclusion: Our results showed that *A. alternata* had more power in sensitizing the patients in comparison with *P. citrinum* and *A. fumigatus*. Also, the protein bands with high molecular weight can be considered as an index of sensitizing in immunoblotting assay.

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Introduction

Asthma is a complex inflammatory disorder affecting many people in the world (1). Both environmental and genetic factors can have an influence on multifactorial diseases (2). Recently, 300 million cases of asthmatic patients were estimated (3).

Allergy to fungi is an important environmental risk factor for the development of asthma. In addition, evidence shows that fungal allergy is associated with severe asthma (4). Up to 80% of asthmatic patients have been reported to have allergic sensitivity to fungi (5).

Some saprophytic fungi, such as *Alternaria*, *Penicillium* and *Aspergillus* species were investigated as the most prevalent fungal allergens in the world. In this regard, several studies reported that there was relevance between fungal antigens and asthma (6).

More than 12 allergens were already found for *Alternaria alternata* in which their characters have been described, but some of these allergens are still unknown (7). Among the *A. alternata* allergens, two allergens are known as important allergic agents called Alt a1 and Alt a2, respectively (8).

Evidence shows that in more than 80% of *A. alternata*-sensitized patients, Alt a1 allergic agent reacts to serum IgE (9). Other *A. alternata* allergens such as Alt a2, Alt a4, Alt a6, Alt a7, Alt a8, Alt a10, Alt a11, Alt a12 and Alt a NTF2 have also been evaluated (10).

Among *Aspergillus* species, *A. fumigatus* was recognized as the most important pathogenic agent with strong allergens. One of the *A. fumigatus* major allergenic proteins is Asp f1 that acts as a specific virulence factor. Asp f3 is the other important allergen of this fungus, which binds to serum IgE in 94% of sensitized patients (11). Moreover, more than twenty *A. fumigatus* allergens including Asp f1 to Asp f22 were identified (12).

Allergens are in several *P*enicillium species, but the majority of them are in *Penicillium citrinum.* Pec c19, Pec c2, Pec c18, Pec c13, Pec c1, Pec c22 allergens and also Pec c24, as an elongation factor have been reported in *Penicillum citrinum* (8).

Identifying the profile of fungi allergens can be used for clinical diagnosis of the allergic diseases; however, this profile may be different from geographical area. Several studies in different countries and Iran have demonstrated that there is an association between fungal allergens and asthma (6,13,14,15). In the present study, we investigated the IgE reactivity of asthmatic patients' serum to *A. alternata*, *P. citrinum* and *A. fumigatus* antigens. Moreover, we compared allergenic power among *A. alternata*, *P. citrinum* and *A. fumigatus.*

Material and Methods

Fungi isolates and cultivation

A. fumigatus, *P. citrinum* and *A. alternata* isolates of campus air in Iran were obtained from fungal Collection of Mycology Research Center, University of Tehran, and grown in Sabouraud glucose agar in sterile conditions by incubation at 27°C for 48-72 hours. Grown colonies were sub-cultured in 500 ml of Sabouraud glucose broth containing chloramphenicol and incubated in a shaker incubator (150 rpm) at 25°C for 8-10 days. In the next step, fungal colonies containing both mycelia and spores were harvested by Whatman filter paper (No. 1, Clifton, USA), washed three times with sterile PBS, and stored at -20°C until use.

Protein extraction

A suspension of wet fungal colonies was prepared in breaking buffer (62.5 M Tris, 1 mM Dithioteritol, 0.2 mg/ml PMSF, 15% Glycerol, pH 6.8) and the cells were disrupted using glass beads (1 mm diameter) on a vortex mixer during 20 min with 5 min intervals by keeping on ice bath to prevent overheating. After cell disruption, the crude extracts were separated from intact cells and debris by centrifugation at 7000 g for 15 min, and recentrifugation of supernatant at 26,000 g for 40 min. The extracts were sterilized with filter (0.2 m, Sartorius, USA) and stored at _20 °C until use. Protein concentration of

these final extracts was determined after reconstituting in distilled water by Bradford method (16).

Patients and controls

Forty-eight serum samples from patients (25 females and 23 males, aged up to 70 years) with asthma attending the Tehran Allergy Clinic (Tehran-Iran) were collected in 2017. The diagnostic criteria for asthmatic patients were based on history, clinical symptoms of allergy and defective breathing. To reduce the clinical symptoms of their asthma, all patients in this study were under the corticosteroid treatment for a nearly similar duration (around 6-8 months). For the control group, forty-eight sera from non-atopic adults were also collected. All subjects gave informed consent to participate in the study. Collected serum samples were stored at -70 °C until use. The patients and control subjects were divided into three subgroups based on age (up to 20, 21-40 and 41-70 years old). In order to perform immunoblotting assay, the pooled serum were prepared for patients and controls according to age subgroups.

SDS-PAGE

Protein components of the fungal extracts were separated by 11% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method in a discontinuous buffer system (minitetra cell system Bio-RAD, USA). Briefly, 30 ml of extracts (containing 200 mg protein) were boiled for 5 min with a reducing sample buffer, containing 2-mercaptoethanol and loaded on the gel next to the molecular size marker (Pageruler unstained protein ladder, Fermentas, Lithuania). The gel was visualized after staining with coommassie brilliant blue G-250 (Sigma) and the molecular weight of the protein components in each lane was determined using a standard curve that was plotted based on the migration distance of the size marker bands.

IgE-Immunoblotting assay

Separated protein bands were transferred from the SDS PAGE gel to a nitrocellulose membrane (PROTRAN Whatman GmbH, Germany) using a transfer buffer (25 mM Tris, 192 mM Glycin, 0.03% SDS and 20% Methanol, pH 8.3) and an electro transfer system (Mini Trans-Blot, Bio-RAD, USA) by applying 100 V for 1 h. Stripped membranes were incubated at 48°C for overnight with PBS-Tween 20-BSA buffer (137 mM Nacl, 2.7 mM KCl, 10 mM Na2HPO4, 1.4 mM KH2PO4, 0.05% Tween 20 and 1.5% BSA, pH 7.2). After three times of washing steps with PBS-Tween 20 buffer at room temperature for 5 min, the strips were incubated with patients and controls' pooled sera (1:2 to 1:5 dilutions in PBS-Tween 20) for 3 h at room temperature, while being shacked very gently. Following 3 times of washing, alkaline phosphatase (ALP)-conjugated anti-human IgE (Sigma, 1:1000 dilution in PBS-Tween 20) was added to each strip and incubated for 3 h. Strips were again washed and the bands were revealed in the presence of nitroblue tetrazolium (NBT), 5 bromo-4-chloro-3-indolyl phosphate (BCIP) mixture. Immunoblot was considered positive if at least one protein band was visible (8).

Results

In this study, 48 asthmatic patients and 48 healthy controls were recruited. The distribution of patients and controls are shown in Table 1. Immunoblotting assay for asthmatic patients and controls' pooled serum was performed, so that the presence of IgE against *A. alternata, P. citrinum* and *A. fumigatus*

antigens was detected. Whereas all the controls sera were negative against *A. alternata*, *P. citrinum* and *A. fumigatus* antigens, several protein bands that was reacted with IgE in asthmatic patients sera were identified.

Table 1. Frequency of asthmatic patients and healthy controls based on age and gender

	Asthmatic Patients		Controls	
Total	**48**		**48**	
Age	Male	Female	Male	Female
<20	6	7	6	7
21 – 40	8	9	8	9
41 – 70	9	9	9	9
Total	23	25	23	25

The bands frequency and size of *A. fumigatus*, *P. citrinum* and *A. alternata*, are demonstrated in Table 2, 3 and 4, respectively. As the results show the most allergenic bands consist to *A. alternata* with 17 bands, and the *P. citrinum* and *A. fumigatus*' band frequency was almost similar.

Table 2. Molecular weights of allergenic protein bands of A.fumigatus that reacted with the pooled serum of asthmatic patients

Age	**<20**	**21 – 40**	**41 – 70**	**Total bands**
Molecular weights of detected proteins (kDa)	71 44	112 80 71	112 80 71 54 31	
Total number of detected bands (%)	2 (20%)	3 (30%)	5 (50%)	10 (100%)

Table 3. Molecular weights of allergenic protein bands of P.citrinum that reacted with the pooled serum of asthmatic patients

Age	**<20**	**21 – 40**	**41 – 70**	**Total bands**
Molecular weights of detected proteins (kDa)	127 91 74	127 91 74	127 91 74 64 55	
Total number of detected bands (%)	3 (27.3%)	3 (27.3%)	5 (45.4%)	11 (100%)

Table 4. Molecular weights of allergenic protein bands of A.alternata that reacted with the pooled serum of asthmatic patients

Age	<20	21 – 40	41 – 70	Total bands
Molecular weights of detected proteins (kDa)	178 115 83 67 61 49	178 115 83 67	178 115 83 67 61 56 49	
Total number of detected bands (%)	6 (35.3%)	4 (23.5%)	7 (41.2%)	17 (100%)

Our results showed that, patients in the age range of 41 to 70 years were more sensitive than others. Comparison between different age groups was determined with more protein bands (Table 5).

Table 5. Comparison of the results of immunoblotting assay to A.fumigatus, P.citrinum and A.alternata allergens with respect to age characteristics in patients. The frequency of allergenic bands with percentage is also shown.

Age	<20		21 – 40		41 – 70		Total bands	
Frequency percentage	Freq.	Per.	Freq.	Per.	Freq.	Per.	Freq.	Per.
A.fumigatus	2	18.2%	3	30%	5	29.5%	10	26.3%
P.citrinum	3	27.3%	3	30%	5	29.5%	11	29%
A.alternata	6	54.5%	4	40%	7	41%	17	44.7%
Total number of detected bands (%)	11	100%	10	100%	17	100%	38	100%

Our findings showed that some extracted bands reacted stronger than others. 71 kDa band for *A. fumigatus*, 127, 91, 74 kDa bands for *P. citrinum* and 178, 115, 83, 67 kDa bands for *A. alternata* reacted with patients' sera in the all age groups (Figure.1). While some of the allergen bands reacted only with one of the age groups.

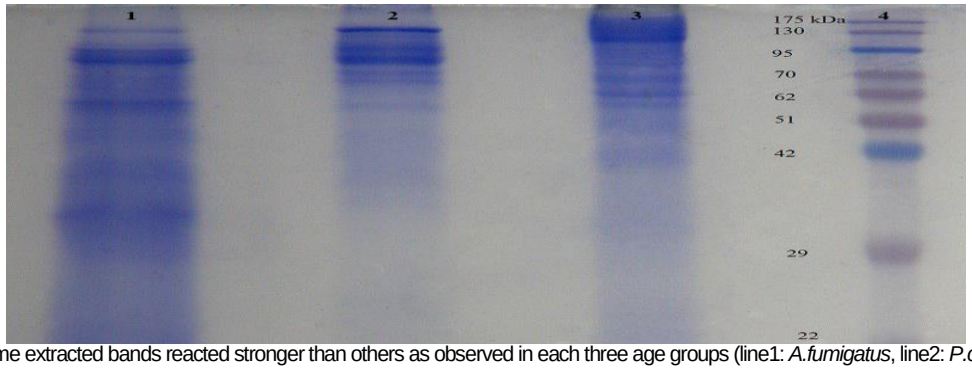

Figure 1. Some extracted bands reacted stronger than others as observed in each three age groups (line1: *A.fumigatus*, line2: *P.citrinum*, line2: *A.alternata*)

Discussion

Many studies have demonstrated an association between *A. alternata* and asthma (5,13). Khosravi et al. in 2009 showed that 50% of patients' sera strongly reacted to *A. alternata* extract (14). Our study indicates that the sera of patients with asthma (aged 41 to 70 years) have the most allergen bands (41.2%) in comparison with other age groups (Table 4). More than 10 approved allergens for *A. alternata* have been reported and we could identify 7 allergen bands for *A. alternata* (2). Interestingly we observed four heavy protein bands in all age groups.

A. alternata, *P. citrinum* and *A. fumigatus* as saprophytic fungi exist extensively in air and can enter the lower respiratory system through inhalation and under specific conditions. They may result in diseases such as asthma, rhinitis, allergic bronchopulmonary aspergillosis and allergy (17). In recent years, fungal allergenic extracts have been evaluated by several investigators and recently, many fungi allergens have been identified and classified (1,18). The evaluation of these allergenic fungi profiles can be useful to enhance our allergen role information in the development of allergy and asthma (11).

A. fumigatus and *P. citrinum* are known as important allergens species (8,17). We have previously shown all asthmatic patients' sera reacts to *A. fumigatus* and IgE available in patients' sera recognized by Immunoblitting assay (19). In this study, we demonstrated that patients aged 41-70 years were more sensitive in comparison to other age groups and their IgE sera contained 50% and 45% of *A. fumigatus* and *P. citrinum* extracts, respectively (Table 2,3).

In this study, several allergenic bands were detected in a wide range of molecular weight using immunoblotting assay. By evaluating the sera of the same patients against the three fungi extracts, we were able to compare the IgE reactivity in response to these fungi. As our results have demonstrated, the most allergenic bands were related to *A. alternata* with 17 bands (44.7%) against *A. fumigatus* and *P. citrinum* with 10 and 11 bands (26.3%, 29%), respectively.

Table 5 indicates that the most reaction occurred between sera' IgE with fungi extracts among 41-70 years old patients in each of three fungi and also decreased patients' age was association with decreased the reacted bands. Although, it seems that *A. alternata* is exclusion with 6 bands (54%) in the patients up to 20 years old, while *A. fumigatus* and *P. citrinum* extracts have only reacted to 2 and 3 bands, respectively.

These results show that some high molecular weight's bands were observed in each three age groups, and 178, 115, 83 and 67 kDa bands for *A. alternata*, 127, 91 and 74 kDa for *P. citrinum* and 71 kDa band for *A. fumigatus* were identified, respectively. Their size and frequency of identified bands were compared with other studies, some different results showed that may be related to Iran's regional isolates, geographical and environmental conditions, and also difference in the extraction methods was showed (4,12,15,18]. In conclusion, this study shows that the chance of the development of asthma is elevated with increasing age. Indeed, this finding may be due to the older people which have more experience in the exposure to fungal allergenic extracts relative to younger patients. Taken together, our results suggest that *A. alternata* had more power in sensitizing the patients in comparison with *P. citrinum* and *A. fumigatus*. In addition, the protein bands with high molecular weight can be considered as an index of sensitizing in immunobloting assay. We recommend an investigation similar to our study on children.

Conclusion

Our results showed that A. alternata had more power in sensitizing the patients in comparison with P. citrinum and A. fumigatus.

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