

Determining Allergenic Bands for *Penicillium* Species Isolated from Outdoor Air in Iran Using Patients' Sera with Asthma

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Abstract: Recent studies have indicated that fungal spores on the air contribute to creating various types of allergy and in particular respiratory allergy. *Penicillium* also is known as one of the allergenic fungi and has a great important role in appearing asthma and allergic rhinitis in human. The aim of present study was classification of different *Penicillium* species according to allergenic bands. In this study, for obtaining allergenic bands of *Penicillium* isolates were used four fungus species including *Penicillium citrinum*, *Penicillium oxalicum*, *Penicillium notatum* and *Penicillium frequentans* isolates, from outdoor air in Iran and also was used for 30 patients sera with asthma attending the Tehran Allergy Clinic. First, these fungi were grown in saboraud glucose agar and then they were cultured in Capek's agar medium to prepare fungi extracts. Isolates were transferred to saboraud glucose broth for obtaining protein and mass-produce. After cells disruption, obtaining supernatants by centrifugation were kept and used as crude antigens. In this study, immunoblotting test was used for identification of allergenic components of the mentioned fungi and anti-IgE conjugated with alkaline phosphatase and BCIP and NBT substrates were used to demonstrate reaction results. Among 34 protein bands exist in *Penicillium* extracts, 21 bands with molecular weight of 45 to 128 KD reacted with specific IgE from pooled sera of asthmatic patients. IgE binding components included: 45, 53, 55, 56.5, 59.5, 63, 65, 66.5, 76, 84, 88, 90, 92, 93, 94, 95, 97, 107, 116, 123 and 128 KD. Bands 45, 53, 56.5, 59.5, 76, 84 and 97 KD were among those which showed more frequencies. It was concluded that in all of the *Penicillium* isolates were existed allergen bands (minimum 13 bands) and in this study none of *Penicillium* species lacked allergenic band. Thus, classifying these funguses as a weak allergen fungus and non allergen is not possible.

Key words: *Penicillium* % Allergy % Immunoblotting

INTRODUCTION

Allergy to different species of fungi has been taken into consideration since many years ago and many different studies have been accomplished on identification and determination of allergenic components of the fungi. Researchers have studied the roles of them in pathology of allergic disorders, especially in atopic individuals [1-3]. Allergic symptoms are prevailing in Iran, though, there is not any precise statistics about the rate of asthma incidence here but according to published statistic, there are 3 million people are suffering from asthma in Iran.

Penicillium species are one of the allergenic fungi and have been identified to be important causative agents of asthma and allergic rhinitis. Researchers on this field have established the role of this fungus in appearance of respiratory allergy symptoms [4, 5]. Studies have accomplished about the *Penicillium* in some of the institutes around the world and some allergenic components have been characterized [6-12]. For obtaining allergenic bands for 30 *Penicillium* isolates understudy and classification of various species of *Penicillium* based on allergenic bands, in which the method of immunoblotting along with the serum of 30 patients with asthma were applied.

MATERIALS AND METHODS

Isolates: 30 proven isolated *Penicillium*, taken from the air of different areas of Iran, were investigated in this research. Table 1 shows the list of these afore fungi.

Preparing Fungus Extracts: First, isolates stored in distilled water were grown in saboraud glucose agar, after appropriate growth of fungi were cultured in Capek's agar medium at 30°C for 48-72 hours. For obtaining mass-produce, some fragments of each isolate were transferred to erlens with 600 ml of saboraud glucose broth in condition completely sterile and were preserved in rotative incubator at 25°C for 10 days. After separating fungal colonies for broth medium by using whattman filter paper number 1 and washing them with distilled water, three times. For cells disruption, first Freeze and Tow was used and then we did mechanical triturating with glass beads which cause to breaking the cells of each isolates. For this stage breaking buffer was prepared: 62.5 mol/L Tris, 1 mmol/L Dithioeritol, 0.2 mg/ml PMSF, 15% Glycerol, pH=6.8. After cells disruption, the fungi extracts were separated from mycelium and broke spores by centrifugation at 25000 rpm for 45 min at 4°C through four times. The supernatants were isolated and kept as crude antigens [13,14].

Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis: The fungi extracts were analyzed by using SDS-PAGE method with 10% separating gel. Along with the samples, standard marker (sigma) was also electrophoresis. Standard marker including: Myosine 205 Da, Beta-galactosidase 116 Da, Phosphorilase b 97Da, Fructose-6-phosphate kinase 84 Da, Bovine serum albomin 66 Da, Glutamic dehydrogenase 55 Da, Ova albumin 45 Da, Glyceraldehyde-3- phosphate dehydrogenase 36 Da, Carbonic anhydrase 29 Da, Thrypsinogen 24 Da and Thrypsin 20 Da [15, 16].

Immunoblotting Test: For immunoblotting test was used nitrocellulose paper. A transphor apparatus was also used for transferring proteins to nitrocellulose membrane. Nitrocellulose paper and gel put into the system and the system was filled with transfer buffer. Transfer buffer (25 mmol/L Tris, 192 mmol/L Glycin, 0.03% SDS and 200 ml Methanol, pH=8.3). Transferring was done with voltage 400 for an hour in a cooling system (refrigerator). The sheets were incubated in covering buffer at 4°C for overnight. Covering buffer: PBS 15% mol/L with pH=7.2 that contains 0.05% Tween 20 and BSA 1.5% weight-

Table 1: List of fungi used in immunoblotting

Number	Fungi
1	<i>P.citrinum</i> C1
2	<i>P.citrinum</i> C2
3	<i>P.citrinum</i> C3
4	<i>P.citrinum</i> C4
5	<i>P.oxalicum</i> O1
6	<i>P.oxalicum</i> O2
7	<i>P.oxalicum</i> O3
8	<i>P.oxalicum</i> O4
9	<i>P.oxalicum</i> O5
10	<i>P.oxalicum</i> O6
11	<i>P.notatum</i> N1
12	<i>P.notatum</i> N2
13	<i>P.notatum</i> N3
14	<i>P.notatum</i> N4
15	<i>P.notatum</i> N5
16	<i>P.notatum</i> N6
17	<i>P.notatum</i> N7
18	<i>P.notatum</i> N8
19	<i>P.frequentans</i> F1
20	<i>P.frequentans</i> F2
21	<i>P.frequentans</i> F3
22	<i>P.frequentans</i> F4
23	<i>P.frequentans</i> F5
24	<i>P.frequentans</i> F6
25	<i>P.frequentans</i> F7
26	<i>P.frequentans</i> F8
27	<i>P.frequentans</i> F9
28	<i>P.frequentans</i> F10
29	<i>P.frequentans</i> F11
30	<i>P.frequentans</i> F12

volume (PBS- T- BSA 1.5%). The nitrocellulose sheets were washed with washing buffer for 5 minutes. Washing buffer: first, PBS buffer 15% mol/L, pH=7.2 was prepared and afterwards 0.05% Tween 20 was added. PBS buffer 15% mol/L: 8gr NaCl, 1.2 gr KCl, 1.15 gr N₂HPO₄, 0.2 gr KH₂PO₄, then 1: 2 of serum of patients with asthma was diluted by washing buffer and added to nitrocellulose papers and were preserved at room temperature for 1 hour. After the strips had been washed 1 time with washing buffer, antihuman IgE conjugated with ALP (sigma) diluted 1:1000 in washing buffer was added to each strip at room temperature for 1 hour. After the period time, the strips were washed with alkaline phosphatase buffer for 5 min, then a degree of appearance substance including: BCIP (5 bromo- 4 chloro-3 indolyl phsphatase) and NBT (nitroblue tetrazolium) in alkaline phosphatase buffer was added and after several minutes of adjacency, brown lines, used for antibodies' reaction against antigen's band, were observed. After the appearance of the bands, substrates product was eliminated and 20 mmol/L EDTA in PBS was added to stop the reaction, then nitrocellulose sheet was removed and dried and preserved in appropriate place [17, 18].

RESULTS

Binding components of *Penicillium* extracts were different with specific IgE of asthmatic patients' sera.

Immunoblotting results using pooled serum of the patients with asthma showed that among 34 protein bands exist in *Penicillium* extract, 21 bands with molecular weight of 45 to 128 KD reacted with sera specific IgE of patients (Figure 1).

IgE Binding Components Included: 45, 53, 55, 56.5, 59.5, 63, 65, 66.5, 76, 84, 88, 90, 92, 93, 94, 95, 97, 107, 116, 123 and 128 KD.

Bands 45, 53, 56.5, 59.5, 76, 84 and 97 KD were among those which showed more frequencies and bands 55, 65, 66.5, 88, 94, 107, 116 and 123 KD with 50% frequency were seen in species (Table 2, Figure 2).

Many bands, which had more responded in terms of banding together with specific IgE, were strong and definite bands. In all of the *Penicillium* isolates were existed allergen bands (minimum 13 bands) and in this present study none of *Penicillium* species lacked allergenic band. Therefore, classifying these fungi as a weak allergen fungus and non allergen is not possible.

Table 2: Allergenic components of *Penicillium* species understudy reacting with specific IgE from pooled sera of asthmatic patients

Binding Components																						
Number of Isolates	45	53	55	56.5	59.5	63	65	66.5	76	84	88	90	92	93	94	95	97	107	116	123	128	Total
1	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	-	-	+	14
2	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	-	-	+	14
3	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	-	-	+	14
4	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	+	+	+	-	15
5	+	+	-	+	+	+	-	+	+	+	+	-	-	+	-	+	+	+	+	-	+	15
6	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	+	-	+	-	14
7	+	+	+	+	+	-	+	-	+	+	+	+	-	-	+	-	+	+	-	+	-	14
8	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+	-	+	-	+	+	-	14
9	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	-	+	+	-	+	-	15
10	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	-	+	-	14
11	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	+	-	+	-	14
12	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	+	+	-	14
13	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	+	+	-	14
14	+	+	-	+	+	+	-	+	+	+	+	-	-	+	-	+	+	+	+	-	+	15
15	+	+	-	+	+	+	-	+	+	+	+	-	-	+	-	+	+	+	+	-	+	15
16	+	+	+	+	+	-	+	-	+	+	+	+	-	-	+	-	+	+	-	+	-	14
17	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	-	+	-	14
18	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	+	-	+	-	14
19	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	-	+	-	14
20	+	+	+	+	+	-	+	-	+	+	-	-	+	-	+	-	+	+	+	-	+	14
21	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	+	+	-	14
22	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	-	+	16
23	+	+	+	+	+	-	+	-	+	+	-	-	+	-	+	-	+	+	-	+	-	13
24	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	+	+	-	+	+	-	14
25	+	+	-	+	+	-	+	+	+	+	-	-	-	+	-	+	+	-	+	-	+	13
26	+	+	+	+	+	-	+	+	+	+	-	-	+	-	+	-	+	-	+	+	-	14
27	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	15
28	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	14
29	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	14
30	+	+	-	+	+	+	+	-	+	+	+	-	-	+	-	+	+	+	-	-	+	14
Total	30	30	23	30	30	7	19	15	30	30	26	5	13	12	18	12	30	20	16	17	13	
%*	100	100	76.6	100	100	23.3	63.3	50	100	100	86.6	16.6	43.3	40	60	40	100	66.6	53.3	56.6	43.3	

*Frequency in 30 isolates of mentioned fungi

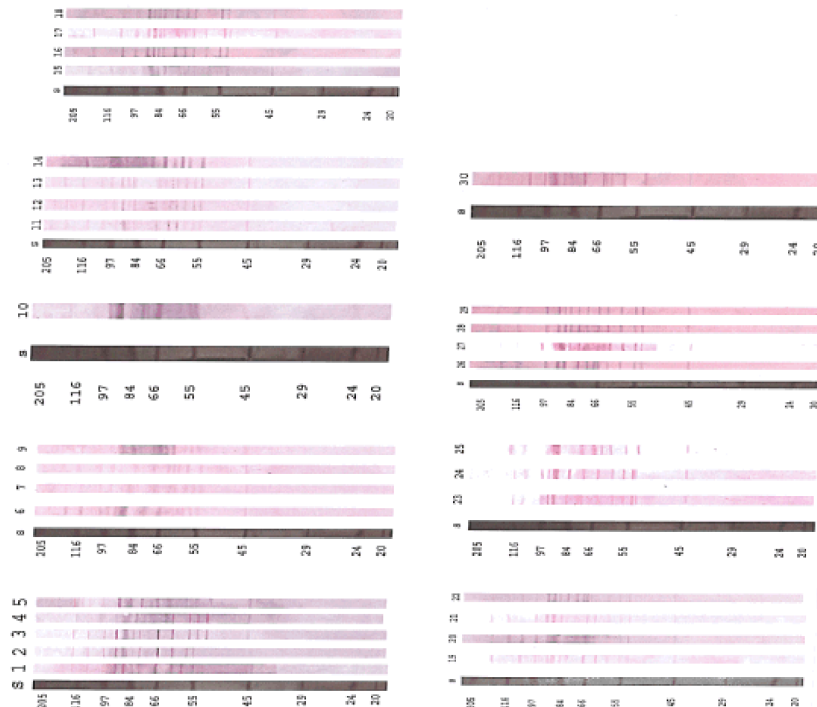


Fig. 1: Immunoblotting results pooled sera of asthmatic patients with antigens of *Penicillium* species under study

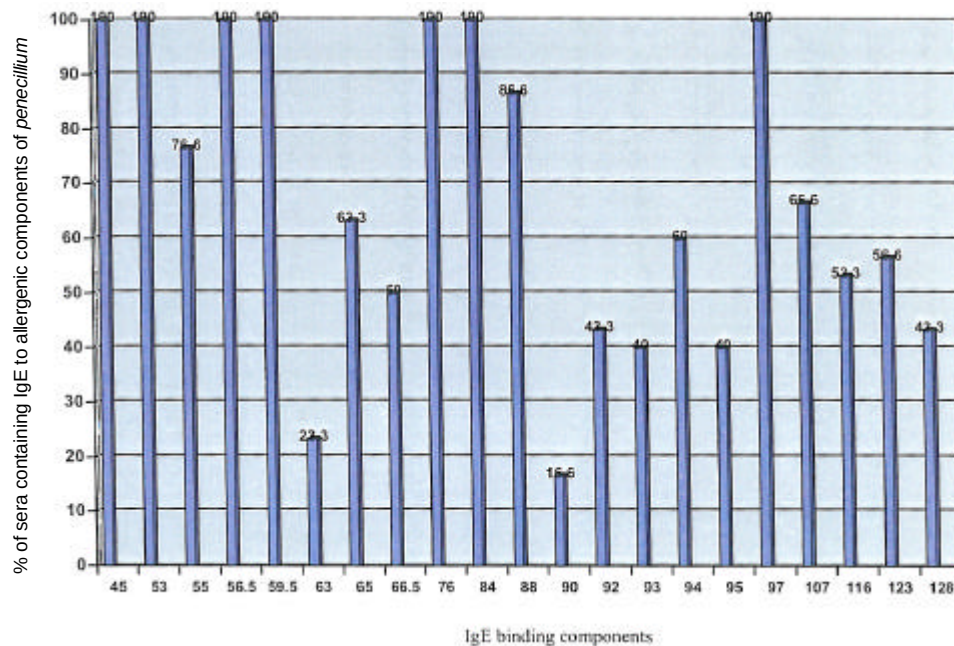


Fig. 2: Histogram of protein bands reacting with specific IgE from pooled sera of asthmatic patients in immunoblotting

DISCUSSION

Saprophytic fungi are various and scattered throughout the nature. The spores of these fungi are distributed in air and humans are in contact with them through breathing [19, 20]. Allergy to fungi has been

noticed from long times ago. Allergy is defined as the response of immune system to external proteins and people who are ready for allergy may be affected with the symptoms of responses of respiratory allergy as a result of breathing conidia of these fungi [21, 22]. According to the studies, the incidence of fungal respiratory allergy

in atopic patients has estimated between 20% to 30%, which in non-atopic people, this is about 6% [23, 24]. In addition, if fungal spores are tiny (usually smaller than 10 micrometer), the majority of them can penetrate into the air passages of the bottom of respiratory system, which cause allergy reaction [25]. Similarly, *Penicillium* has various species in nature. Its conidia is scattered in air and as a results of the smallness of conidia diameter, it often reaches the pulmonary alveolus through upper respiratory passage of human and animal which may create infection in specific situations and give rise to an allergic reaction in the form of asthma [25-27]. Accordingly, a number of different studies have been done through immunoblotting and SDS-PAGE method to identify allergenic components of these fungi. Hitherto, allergenic components of *Penicillium* have been examined. A number of studies, performed on *P. citrinum*, *P. notatum*, *P. oxalicum* and *P. brevicompactum* by Shen, *et al.* suggested that proteins 30-34 KD are major allergens which reacts with specific IgE of asthmatic patients; which was defined by using N-terminal amino acid sequence, this 33 KD allergen is an alkaline serine proteinase [6-9]. Another study performed on *P. notatum* by Shen, *et al.*, using 39 serums of asthmatic patients, identified 11 allergenic bands for these fungi, which the bands with molecular weight 64 and 68 KD with frequencies 46% and 56%, respectively, reacted with 39 studied patients with asthma [28]. In another research on 70 serums of asthmatic patients, the major allergen of *P. notatum* was 32 and 34 KD and it was 30 KD for *P. oxalicum* [29]. The significant point in our study is the presence of various allergens including 45, 53, 56.5, 59.5, 76, 84 and 97 KD. Identifying these allergens was done by using pooled serums of the patients with asthma and immunoblotting method. Although, band 32 KD existed in studied *Penicillium*, It did not react with the serums of asthmatic patients. The existence of *Penicillium* in the atmosphere of different regions of Iran has been proved and considered as the important saprophytic fungi existed, in air [30, 31]. The repetition of their contact with these fungi can be either the presence of various allergens reacting with the serum of patients with asthma or the presence of cross reactions with known and unknown agents. The same is true with *Candida albicans* and *Malassezia furfur* allergens in Iran [32]. Another important point is the conditions of the home atmosphere or that of outside. In many countries, changeable syndromes have been reported as being related to the environment. The presumption is that, the novel methods of living,

air conditioning system, wall-paper and dampness give rise to fungal allergen factors which interfere in mentioned syndromes. In Iran, in addition to above-mentioned factors, keeping moldy food stuffs, including bread and fruits, at home and lack of presence appropriate air conditioning can increase the presence of aero-fungi and these patients are involved in asthma's symptoms at home, of course, it needs more investigation that why the specific anti-allergens IgE, indentified in the serums of our patients, does not exist in the other countries.

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