

ALLERGENS FROM *FUSARIUM SOLANI* IDENTIFIED BY IMMUNOBLOTTING IN ASTHMA PATIENTS IN IRAN

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We extracted *Fusarium solani* antigens to evaluate specific anti-*F. solani* IgE in fifty-one patients with asthma (33 men and 18 women) and in 22 non-atopic healthy subjects (15 men and 7 women). *F. solani* strains were cultured in Sabouraud glucose agar and subjected to cell disruption using the freeze-and-thaw method. The obtained cytoplasmic extracts were analysed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Sensitisation to *F. solani* antigens has been evaluated in asthmatic patients using the immunoblotting assay. The SDS-PAGE identified 29 protein bands in the cytoplasmic extracts of *F. solani* isolates, with molecular weights ranging from 24 kDa to 112 kDa. Immunoblotting detected specific anti-*F. solani* IgE antibody in all asthma patients, but not in the control group. The predominant reactive allergens in patients corresponded to the bands with molecular weights of 24 kDa, 58.5 kDa, 64.5 kDa, 69 kDa, 72 kDa, and 97 kDa. Our results suggest that various allergenic components of *F. solani* may produce symptoms of asthma in susceptible individuals and they call for further research.

KEY WORDS: *airborne spores, allergenic sensitisation, fungal allergy, IgE, SDS-PAGE*

Today, more than one fifth of the world population faces IgE-mediated allergic diseases such as asthma, rhino-conjunctivitis, and eczema (1). Asthma is a complex illness resulting from an interplay of several allergenic and non-allergenic factors, and is characterised by airway obstruction and inflammation (2).

Sensitisation to allergens has an important role in the development of asthma (3). Fungal spores and mycelia are well known respiratory allergens. It is estimated that 3 % to 40 % of allergic patients worldwide suffer from fungal allergy (4). By now, the role of fungi in the development of asthma has not been fully investigated. *Alternaria alternata*, *Aspergillus fumigatus*, and *Cladosporium herbarum* have been reported as the most common causes of fungal allergies, and many allergens of these fungi

have been described at the molecular level (5, 6). In addition, airborne spores of the *Fusarium* species are also widely dispersed and common in many environments (7, 8). These saprophytic or parasitic fungi often contaminate crops, fruit, and vegetables. They also frequently affect the eyes, skin and nails, and cause systemic infections in immunocompromised hosts (9, 10). Among the *Fusarium* species, *F. solani* plays the most important role in IgE-mediated allergic reaction in patients with nasobronchial allergy (11). O'Neil et al. (12) found that about 24 % of atopic individuals had the skin test and radioallergosorbent test positive to *F. solani* extracts.

The culture filtrate, mycelium, and spore extracts of *F. solani* contain various allergens, either species-specific such as the 65-kDa glycoprotein (13) or shared with related species or genera.

The diagnosis of allergy is usually based on the medical history of the patient, skin prick tests, and *in vitro* tests for allergen-specific IgE in the patient serum. However, most commercial fungal allergen extracts used for *in vivo* and *in vitro* diagnostics are neither pure enough nor standardised. Our study is an attempt to investigate potential allergenic proteins from *F. solani* that could improve screening for asthmatic patients by widening the array of known fungal allergens.

MATERIALS AND METHODS

Chemicals and reagents

All general chemicals and reagents used in the study were purchased from Merck Co. (Darmstadt, Germany), unless specified otherwise.

Strain and cultivation

A total of 12 *F. solani* strains were isolated from the air and foodstuffs from Iran. First the samples were cultured in Sabouraud glucose agar supplemented with 0.5 % chloramphenicol (Sigma Chemical Co., St. Louis, Mo., USA) at 30 °C for 5 days. All *Fusarium* sp. isolates were further subcultured on potato dextrose agar (PDA), Spezieller Nährstoffarmer agar (SNA), and carnation leaf agar (CLA). PDA cultures were incubated at 30 °C for 5 to 7 days, while CLA and SNA cultures were incubated at 25 °C for 2 to 4 weeks.

The cultures were evaluated both by eye and microscopically. For morphological identification of the isolates we followed the criteria of Leslie and Summerell (14). Colony morphology was observed from cultures cultivated on PDA. To assess the morphology of macroconidia, microconidia, conidigenous cells, and the chlamydospores we used the cultures incubated on SNA and CLA.

Cell fractionation and crude extract preparation

The fungal colonies were cultured in 500 mL of Sabouraud glucose broth and agitated in the shaker at 30 °C for 10 days. At the end of incubation, the fungal mats of each strain containing spores and mycelia were harvested using the filtration technique (Whatmann filter paper; no. 0.45 µm) and washed three times with the PBS buffer (0.15 mol L⁻¹, pH 7.2). A suspension of wet fungal colonies was prepared in a lysing buffer

(62.5 mmol L⁻¹ Tris, 1 mmol L⁻¹ dithiothreitol, 0.2 mg mL⁻¹ of phenyl sulfonyl fluoride and 15 % glycerol, pH 6.8). Cells were disrupted by freezing them to -70 °C for 30 min and thawing them over 20-minute intervals, repeating the procedure six times in a row (the so called freeze-and-thaw method). The samples were finally ground by a sterilised mortar for 20 min. Crude antigens were separated from other cell components by centrifugation at 20000g for 20 min, and subsequently centrifuged at 25000g for 30 min. Clear supernatants were obtained and stored at -20 °C until used.

Protein determination

Protein concentration of fungal extracts was measured after reconstituting the extracts in 50 µL and 100 µL of distilled water using the Bradford method (15).

SDS-PAGE analysis

To separate the fungal extracts we used a 17.5 % separation gel with a 5 % stacking gel in a discontinuous buffer system as proposed by Laemmli (16). The extracts were boiled in a reducing sample buffer with 2-β-mercaptoethanol for 5 min. Aliquots of the sample (V=10 µL, which equals to 60 µg of proteins) were loaded on a 0.5 cm gel. Molecular weight standard markers of 14.4 kDa to 93 kDa (Rainbow markers, Amersham International PLC, UK) were also loaded on the gel. The gels were stained with silver nitrate and commassie brilliant blue R-250 (Sigma, St. Louis, USA).

Serum collection

Sera used in this study were pooled from 51 adult patients (33 men and 18 women aged 20 to 60 years) whose asthma had been confirmed by a physician. The majority (62.7 %) were younger than 40 years. They were recruited in the Tehran Allergy Clinic, Iran. The sera were also taken for testing from an age and sex matching control population of 22 healthy subjects (15 men and 7 women) with no personal or family history of atopy. All subjects gave informed consent to participate in the study.

All underwent a detailed clinical examination with special attention to the respiratory system. All subjects also completed a questionnaire with personal data and information related to possible confounding factors such as smoking, drugs used, history of diseases, respiratory and non-respiratory allergic symptoms,

history of oral corticosteroid use and other therapies. All patients had a history of bronchitis, difficulties breathing, and limited lung capacity. None took any kind of medications. Only five patients smoked.

Blood was collected by venipuncture in BD vacutainers (Becton Dickinson, UK) and allowed to clot to facilitate serum collection. Sera collected from each subject were pooled into one sample for each study group, frozen, and stored until use.

Serum IgE against *F. solani* antigens was determined using the immunoblotting assay.

IgE immunoblotting

The isolated *Fusarium* sp. proteins were transferred to nitrocellulose sheets (Schleicher and Shuell, Dassel, Germany) with a transphor apparatus (Hoefer, San Francisco, USA) at 100 V for 2.5 h using a transfer buffer (25 mmol L⁻¹ Tris, 192 mmol L⁻¹ glycine, 0.03 % SDS, and 20 % methanol, pH 8.3). The nitrocellulose sheets were cut into 0.5 cm strips and incubated overnight at 4 °C with the TBS-Tween 20-BSA buffer (20 mmol L⁻¹ Tris, 0.15 mol L⁻¹ NaCl, 0.05 % Tween 20, and 1.5 % BSA, pH 7.5). The strips were washed three times with the TBS-Tween 20 buffer at room temperature for 15 min.

Pooled serum was diluted to 1:2 in TBS-Tween 20-BSA and incubated at room temperature for 16 h. After the strips had been washed three times with TBS-Tween 20-BSA, each was added anti-human IgE conjugated with alkaline phosphatase (Sigma, St. Louis, USA) diluted to 1:1000 in TBS-Tween 20-BSA. The strips were then incubated for 5 h and then washed in the alkaline phosphatase buffer (100 mmol L⁻¹ Tris-HCl, 100 mmol L⁻¹ NaCl, and 5 mmol L⁻¹ magnesium chloride) for 10 min. The immunoblot with at least one visible protein band was considered positive (17).

RESULTS

The SDS-PAGE electrophoregram obtained from cytoplasmic extracts of *F. solani* isolates has shown 29 protein bands with molecular weights ranging from 24 kDa to 112 kDa (Table 1 and Figure 1). The gel showed a moderate degree of similarity between the protein banding patterns of various fungal isolates. Overall, the isolates differed in 27 bands whereas two bands were common in all isolates. The most common protein bands were 24 kDa and 32 kDa, followed by 72 kDa and 50 kDa, whereas bands with molecular

weights of 108 kDa, 87 kDa and 40 kDa were the least common bands within the *F. solani* isolates.

As isolates 1, 4, 8, and 12 had the highest number of protein bands, they were selected for immunoblotting to detect the presence of IgE against *F. solani* antigens. Immunoblotting of the pooled sera of the asthmatic patients showed a strong reaction to fungal extracts. At the same time, no positive result was observed in controls. IgE responded to 29 different bands in asthmatic patients (Figure 2). The strongest response was to the following six allergens: 24 kDa, 58.5 kDa, 64.5 kDa, 69 kDa, 72 kDa, and 97 kDa.

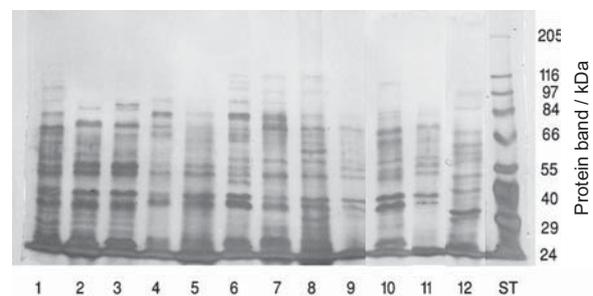


Figure 1 Protein components of crude extracts of *Fusarium solani* isolated with SDS-PAGE.

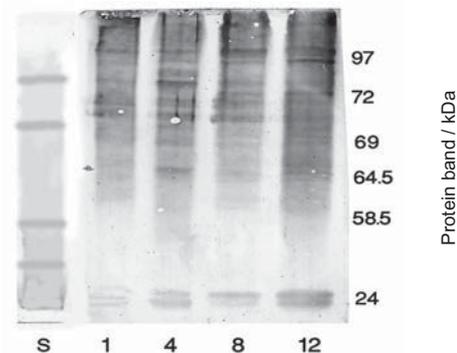


Figure 2 IgE binding to *Fusarium solani* allergen extracts in the immunoblotting test with pooled serum of 51 patients with asthma.

DISCUSSION

Members of the genus *Fusarium* are common indoor and outdoor airborne fungi, confirmed to cause bronchial asthma (18). Earlier studies (19, 20) suggest that sensitivity to fungi is common, especially in patients with asthma.

Polyacrylamide gel electrophoresis is frequently used to isolate and identify proteins from different fungal species (21). The same method was successfully employed in the present study. The most important finding is that *F. solani* isolates showed several similar

Table 1 Bands identified with the SDS-PAGE in each of the 12 isolates of *Fusarium solani*.

Isolate No.	Protein bands / kDa	Total number of bands
1	24, 26, 27, 32, 38, 45, 55, 58.5, 64, 69, 72, 81, 94, 100	14
2	24, 26, 28.5, 32, 37, 50, 55, 60, 63, 72, 81	11
3	24, 26, 28.5, 32, 37, 50, 53, 60, 66, 72, 87	11
4	24, 32, 35, 40, 50, 53, 58.5, 66, 72, 81, 94, 100, 112	13
5	24, 32, 35, 50, 53, 64, 72, 81	8
6	24, 32, 35, 61, 64, 66, 72, 81	8
7	24, 28.5, 32, 35, 50, 58.5, 75, 81, 97	9
8	24, 28.5, 32, 35, 50, 58.5, 61, 64, 69, 72, 81	13
9	24, 32, 35, 50, 53, 75	6
10	24, 27, 32, 38, 45, 55, 63, 66, 81, 108	10
11	24, 28, 32, 38, 45, 55, 63, 66	8
12	24, 27, 32, 38, 55, 60, 64, 69, 81, 97	10

protein profiles in cytoplasmic extracts within the range of 24 kDa to 112 kDa. In a previous study by Aghamirian and Zaini (22), somatic proteins UAMH 3317 and UAMH 7419 extracted from *F. solani* produced 16 and 21 protein bands, respectively, with molecular weights of 14 kDa to 100 kDa. In a cytoplasmic extract of *F. solani* isolated from the soil, Bahrain Mandeel et al. (23) identified 18 protein bands ranging from 21 kDa to 100 kDa. In addition, using the SDS-PAGE method, Verma and Gangal (24) demonstrated 18 cytoplasmic protein bands from *F. solani* in India, with the 65-kD protein component as a major allergen. It is difficult to identify a protein in a complex antigen extract on the basis of its molecular weight, and sometimes the same antigen differs in molecular weight between studies. This discrepancy may be related to differences in the calculation of molecular weight, differences in medium composition and incubation temperature or to the presence of several antigens with the same molecular weight (25). Investigation should be continued to further specify the bands.

For clinical diagnosis it is essential to identify the allergenic profiles of the *Fusarium* species, *F. solani* in particular, and see if there are any IgE-cross reactivities between major allergens of this widespread fungus. Immunoblotting is a readily available and simple screening test for IgE binding to major *F. solani* allergens. In this study, we identified six major allergens of *F. solani* with molecular weights of 24 kDa, 58.5 kDa, 64.5 kDa, 69 kDa, 72 kDa, and 97 kDa. In two studies conducted by Verma et al. (11, 26), a 65-kDa allergen of *F. solani* bound IgE from all the sera of asthmatic patients. Verma et al. (27) also

demonstrated that peptide named IV-1, a 3.4-kDa fragment of the 65-kDa allergen, was highly allergenic and bound to IgE in the sera of most *F. solani*-positive patients. In another study (28), the same researchers found that a 45-kDa allergen of *F. solani* reacted with patients' sera sensitive to many fungi.

In general, IgE response seems to vary from country to country. Our results disagree with the findings of O'Neil et al. (12) and Hoff et al. (29), who established IgE response to extracts of *Fusarium* species in 24 % and 50 % of atopic patients from the USA and Germany, respectively. The reasons for these differences are not clear, but point to differences in exposure between these countries that may involve climate/geography, *Fusarium* strains, heating, ventilation, and air-conditioning. In addition, the study procedures may have varied, including the allergen extraction methods, patient recruitment criteria, and assays used. Furthermore, immune responses to the allergens may vary between different populations and even races (30), as allergies result from an interplay of various environmental and genetic factors. Nevertheless, this discrepancy between our and the reported studies needs further clarification.

In conclusion, we identified six allergenic bands of *F. solani* (corresponded to 24 kDa, 58.5 kDa, 64.5 kDa, 69 kDa, 72 kDa and 97 kDa proteins), which showed the highest IgE-binding frequency in asthmatic patients. These results suggest that different allergenic components of *F. solani* may produce symptoms of asthma in susceptible individuals and call for further investigation.

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Sažetak

ALERGENI IZ PLIJESNI *FUSARIUM SOLANI* U IRANSKIH BOLESNIKA S ASTMOM UTVRĐENI S POMOĆU IMUNOBLOT-TESTA

Ekstrahirali smo antigene plijesni *Fusarium solani* kako bismo izmjerili imunosni odgovor, tj. razine anti-*F. solani* IgE u 52 bolesnika s astmom (33 muškarca i 18 žena) te u 22 zdrava ispitanika bez atopija (15 muškaraca i sedam žena). Sojevi *F. solani* uzgojeni su na podlozi glukoznoga Sabouraudova agara i podvrgnuti razbijanju stanica s pomoću metode smrzavanja i odmrzavanja. Dobiveni citoplazmatski ekstrakti analizirani su s pomoću elektroforeze u poliakrilamidnom gelu u prisutnosti natrijeva dodecilsulfata (engl. *sodium dodecyl sulphate-polyacrylamide gel electrophoresis*, krat. SDS-PAGE). Senzibilizacija na antigene *F. solani* u bolesnika s astmom utvrđena je s pomoću imunoblot-testa. S pomoću SDS-PAGE-a u citoplazmatskim ekstraktima izolata *F. solani* dokazano je 29 proteinskih vrpca. Molekulske mase razdvojenih proteina kretale su se u rasponu od 24 kDa do 112 kDa. Imunoblot-test otkrio je specifično anti-*F. solani* IgE- protutijelo u svih bolesnika s astmom, ali niti u jednog ispitanika iz kontrolne skupine. Najčešći su alergeni u bolesnika s astmom odgovarali proteinima ovih molekulskih masa: 24 kDa, 58,5 kDa, 64,5 kDa, 69 kDa, 72 kDa i 97 kDa.

Naši nalazi upućuju na to da simptome astme u osjetljivih pojedinaca može izazvati više alergena soja *F. solani* te da ih treba podrobnije istražiti.

KLJUČNE RIJEČI: *alergija na plijesni, IgE, SDS-PAGE, senzibilizacija na alergene, spore u zraku*

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