

Studies on shared antigenic/allergenic components among fungi

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Fungal allergens have been found to be one of the most prevalent aeroallergens in India. Knowledge of shared/unique components among different fungi is necessary for proper diagnosis and treatment of patients allergic to fungi. In the present study, crude extracts (CE) of 11 common fungi (*Alternaria alternata*, *Aspergillus flavus*, *Asp. fumigatus*, *Asp. niger*, *Asp. tamarii*, *Asp. versicolor*, *Cladosporium herbarum*, *Curvularia lunata*, *Mucor hiemalis*, *Penicillium citrinum*, and *Fusarium solani*) were characterized by isoelectric focusing (IEF), SDS–PAGE, and immunoblot. On IEF (pI 3–9), the number of protein bands was found to be greatest (46) in *M. hiemalis* extract. SDS–PAGE exhibited a varied number of bands, generally 18–40, with mol. mass ranging from 14 to 100 kDa. IgG-specific immunoprint using rabbit anti-*F. solani* CF antibodies demonstrated a mol. mass distribution of shared antigenic proteins of 14–100 kDa in most of the fungi. Shared allergenicity was observed in a number of allergenic proteins in fungal extracts with mol. mass ranging between 14 and 70 kDa on IgE-specific immunoblot using pooled sera of patients allergic to *Fusarium*. A 45-kDa protein was found to be common among these fungi on immunoblot with patients as well as with rabbit antibodies. *F. solani* CF extract contained more antigenic/allergenic proteins than *F. solani* CE. It was concluded that *F. solani* CF shared several antigenic/allergenic components with CE of other common fungi. This fact needs to be taken into account when fungal extracts are used in diagnosis and immunotherapy of allergic patients.

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Fungi have been shown to be involved in various allergic manifestations (6, 15). *Fusarium*, *Aspergillus*, *Alternaria*, *Cladosporium*, and *Penicillium* spp. have been reported to be important allergenic fungi throughout the world as well as India (1–5, 12, 18, 19). It has been shown that culture filtrate, mycelium, and spores contain a number of allergenic proteins. They may be unique to one species or may show shared allergenicity with related species or genera (23–26).

Some authors (9, 10, 25) have demonstrated cross-reactivity among different species of the same genus. Verma & Gangal (22) have shown significant cross-reactivity among *Fusarium* species (*F. solani*, *F. equiseti*, and *F. moniliforme*). Vijay et al. (24) reported both shared and unique antigenic/allergenic components among *Alternaria tenuis* and *A. solani*. Earlier studies did not find significant cross-reactivity among *Alternaria*, *Cladosporium*, and *Aspergillus* species (5, 10) while Agarwal et al. (3) demonstrated that

Alternaria and *Stemphylium* extracts contain multiple shared antigenic determinants with rabbit antisera. Hoffman et al. (8) demonstrated extensive cross-reactivity among *Alternaria*, *Drechslera*, *Spondylocodium*, *Stemphylium*, and *Curvularia*. Similarly, *Stachybotrys* appeared to cross-react with the genera *Alternaria*, *Stemphylium*, and *Curvularia*. Although many reports have indicated intrageneric and intergeneric cross-reactivity among fungi, there is no detailed study of cross-reactivity among various genera.

Among the Fungi Imperfecti, *Fusarium* has been found to be one of the most prevalent fungi in India. Its incidence in allergy was reported by intradermal skin testing in 3–3.8% of patients suffering from nasobronchial allergy (17–19, 22, 23). Frankland (7) also demonstrated the allergenicity of *Fusarium* mycoprotein in those patients who were already sensitive to mold spores. *F. solani* has been used in combination with other mold extracts for immuno-

therapy. In our earlier studies (23), culture filtrate (CF) extract of *Fusarium solani* was found to be an excellent source of allergens, as most of the allergens of mycelial and spore extracts were present in *F. solani* CF.

In the present study, crude extracts (CE) of 11 common fungi used for diagnostic purposes were characterized by isoelectric focusing (IEF), SDS-PAGE, and immunoblot. To determine the shared allergens and antigens in fungi, we prepared a standardized extract of *F. solani* CF against which the cross-reactivity of other fungi was tested.

Material and methods

Preparation of CF extract of *F. solani*

F. solani (3596) strain obtained from the Indian Agricultural Research Institute, Delhi, India, was used for the culture. It was grown at 30°C for 5, 7, 9, 11, 13, 16, 21, and 26 days in synthetic medium (11) under constant agitation (125 rpm) to prevent sporulation. The maximum protein and dry weight of mycelium were observed in 11-day-old culture. On IEF (pI 3–9), the number of protein components in CF was also found to be greatest at the same age of culture (23). After that period, a decrease in the number of protein components, dry weight of mycelium, and protein content was observed. Hence, 11-day-old culture was selected for the preparation of CF extract of *F. solani*. The CF supernatant obtained after centrifugation was brought to 90% saturation with ammonium sulfate. After centrifugation, the pellet was dissolved in a minimum quantity of water, dialyzed against water (Spectrapor membrane, mol. wt. cutoff 3500), and lyophilized.

Preparation of crude extracts (CE) of other fungi

Powder containing mixed mycelium and spores of each of the fungi (*Mucor hiemalis*, *Alternaria alternata*, *Curvularia lunata*, *Cladosporium herbarum*, *Penicillium citrinum*, *Aspergillus fumigatus*, *Asp. flavus*, *Asp. tamarii*, *Asp. versicolor*, *Asp. niger*, and *F. solani*) obtained from the Antigen Production Division, Centre for Biochemical Technology, Delhi, India, was dissolved in carbonate buffer (1:20 w/v) containing 50 mM ammonium bicarbonate, 0.15 M sodium chloride, 5 mM ethylenediaminetetraacetate, and 1 mM phenyl methyl sulfonyl fluoride. They were sonicated separately for a total time of 15 min (predetermined) and left overnight at 4°C to extract the proteins. After centrifugation, supernatants were brought to 90% saturation with ammonium sulfate. The precipitate was dissolved in a minimum quantity of water, dialyzed against water, and lyophilized.

Isoelectric focusing (IEF)

IEF was carried out on ready-made gel of pI range (3–9) (Pharmacia). Using the Phast system, 3 µg proteins of each fungal extract was resolved in separate lanes on the gel according to the program given in the instruction manual. The separated protein components were fixed in 20% tricarboxylic acid and stained with a silver-staining kit (Pharmacia).

Immunologic analysis

Fifteen patients' sera were collected on the basis of clinical history and 2+ to 4+ skin test reactions to *Fusarium* antigen. All 15 patients demonstrated the presence of *F. solani* CF-specific IgE as tested by RAST (Pharmacia Diagnostics). Normal human serum showing negative skin test and RAST for *F. solani* was included in the study as a negative control. Sera of nine patients sensitive to *Fusarium* extract only (skin reaction 2+ to 3+), and not to other fungal extracts (in study), were pooled and used for immunochemical studies. They also exhibited class 2 antibody response and percent RAST binding (7–11.9) to *F. solani* CF extract.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

SDS-PAGE of CF extract of *F. solani* and CE extracts of other fungi were performed on 10% gel. Amounts of 250 µg protein of each extract were loaded in separate lanes. Resolved proteins of all fungal extracts were transferred electrophoretically onto a nitrocellulose membrane (21) using dry-blot apparatus (Pharmacia). The membrane was air-dried after blotting, and free sites were blocked by incubation in blocking buffer (PBS with 1% BSA) for 1 h at 37°C. The membrane was washed with buffer (PBS-Tween) and incubated overnight at 4°C with (1:10 diluted) patient serum pool. The membrane was washed with rinse buffer and incubated with horseradish peroxidase-conjugated antihuman IgE (1:1000 v/v) for 4 h at 37°C. The membrane was then washed three times with rinse buffer, and color was developed with diaminobenzidine and hydrogen peroxide, dissolved in sodium acetate buffer (pH 5.0). The reaction was stopped by washing the membrane with distilled water.

Results

Intradermal skin test results of patients and their sensitivity to different allergen sources are given in Table 1. Most of the patients suffering from naso-bronchial allergy were in the age group 25–35 years

Table 1. Skin test data of patients showing sensitivity to various fungal allergens

Patient no.	Age (years)	Skin test positivity
1.*	35	Fus (++), Epi (++), Can (+)
2.	18	Fus (+++), Rhiz (++), Clad (+)
3.	30	Fus (+++), Cur (+), Muc (++)
4.	24	Fus (+++), A. fum (++)
5.*	28	Fus (+++), Cur (+)
6.*	32	Fus (+++), Helmin (+)
7.	30	Fus (+++), Clad (++)
8.*	26	Fus (+++), Tricho (++), Rhiz (+)
9.*	29	Fus (+++), Epi (+)
10.*	27	Fus (+++)
11.*	23	Fus (+++)
12.*	31	Fus (+++), Rhiz (+), Can (++)
13.	30	Fus (+++)
14.*	30	Fus (+++), Can (++)
15.	28	Fus (+++), A. fum (+), Can (++)

Symbols: Fus – *Fusarium*, Epi – *Epicoecum*, Can – *Candida*, Rhiz – *Rhizopus*, Clad – *Cladosporium*, Cur – *Curvularia*, Muc – *Mucor*, A. fum – *Asp. fumigatus*, Helmin – *Helminthosporium*, Tricho – *Trichoderma*.

* Sera used for immunologic studies.

and exhibited 2+ to 3+ skin reactions to *Fusarium* extract. On RAST analysis using *F. solani* CF coupled CNBr activated paper disks, class 2 antibody response was observed. The sera of nine patients showing class 2 antibody response in RAST and skin test positivity to *Fusarium* extract (but not to other fungal extracts in the study) were pooled for immunologic investigations.

Isoelectric focusing

IEF pI (3–9) patterns of all fungal extracts are shown in Fig. 1. The number of protein components was greater in *Mucor hiemalis* extract than in other fungal extracts.

SDS-PAGE and immunoblots

All fungal extracts were resolved in the range 14–100 kDa on SDS-PAGE, as shown in Fig. 2. The immunoprint analysis showed shared antigenic components between *F. solani* CF and other fungal extracts with rabbit anti-*F. solani* CF antibodies (Fig. 3). The crude extracts of *Asp. fumigatus*, *Asp. flavus*, *Asp. niger*, *Asp. versicolor*, *Asp. tamarii*, *M. hiemalis*, *Cladosporium herbarum*, *Curvularia lunata*, *Penicillium citrinum*, *Alternaria alternata*, *F. solani*, and *F. solani* CF revealed 6–17 antigenic proteins with corresponding rabbit antiserum, while with *F. solani* CF rabbit antibodies shared antigenicity could be demonstrated with a number of proteins of the other fungal extracts (Table 2). A 45-kDa antigenic protein was found to be common among all these fungal extracts except in that of *Asp. tamarii*.

IgE-binding protein components of different fungal extracts on immunoblot using a correspondingly sensitive patients' serum pool were studied (Fig. 4). *Asp. flavus*, *Asp. niger*, *Asp. versicolor*, *Asp. tamarii*, *M. hiemalis*, *P. citrinum*, *A. alternata*, *F. solani* CF, and *F. solani* CE showed 7–18 IgE-binding proteins.

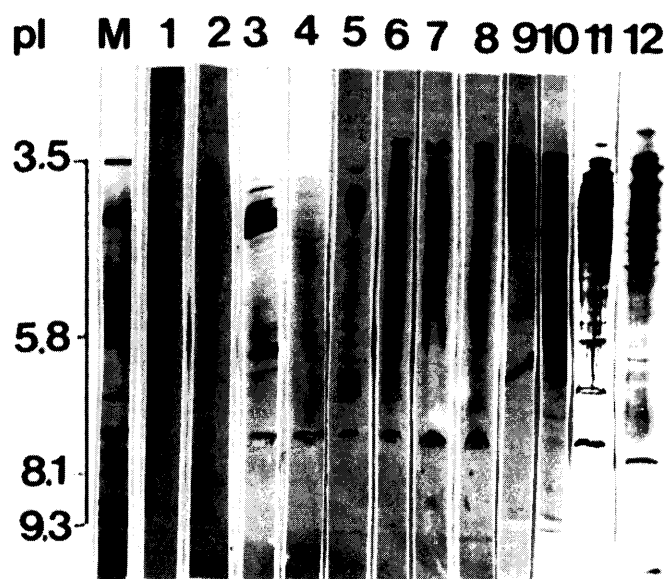


Fig. 1. IEF (pI 3–9) patterns exhibiting protein components in various fungal extracts. 1) *A. alternata*, 2) *P. citrinum*, 3) *C. lunata*, 4) *C. herbarum*, 5) *M. hiemalis*, 6) *Asp. fumigatus*, 7) *Asp. flavus*, 8) *Asp. tamarii*, 9) *Asp. versicolor*, 10) *Asp. tamarii*, 11) *F. solani* CE, 12) *F. solani* CF.

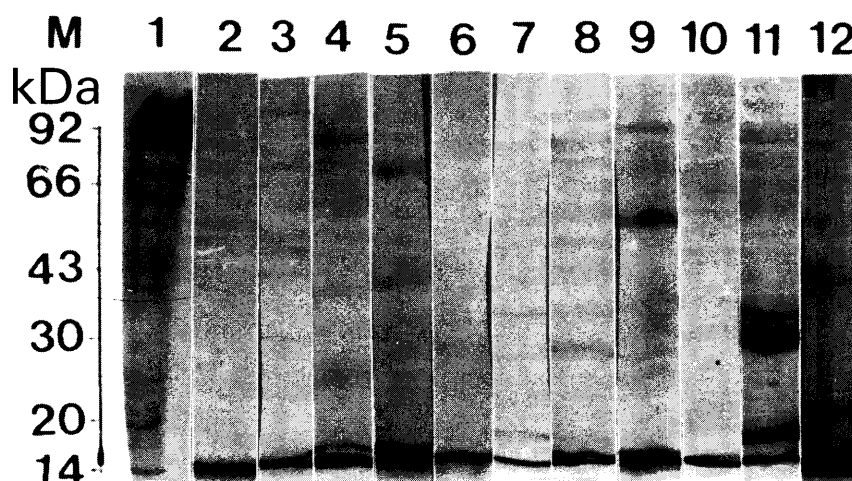


Fig. 2. SDS-PAGE pattern showing protein components in various fungal extracts. 1) *F. solani* CF, 2) *A. alternata*, 3) *P. citrinum*, 4) *C. lunata*, 5) *C. herbarum*, 6) *M. hiemalis*, 7) *Asp. fumigatus*, 8) *Asp. flavus*, 9) *Asp. tamarii*, 10) *Asp. versicolor*, 11) *Asp. niger*, 12) *F. solani* CE.

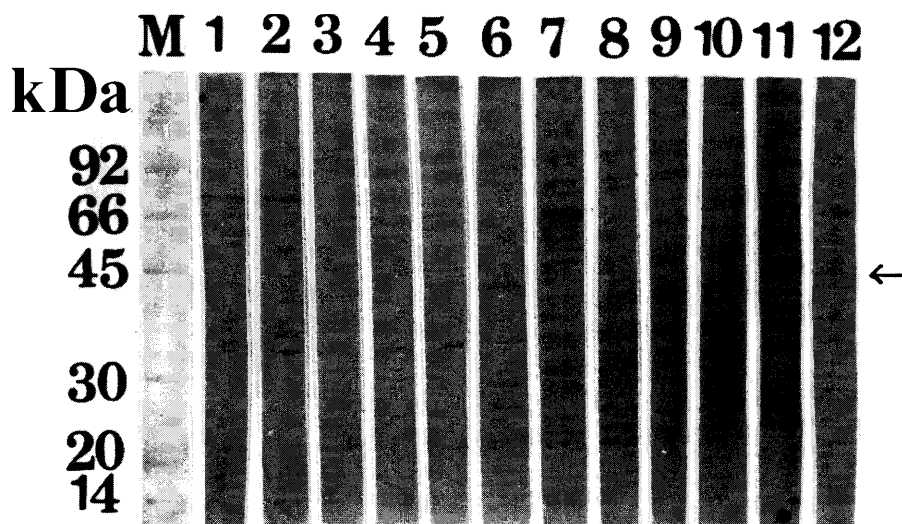


Fig. 3. IgG-binding components in various fungi exhibiting shared antigenicity with *F. solani* CF on immunoblot with rabbit anti-*F. solani* CF antibodies. 1) *Asp. tamarii*, 2) *F. solani* CE, 3) *Asp. niger*, 4) *Asp. versicolor*, 5) *Asp. flavus*, 6) *Asp. fumigatus*, 7) *M. hiemalis*, 8) *C. herbarum*, 9) *C. lunata*, 10) *P. citrinum*, 11) *A. alternata*, 12) *F. solani* CF.

Table 2. Protein components present in different fungal extracts showing shared antigenicity with *F. solani* (culture filtrate) using rabbit anti-*F. solani* (culture filtrate) antibodies on immunoblot

Fungal species	No. of bands	Mol. mass (kDa)
<i>Asp. fumigatus</i>	7	70, 65, 55, <u>45</u> *, 35, 25, 20
<i>Asp. flavus</i>	3	65, <u>45</u> , 35
<i>Asp. niger</i>	5	55, <u>45</u> , 35, 25, 20
<i>Asp. versicolor</i>	4	65, 55, 50, <u>45</u>
<i>Asp. tamarii</i>	3	70, 65, 35
<i>M. hiemalis</i>	9	70, 55, 50, <u>45</u> , 38, 35, 30, 24, 20
<i>C. herbarum</i>	6	70, 65, <u>45</u> , <u>38</u> , 30, 24
<i>P. citrinum</i>	7	50, <u>45</u> , <u>38</u> , 35, 30, 24, 20
<i>A. alternata</i>	4	55, 50, <u>45</u> , 38
<i>F. solani</i> (crude extract)	9	70, 55, <u>45</u> , 41, 38, 35, 19, 17, 14

* Common antigenic protein among fungi indicated by underlined numbers.

However, using an *F. solani*-sensitive patient serum pool, 5–10 allergenic proteins of *Asp. flavus*, *Asp. niger*, *Asp. versicolor*, *Asp. tamarii*, *M. hiemalis*, *P. citrinum*, *A. alternata*, and *F. solani* CE demonstrated shared allergenicity with *F. solani* CF extract (Table 3). A 45-kDa allergenic protein was present in most of the fungal extracts studied.

Discussion

A number of fungal extracts have been used for the diagnosis and immunotherapy of allergic disorders. Many patients show multiple sensitivity to different fungal extracts by skin testing (Table 1). This could

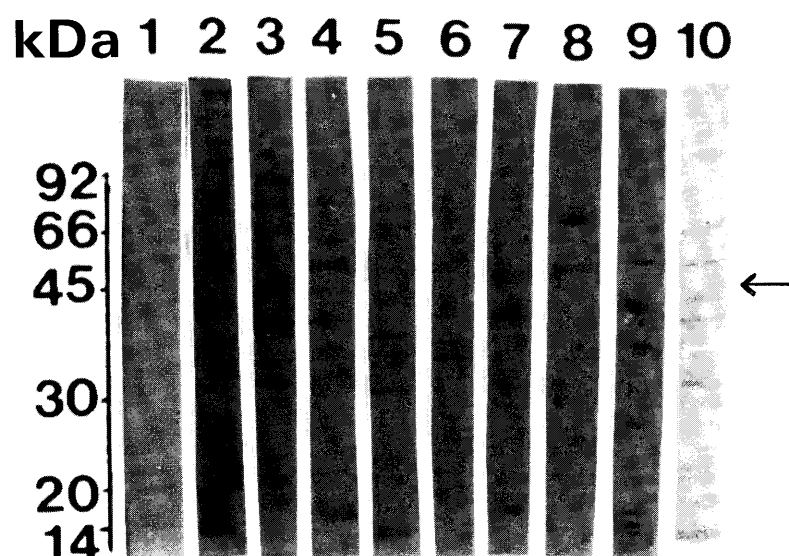


Fig. 4. Identification of IgE-binding components in various fungal extracts exhibiting shared allergenicity with *F. solani* CF using immunoblot analysis. 1) Mol. mass markers, 2) *Asp. flavus*, 3) *Asp. niger*, 4) *Asp. versicolor*, 5) *Asp. tamaritii*, 6) *M. hiemalis*, 7) *P. citrinum*, 8) *A. alternata*, 9) *F. solani* CE, 10) *F. solani* CF.

Table 3. Protein components present in different fungal extracts showing shared allergenicity with *F. solani* (culture filtrate) on immunoblot using pooled serum of patients allergic to *Fusarium*

Fungal species	No. of bands	Mol. mass (kDa)
<i>Asp. flavus</i>	5	65, <u>45</u> *, 35, 18, 14
<i>Asp. niger</i>	5	<u>45</u> , 38, 20, 16, 14
<i>Asp. versicolor</i>	2	<u>30</u> , 16
<i>Asp. tamaritii</i>	10	70, 68, 65, <u>45</u> , 38, 35, 30, 28, 20, 16
<i>M. hiemalis</i>	9	68, 65, <u>45</u> , 41, 38, 35, 30, 28
<i>P. citrinum</i>	7	70, 68, <u>45</u> , 38, 35, 30, 18
<i>A. alternata</i>	5	70, 65, <u>45</u> , 30, 28
<i>F. solani</i> (crude extract)	10	70, 65, <u>55</u> , 50, <u>45</u> , 35, 30, 28, 25, 20, 14

* Common allergenic protein among fungi indicated by underlined numbers.

be due to the unique or shared allergenic components in these extracts.

Some authors (7, 14, 16) have reported cross-reactivity among various genera of molds by ELISA, RAST inhibition, and skin prick tests, while other workers (9, 22, 24) used immunodiffusion, crossed immunoelectrophoresis, and fused rocket immunoelectrophoresis to study the cross-reactivity among some species of fungi.

Aerobiologic studies conducted in India (17–19) have shown that *Alternaria*, *Penicillium*, *Mucor*, *Cladosporium*, and *Aspergillus* are present ubiquitously and have been a source of allergens causing allergic disorders. Earlier, we have reported the characteristics of the fungal extracts of *Rhizopus nigricans* and *F. solani* (20, 23). It has been reported that *F. solani* CF extract contained most of the major allergens present in the mycelial and spore extracts. In this

study, a standard *F. solani* CF extract was used to study the shared antigenic/allergenic cross-reactivity of its components with crude extracts of various common fungi identified by aerobiologic and clinico-immunologic studies conducted in India (1, 18, 19).

For cross-reactivity studies, various immunochemical methods, including immunoblot analysis, were used (Figs. 3 and 4). IgG-specific immunoblot of crude extracts of *A. alternata*, *M. hiemalis*, *P. citrinum*, *C. lunata*, *C. herbarum*, *Asp. fumigatus*, *Asp. niger*, *Asp. flavus*, *Asp. tamaritii*, and *Asp. versicolor* with rabbit anti-*F. solani* CF antibodies have demonstrated several shared antigenic proteins in the 14–100-kDa range (Table 2). Earlier studies (3, 7, 12) have shown that *Fusarium* antigenic proteins cross-react with those of *Alternaria*, *Penicillium*, *Aspergillus*, *Cladosporium*, and *Stemphylium*. O'Neil et al. (13) have reported that *F. solani*, *Penicillium notatum*, and *Asp. glaucus* share several antigenic and allergenic determinants.

Among the species of the same genus, *F. solani*, *F. equiseti*, and *F. moniliforme* were reported to possess common allergenic determinants (22). Both shared and unique antigens were also demonstrated among extracts of *Alternaria solani* and *A. tenuis* by Vijay et al. (24). However, Karr et al. (10) did not find cross-reactivity of *Alternaria* with three species of *Aspergillus* by RAST inhibition.

Allergenic cross-reactivity among six basidiomycetes species (*Calvatia cyathiformis*, *Coprinus quadrididus*, *Psilocybe cubensis*, *Pleurotus ostreatus*, *Ganoderma meredithae*, and *Pisolithus tinctorius*) was studied by Zubiria et al. (26) using the immunoprint inhibition method with proteins resolved on IEF (pI

3.5–9.5). Using a similar method, Wedner et al. (25) evaluated the existence of cross-reactivity between *Epicoccum nigrum* and dark-spored fungi, namely, *Alternaria*, *Curvularia*, *Nigrospora*, and *Stemphylium* and observed no significant cross-reactivity among them.

In the present study, a number of proteins in the 14–70-kDa range of *M. hiemalis*, *A. alternata*, *P. citrinum*, *Asp. niger*, *Asp. flavus*, *Asp. tamarii*, and *Asp. versicolor* were found to have shared allergenicity with the components of *F. solani* CF on immunoblot using pooled sera of patients sensitive to *Fusarium* (Table 3). A 45-kDa protein in most of the fungal extracts demonstrated shared antigenicity/allergenicity with the 45-kDa protein of *F. solani* CF extract.

This study provides strong evidence of a significant amount of cross-reactivity among fungal antigens. It is also possible that a mixture of purified protein components of different fungi showing shared allergenicity among the fungi could be used for diagnosis or immunotherapy of allergic disorders.

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