

## ORIGINAL ARTICLE

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# Identification of *Aspergillus* (*A flavus* and *A niger*) Allergens and Heterogeneity of Allergic Patients' IgE Response

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## ABSTRACT

*Aspergillus* species (*A flavus* and *A niger*) are important sources of inhalant allergens. Current diagnostic modalities employ crude *Aspergillus* extracts which only indicate the source to which the patient has been sensitized, without identifying the number and type of allergens in crude extracts. We report a study on the identification of major and minor allergens of the two common airborne *Aspergillus* species and heterogeneity of patients' IgE response to them.

Skin prick tests were performed on 300 patients of bronchial asthma and/or allergic rhinitis and 20 healthy volunteers. Allergen specific IgE in patients' sera was estimated by enzyme allergosorbent test (EAST). Immunoblots were performed to identify major/minor allergens of *Aspergillus* extracts and to study heterogeneity of patients' IgE response to them.

Positive cutaneous responses were observed in 17% and 14.7% of patients with *A flavus* and *A niger* extracts, respectively. Corresponding EAST positivity was 69.2% and 68.7%. In immunoblots, 5 allergenic proteins were identified in *A niger* extract, major allergens being 49, 55.4 and 81.5 kDa. Twelve proteins bound patients' IgE in *A flavus* extract, three being major allergens (13.3, 34 and 37 kDa). The position and slopes of EAST binding and inhibition curves obtained with individual sera varied from patient to patient. The number and molecular weight of IgE-binding proteins in both the *Aspergillus* extracts varied among patients.

These results gave evidence of heterogeneity of patients' IgE response to major/minor *Aspergillus* allergens. This approach will be helpful to identify disease eliciting molecules in the individual patients (component resolved diagnosis) and may improve allergen-specific immunotherapy.

**Keywords:** Allergens; *Aspergillus flavus*; *Aspergillus niger*; Heterogeneity; IgE response; Immunoblotting

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## INTRODUCTION

Allergic diseases of respiratory system, such as

bronchial asthma and allergic rhinitis, are increasingly becoming global health problems.<sup>1-3</sup> Airborne fungi are significant environmental components involved in the aetiology of allergic respiratory diseases.<sup>4,5</sup> *Aspergillus*, *Cladosporium*, *Alternaria*, *Curvularia* and *Fusarium* species have been reported as the most prevalent components of indoor and outdoor aeromycota around the world.<sup>6-11</sup> The incidence of fungal sensitization in patients of allergic respiratory diseases has been reported from 2.3% to even 80% in various studies worldwide.<sup>12-15</sup>

Crude fungal extracts used for diagnostic purposes only provide information of the allergen source to which the patient has been sensitized.<sup>16-19</sup> However, the clinical sensitivity of patients depends on the number and type of IgE-binding proteins present in these extracts.<sup>18,19</sup> Hence, it is crucial to determine the profile of allergenic proteins in crude fungal extracts and identify the IgE reactivity profile of each patient.

Of the various fungi, *Aspergillus* species have been reported to be one of the important inhalant allergens in different geographical regions of the world.<sup>6-10,13-16,18,20-22</sup> The reported frequency of sensitization with various species of *Aspergillus* in patients of allergic respiratory diseases varies from 15.3-38% worldwide.<sup>18,23-26</sup> A number of *A. fumigatus* allergens have been cloned from cDNA/phage display libraries, characterized and purified as recombinant proteins.<sup>4</sup> Recently, major and minor allergens of *A. tamarii* have also been reported by the authors.<sup>25</sup> Serine proteases and amylases have been identified as major allergens of *A. flavus* and *A. niger*, respectively.<sup>4,20-22</sup> However, a detailed analysis of *A. flavus* and *A. niger* allergens has not yet been reported. The aim of the present study was to investigate the allergenic potential of *A. flavus* and *A. niger* extracts; identification of their major/minor allergens and evaluation of heterogeneity of patients' IgE response to them.

## MATERIALS AND METHODS

### *Aspergillus* Extracts

Lyophilized *A. flavus* and *A. niger* extracts were procured from a commercial manufacturer (All Cure Pharma Pvt. Ltd., Haryana, India). The manufacturer has been duly licensed by Drugs Controller General of India for the production of fungal extracts. The fungi were cultured in a chemically defined medium for 3 weeks at  $28 \pm 2^\circ\text{C}$  (stationary culture). Fungal mats

were separated from medium by filtration, washed with double distilled water and lyophilized. The dried mats were finely powdered, defatted with diethyl ether, extracted in ammonium bicarbonate (0.1 M; pH 7.8), dialyzed and lyophilized. Protein content of the crude extracts was quantified by modified Lowry's method using phosphotungstic acid (15% in 10% HCl) as precipitating reagent.<sup>27</sup> Carbohydrate content was estimated by Anthrone method.<sup>28</sup>

### Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis

Protein profiles of the fungal extracts were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Hoefer miniVE SE300, MA, USA) using vertical slab gel in discontinuous buffer (0.025 M Tris, 0.2 M glycine and 0.1% SDS) system with stacking and resolving gels containing 5% and 12% polyacrylamide, respectively.<sup>29</sup> Lyophilized extracts were reconstituted in sample buffer containing  $\beta$ -mercaptoethanol followed by boiling at  $100^\circ\text{C}$  for 5 minutes. The samples containing 100  $\mu\text{g}$  of protein were loaded in the wells. Electrophoresis was carried out at 80 volts for 30 minutes followed by 120 volts for 2 hours. The bands were detected by staining with 0.1% Coomassie brilliant blue R-250 stain. Protein standards (19.8-103 kDa; Bio-Rad, CA, USA) were used to determine the molecular weights of proteins.

### Patients and Healthy Controls

A total number of 300 patients suffering with allergic respiratory diseases i.e. bronchial asthma and/or allergic rhinitis were studied. These study subjects were selected from the patients attending the Outpatients Department of Vishwanathan Chest Hospital, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India. The diagnosis of bronchial asthma and allergic rhinitis was ascertained as per GINA and ARIA guidelines, respectively.<sup>1,2</sup> Besides, 20 non-allergic, healthy volunteers (NHV) with no personal or family history of any allergic diseases were included as negative controls. Before performing diagnostic tests, phenothiazines and tricyclic antidepressants were discontinued for 1-2 weeks. Hydroxyzine, cetirizine and loratadine were discontinued for 3-10 days.<sup>30</sup> The protocol of the study was approved by Institutional Ethics Committee of Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India. Informed, written consent was obtained

from each subject.

### Skin Prick Tests

Skin prick tests (SPT) were performed on patients and NHV with *Aspergillus* extracts reconstituted in 50% glycerinated phosphate buffered saline (GPBS; 1:20 w/v). Besides, histamine (5 mg/mL in GPBS) and 50% GPBS were tested as positive and negative controls, respectively. Skin wheal diameters were measured after 15 minutes in two axes (one longest and the other perpendicular to it), excluding any pseudopodia and average was taken as wheal diameter for grading the cutaneous response.<sup>25</sup> Allergen induced wheal response, equivalent to wheal response induced by histamine, was graded as 2+. Skin responses of 2 mm less or 2 mm more than histamine reaction were graded as 1+ and 3+, respectively. Wheal response greater than 3+ was graded as 4+.<sup>25</sup> The positive cutaneous responses were confirmed by repeating the tests on the same day. Blood samples (15 ml) were collected from each patient and healthy volunteer, serum separated and stored at -20°C for performing various immunologic studies.

### Enzyme Linked Allergosorbent Test (EAST)

EAST was performed to estimate *Aspergillus* allergen specific IgE in the serum of individual patients.<sup>31</sup> Briefly, *Aspergillus* protein (1 µg/100 µl of 0.1 M carbonate buffer; pH 9.5) was coated in each well of a 96-well microtiter plate (Corning, USA) for 16 hours at 4°C. The wells were rinsed extensively with PBS containing 1% BSA and 0.5% Tween-20 (PBS-BSA-T). The unoccupied binding sites were blocked with PBS-1%BSA for 3 hours at 25°C. This was followed by overnight incubation at 4°C with 100 µl of serum samples diluted 1:4 v/v in PBS-BSA-T. After three washings with PBS-BSA-T, 100 µl of diluted alkaline phosphatase-conjugated monoclonal anti-human IgE (Sigma-Aldrich, MO, USA; 1:1000 v/v in tris buffered saline; TBS) was added in each well for 3 hours at room temperature. Thereafter, the wells were washed 4 times with PBS containing 0.05% Tween-20 and 100 µl of substrate solution (1mg/ml p-nitrophenyl phosphate in 0.1 M diethanolamine; pH 10.3) was added. The reaction was stopped with 100 µl of 0.75N sodium hydroxide after 30 minutes. The optical density (OD) was read at 405 nm using an automated microplate reader (BioRad, CA, USA). Each sample was tested in duplicate. Sera of 20 healthy controls

were used as negative controls. OD 405 values greater than the mean OD value obtained with 20 healthy controls +3 standard deviations (Mean +3SD) were considered positive.<sup>32</sup>

### IgE EAST Binding and Inhibition Assays

For various immunochemical studies, pooled patients' sera (PPS) (*Afl*) was prepared by pooling equal volumes of hypersensitive patients' sera (n = 21), who showed highly positive skin test and EAST response with *A. flavus*. Similarly, PPS (*Ani*) was also prepared by pooling equal volumes of sera from patients' hypersensitive to *A. niger* (n= 12). Another pool of sera from 11 non-allergic healthy subjects (NHS), showing uniformly negative skin test and EAST reactions to both the *Aspergillus* extracts, was also prepared to serve as a negative control.

Volume of PPS to be used for performing *Aspergillus* IgE EAST inhibition was ascertained by performing EAST binding assays using increasing amount of PPS (2-50 µl). The binding curve was plotted and the amount of PPS giving an optimum binding was determined. To evaluate the specificity of *Aspergillus* EAST, IgE binding was inhibited by pre-incubating 50 µl of specific PPS (1:8 v/v in PBS-BSA-T) with 50 µl self extract (1-1000 ng) for 16 hours at 4°C.<sup>25</sup> The pre-incubated mixture (100 µl) was then added to microtiter plate coated with *Aspergillus* extract and EAST was performed. In each assay, specific PPS without any inhibitor (100 µl) was used as a positive control and NHS as a negative control. Percentage inhibition was calculated as under:

$$\text{Inhibition (\%)} = \left[ 1 - \frac{\text{OD of PPS with inhibitor}}{\text{OD of PPS without inhibitor}} \right] \times 100$$

A dose-dependent curve was plotted (amount of allergen extract vs. % inhibition). To check the specificity of inhibition induced by self extracts, inhibition of each *Aspergillus* EAST was also attempted with five unrelated heterologous allergen extracts, pollen- *Prosopis juliflora*, *Ricinus communis*, insect- mosquito (*Culex quinquefasciatus*), moth (*Spodoptera litura*) and horse dander.

Besides, inhibition of *Aspergillus* EAST was also conducted using sera from *Aspergillus* sensitive individuals increasing amounts of homologous extract (0.1-10 µg) as inhibitor. The slopes and position of the curves obtained in each *Aspergillus* EAST were

compared (ANOVA, GraphPad Prism version 4.00, GraphPad Software, San Diego CA USA). For cross-reactivity studies, inhibition of each *Aspergillus* EAST using heterologous extract as liquid phase inhibitor was attempted

### IgE Immunoblots

Proteins of two *Aspergillus* extracts were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Pore size = 0.45  $\mu$ m, Bio-Rad, CA, USA) at 30V in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol; pH 8.3; Hoefer miniVE SE300, MA, USA). Protein markers (19.5-103 kDa; BioRad, CA) were also transferred along with sample proteins. The unoccupied sites were blocked with TBS-3% BSA at 37°C for 1 hour. The strips were washed two times with TBS and incubated at 4°C with specific PPS or individual sensitive patients' sera (1:5 v/v in TBS) for 16 hours. NHS (1:5 v/v in TBS) was used as a negative control. The strips were further washed three times with TBS containing 0.05% Tween-20 (TBST). Monoclonal anti-human IgE-alkaline phosphatase antibody (Sigma-Aldrich, MO, USA; 1:1000 v/v in TBS) was added to each strip for 3 hours at 37°C. The unbound antibodies were removed by washing with TBST and blots were developed by incubating the strips in 5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium chloride substrate (BCIP/NBT; SRL Pvt. Ltd., Mumbai, India) solution for 15 minutes and then washed with 0.5 M ethylenediaminetetraacetic acid in 50.0 ml TBS to stop the reaction.<sup>33</sup> As another control, immunoblot experiments with PPS were performed by substituting *Aspergillus* extracts with a non-specific horse dander extract.

## RESULTS

### Biochemical Characterization

The protein content in *A flavus* and *A niger* extracts was 142 and 185 mg/g of crude extract, respectively. The protein to carbohydrate ratio was 1:2.04 for *A flavus* and 1:3.36 for *A niger* extract. Fig.1 depicts that the total number of proteins detected in *A flavus* and *A niger* extracts were 15 (13.3-105 kDa) and 9 (17-81.5 kDa), respectively.

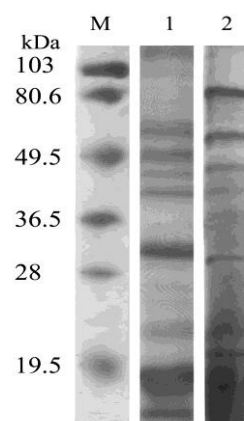
### Skin Sensitization to *Aspergillus* Allergens and Specific IgE Estimation

SPT were performed on 300 patients with different

allergic manifestations (bronchial asthma: 144; allergic rhinitis: 58; bronchial asthma with allergic rhinitis: 98). The age of patients varied from 14 to 57 years (Mean =  $33.9 \pm 13.3$  years) and of 20 healthy volunteers from 20 to 38 years (Mean =  $29 \pm 5.9$  years). Positive cutaneous reactivity with *A flavus* was observed in 17% of patients (1+: 20, 2+: 28 and 3+: 3). *A niger* extract elicited positive skin responses in 14.7% patients (1+: 15, 2+: 24, and 3+: 5). Only one of 20 NHV showed a 1+ reaction with both the extracts. No significant difference was observed in cutaneous responses among patients of bronchial asthma, allergic rhinitis and bronchial asthma with allergic rhinitis ( $p < 0.05$ ). *Aspergillus* specific IgE values were determined by EAST. The cut-off values of normal human sera OD for considering an *A flavus* and *A niger* EAST to be positive were 0.081 and 0.083, respectively. *A flavus*-specific IgE in patients represented by OD ranged from 0.212 to 0.601. Similarly, *A niger*-specific IgE in patients ranged from 0.197 to 0.595. Significantly elevated IgE levels were observed in patients as compared to normal controls ( $p < 0.05$ ).

*A flavus* EAST was positive in 69.2% of SPT positive cases. Of the 32 SPT positive sera, 22 (68.75%) turned out to be positive in *A niger* EAST.

Allergen-specific IgE levels increased with increase in patients' cutaneous response (0% in negative to 100% in 3+/4+). None of the 25 patients showing a negative SPT response to *A flavus* and *A niger* showed a positive EAST result.



**Figure 1. SDS-PAGE profile of the two *Aspergillus* extracts. M, Molecular weight markers (in kilodaltons); 1, *A flavus*; 2, *A niger*.**

### Specificity of IgE Quantification

PPS (*Afl*) and PPS (*Ani*) produced dose dependent binding in homologous *Aspergillus* EAST. Based on

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these binding curves, 12.0 µl and 10.0 µl volume of the PPS (*Afl*) and PPS (*Ani*), respectively was selected for conducting inhibition assays. Homologous extracts produced dose-related inhibition in each *Aspergillus* EAST, giving evidence for its specificity. The concentrations required for 50% inhibition of IgE binding to self extract were 480 ng and 90 ng for *A flavus* and *A niger*, respectively. All the five non-related allergen extracts failed to produce significant inhibition of each *Aspergillus* EAST even with 10 µg. Besides, *A niger* extract failed to produce significant inhibition in *A flavus* EAST even with an amount of 10 µg and vice versa.

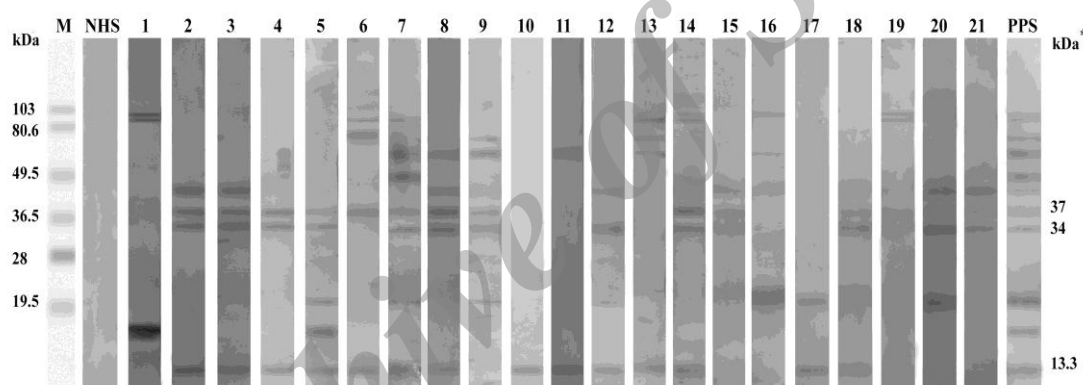
### Major and Minor Allergenic Proteins

The number of allergenic proteins detected in the two *Aspergillus* species were- *A flavus*: 11 (13.3-98.6 kDa) and *A niger*: 5 (34-81.5 kDa) (Figs. 2 and 3; Tables 1 and 2). No IgE-binding proteins were detected

in immunoblots performed with *Aspergillus* extracts and NHS. Besides, no allergenic proteins were detected in immunoblots of non-specific horse dander extract and specific PPS. The molecular weight of major allergens i.e. proteins recognized by more than 50% of individual patients' sera were: *A flavus*-13.3, 34, 37 kDa and *A niger*-49, 55.4, 81.5 kDa.

### Heterogeneity of IgE Response to *Aspergillus* Allergens

Heterogeneity of patients' IgE response to *Aspergillus* allergens was analyzed by EAST binding, inhibition and immunoblot experiments conducted with multiple patients' sera. In *Aspergillus* EAST, position and slopes of the binding curves varied among patients. The results of inhibition assays of *Aspergillus* EAST conducted with the sera of multiple patients and homologous extract as liquid phase inhibitor are



**Figure 2.** IgE immunoblot of *A flavus* extract using hypersensitive patients' sera. M, Molecular weight markers; NHS, probed with normal human serum; 1-21, probed with individual patients' sera; PPS, probed with pooled sera. \*Major allergens.

**Table 1.** The frequency of specific IgE binding proteins in 21 patients sensitized to *A flavus*.

Patient MW kDa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Freq. %
98.6	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	-	-	19.05
89.5	+	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	28.6
76.4	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	14.3
60	-	-	-	-	-	-	+	+	+	-	+	-	+	-	-	+	-	-	-	-	-	28.6
47	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	9.5
43	-	+	+	-	-	-	-	+	-	-	-	+	-	-	+	+	-	-	-	-	+	38.1
37 <sup>a</sup>	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	66.7
34 <sup>a</sup>	-	+	+	+	+	-	+	+	+	-	-	+	-	+	+	-	-	+	-	+	+	61.9
20	-	-	-	-	+	-	+	-	+	-	-	+	-	-	-	+	+	-	+	-	-	33.3
15.4	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9.5
13.3 <sup>a</sup>	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	-	-	-	66.7

MW, Molecular weight of allergenic protein; <sup>a</sup>Major Allergens

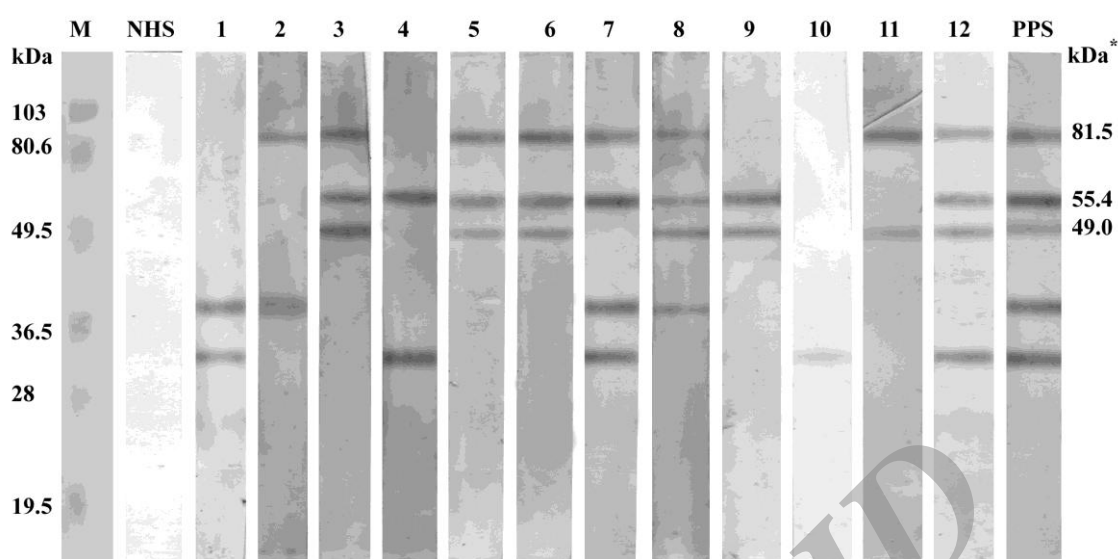


Figure 3. IgE immunoblot of *A niger* extract using hypersensitive patients' sera. M, Molecular weight markers; NHS, probed with normal human serum; 1-12, probed with individual patients' sera; PPS, probed with pooled sera. \*Major allergens.

Table 2. The frequency of specific IgE binding proteins in 12 patients sensitized to *A niger*.

Patient	1	2	3	4	5	6	7	8	9	10	11	12	Frequency %
MW kDa													
81.5 <sup>a</sup>	-	+	+	-	+	+	+	+	-	-	+	+	58.3
55.4 <sup>a</sup>	-	-	+	+	+	+	+	+	+	-	-	+	66.7
49 <sup>a</sup>	-	-	+	-	+	+	-	+	+	-	+	+	58.3
39.5	+	+	+	-	-	-	+	+	-	-	-	-	41.7
34	+	-	-	+	-	-	+	-	-	+	-	+	41.7

MW, Molecular weight of allergenic protein; <sup>a</sup>Major Allergens

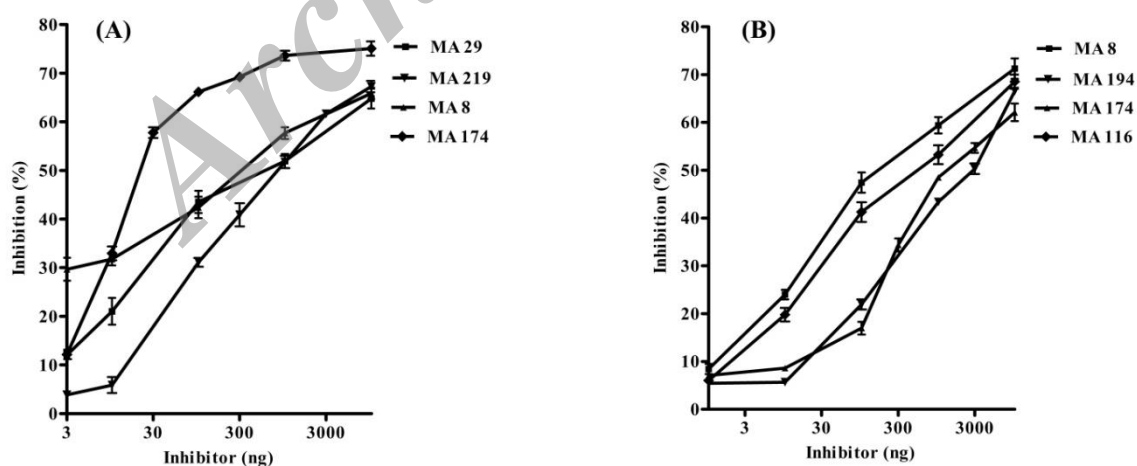


Figure 4. Inhibition of (A) *A flavus* and (B) *A niger* EAST with corresponding homologous extract as liquid phase inhibitor using multiple patients' sera. The values represent Mean  $\pm$  SD of three independent experiments. Slopes of the inhibition curves induced by different sera were significantly different from each other (ANOVA,  $p > 0.05$ ).

presented in Fig. 4. The position and slopes of the inhibition curves were variable with different sera. In immunoblots performed with multiple patients' sera, the number and molecular weight of IgE binding proteins detected in each *Aspergillus* extract varied from patient to patient. Thus, different patients' sera demonstrated distinct allergen-binding IgE profiles with each *Aspergillus* extract (Figs. 2 and 3; Tables 1 and 2).

## DISCUSSION

*Aspergillus* species are recognized as important inhalant allergens.<sup>4-10, 20-26</sup> Maurya and associates reported that *Aspergillus*-sensitized asthmatics had a more severe form of asthma as compared to those with hypersensitivity to some other allergens.<sup>24</sup> The present study demonstrated the allergenic potential of *A flavus* and *A niger* in vivo by skin testing and in vitro by revealing the presence of serum IgE against their allergens in EAST. The positive SPT responses were higher with *A flavus* (17.0%) than *A niger* extract (14.7%). However, in earlier studies, positive intradermal test responses with *A flavus* and *A niger* extracts were observed in 10-17.1% and 2.7-17% of patients, respectively.<sup>18,34</sup> This variability may occur due to various factors such as differences in patients' population, extent of exposure (occupational/non-occupational), characteristics of diagnostic extracts (potency and protein profile), testing methods and criterion used for grading. Homologous extract induced a dose-dependent response in each *Aspergillus* EAST. Besides, non-related heterologous extracts failed to induce any inhibition in EAST, further demonstrating the specificity of EAST inhibition.

In the present study, immunoblots identified the precise identity of disease eliciting molecules present in crude *Aspergillus* extracts. The results revealed the presence of multiple IgE-binding proteins in *A flavus* and *A niger* extracts. These allergens can be considered as promising candidate molecules for component resolved diagnosis of hypersensitive patients. Eleven IgE-binding proteins (13.3-98.6 kDa) were detected in *A flavus* extract using sera of 21 hypersensitive patients. Yu and associates also identified 7 IgE-reactive proteins of 28-65 kDa in *A flavus* extract using sera from only 4 *Aspergillus*-sensitive patients.<sup>35</sup> In *A niger* extract, five IgE-binding proteins (34, 39.5, 49, 55.4 and 81.5 kDa) were identified. Li and Sharma have reported only one (34 kDa) and two (18 and 70

kDa) allergenic proteins in crude *A niger* extract, respectively.<sup>10,36</sup> Thus, allergenic proteins identified in crude *Aspergillus* extracts varied for different study populations. This may have been due to the fact that allergenic potential of a protein is a function of genetic predisposition of different patient populations, level of their environmental exposure (qualitative as well as quantitative), and also due to variations in allergenic potency of the extracts used in different studies.

Major and minor allergens of various *Aspergillus* extracts have been identified in different studies.<sup>35-40</sup> Yu and associates and subsequently some other workers have reported 34 kDa alkaline- and vacuolar-serine proteases as major allergens of *A flavus* extract.<sup>35-37</sup> In the present study, in addition to 34 kDa protein, two proteins of 13.3 and 37 kDa were also observed as major allergens of *A flavus* extract. In *A niger*, 34 kDa serine protease has been reported to be a major allergen by some workers.<sup>36,39</sup> In the present study, two IgE-binding proteins of 55.4 and 81.5 kDa were identified as major *A niger* allergens while 34 kDa allergen was found to be minor for our study population. The major and minor allergens will be further confirmed by immunoproteomics approach i.e. resolving the extracts by two dimensional gel electrophoresis and serological screening.

While dose-dependent binding was obtained in each *Aspergillus* EAST with multiple sera, slopes and position of the binding curves varied from patient to patient. Similarly, slopes and position of *A flavus* and *A niger* EAST inhibition curves with individual patients' sera were also different. In immunoblots, variable IgE binding allergen profiles were obtained with individual patients' sera (n=12-21). These results gave definitive evidence for heterogeneity of patients' IgE response to different *A flavus* and *A niger* allergenic proteins. Gautam and associates also reported varying sensitization profiles of 8 allergic patients to *A fumigatus* proteins.<sup>40</sup> Vermani and associates reported variable IgE-binding profiles using sera from 26 *A tamarii* hypersensitive patients.<sup>25</sup>

The evaluation of heterogeneity of IgE response to different allergens has important clinical and diagnostic implications. The concept of component resolved diagnosis is based on the determination of patient specific allergen profile.<sup>19</sup> A critical analysis of IgE-binding protein profiles of 21 *A flavus* hypersensitive patients revealed that only minor allergens were detected with the sera of patient 1 (98.6, 89.5 and 15.4

kDa) and patient 16 (98.6, 60, 43 and 20 kDa; Fig. 2). Fig. 3 depicts that IgE binding protein profiles of patient 1 (39.5 and 34 kDa) and patient 10 (34 kDa) revealed only minor allergens of *A niger* extract. The allergens which were designated as 'minor' on the basis of study population, were the 'major' causes of allergic diseases in these patients. This observation suggests that current diagnostic approach based on crude extracts is not adequate for diagnosis and treatment of patients especially reacting to minor allergens. Thus, analysis of complete IgE-binding protein profiles (component resolved diagnosis) is crucial for the accurate diagnosis and effective immunotherapy of allergic patients.

In conclusion, our results revealed that crude *A flavus* and *A niger* extracts are composed of multiple major and minor allergens. The number of allergenic proteins detected in the *A flavus* was 11 including three major allergens (13.3, 34, 37 kDa). Five IgE binding proteins were detected in *A niger*, three of them being major allergens (49, 55.4, 81.5 kDa). Heterogeneity of patients' IgE response to various major/minor allergens of each *Aspergillus* extract has been demonstrated. Thus, the present study emphasizes the importance of component resolved diagnosis i.e. identification of allergen binding IgE profile of an individual. This patient-tailored diagnosis will be helpful in identifying actual disease eliciting molecules in allergic patients and may improve allergen-specific immunotherapy.

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