



Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio



Physico-chemical and clinico-immunologic studies on the allergenic significance of *Aspergillus tamarii*, a common airborne fungus

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ARTICLE INFO

Article history:

Received 30 January 2010

Received in revised form 14 June 2010

Accepted 28 June 2010

Keywords:

Inhalant allergens

Aspergillus tamarii

Airborne content

Major allergens

Heterogeneity

Cross-reactivity

ABSTRACT

Aspergillus-derived inhalant allergens play an important role in the etiology of allergic respiratory diseases. In the present study, we investigated the allergenic potential of *Aspergillus tamarii*, quantified its airborne content, identified its major/minor allergens, evaluated heterogeneity of patients' IgE response to its allergens and cross-reactivity of its allergens with other *Aspergillus* allergens. Skin prick tests with *A. tamarii* extract were performed on 300 patients of bronchial asthma/allergic rhinitis and 20 healthy volunteers. Sixty-six patients (22%) elicited positive cutaneous reactions to *A. tamarii* extract. Only one of the 20 non-allergic healthy volunteer showed a mild positive cutaneous reaction. Allergen-specific IgE levels increased with increase in patients' cutaneous response (0% in negative to 100% in 3+/4+). The skin positivity and allergen-specific IgE levels were significantly higher in patients compared to healthy volunteers ($P > 0.05$). However, no differences were found for these two parameters among patients of bronchial asthma, allergic rhinitis and bronchial asthma with allergic rhinitis. The airborne *A. tamarii* allergen content was highest in February and October. *A. tamarii* extract revealed at least 22 proteins (13.3–120 kDa). Seventeen of these proteins bound patients' IgE with six being major allergens (13.3, 23, 25, 34, 39.5, 43 kDa). Three major allergens (13.3, 34, 43 kDa) were found to cross-react with *A. flavus* and one (34 kDa) with *A. niger*. Our results revealed that *A. tamarii* allergen(s) are present in the air, which might serve as important inhalant allergens in IgE-mediated allergic respiratory diseases.

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Introduction

Allergic diseases of respiratory system, such as bronchial asthma and allergic rhinitis, are increasingly becoming global health problems (Masoli et al. 2004; Bousquet et al. 2008). The phenomenal increase in prevalence of allergies in the past decades is a result of changes in environment (Koppelman 2007). Fungi, ubiquitous in occurrence, have a great capacity to colonize many kinds of substrata and may develop in extreme environmental conditions, from where they become airborne. The most important fungi causing

respiratory allergies include *Aspergillus*, *Cladosporium*, *Penicillium*, *Alternaria*, *Epicoccum*, *Curvularia*, *Bipolaris*, *Candida*, etc. (Agarwal et al., 1969; Aggarwal et al., 2000; Agarwal and Shivpuri 1974; Crameri et al. 2008; Horner et al. 1995; Kurup and Banerjee 2000; Shivpuri and Agarwal 1969; Simon-Nobbe et al. 2008).

Crude allergen extracts are used for the diagnosis and immunotherapy of patients suffering with respiratory allergies. These extracts are usually complex mixtures of allergenic (major as well as minor allergens) and non-allergenic molecules (King et al. 1994; Larsen and Dreborg 2008). The precision of diagnosis and efficacy of immunotherapy depends on the quality of extracts used. Hence, it is absolutely necessary to standardize crude fungal extracts with respect to their allergenic proteins as well as their total allergenic potency. For quality control purposes, profile of allergenic proteins in crude extracts (especially major allergens) should be studied (Lockey et al. 2003). Besides, identification of cross-reactive proteins may reduce the number of allergens and thus overcome problems associated with standardizing crude fungal extracts (Lockey et al. 2003; Crameri et al. 2008).

Among the fungi, airborne *Aspergillus* species are sizable environmental components involved in the pathogenesis and persistence of allergic respiratory diseases (Aggarwal et al. 2000;

Abbreviations: *A. tamarii*, *Aspergillus tamarii*; BSA, bovine serum albumin; GPBS, 50% glycerinated phosphate buffered saline; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium chloride; NHV, non-allergic healthy volunteers; NHS, normal human serum; OD, optical density; PPS (*Ata*), pool of sera from *A. tamarii* hypersensitive patients; EAST, enzyme allergosorbent test; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SPT, skin prick test; TBS, Tris buffered saline.

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Cramer et al. 2008; Kurup and Banerjee 2000; Simon-Nobbe et al. 2008). The fungi grow outdoors as well as indoors, release large quantities of conidiospores and hyphal fragments, which act as sources of inhalant allergens (Cramer et al. 2008; Kurup and Banerjee 2000; Simon-Nobbe et al. 2008). The allergenic significance of various airborne *Aspergillus* species like *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans*, *A. versicolor* and *A. oryzae* has been extensively studied (Aggarwal et al. 2000; Kurup and Banerjee 2000; Simon-Nobbe et al. 2008; Shen et al. 1999). A number of *A. fumigatus* allergens have been cloned from cDNA/phage display libraries, characterized and purified as recombinant proteins (Cramer and Blaser 1996; Cramer et al. 2006; Vailes et al. 2001). Alkaline- and vacuolar-serine proteases have been identified as major allergens of *A. flavus* (Shen et al. 1999, 2007; Kurup and Banerjee 2000). Shared and specific allergens among these species have also been reported (Aggarwal et al. 2000; Shen et al. 1999, 2007; Aalberse 2007).

Aspergillus tamarii has also been identified as an important source of allergens (Agarwal et al., 1969; Aggarwal et al., 2000; Shivpuri and Agarwal 1969; Khan et al. 1999). However, to the best of our knowledge, a detailed analysis of its allergens has not been reported. The aim of the present study was to investigate the allergenic potential of *A. tamarii*, quantify its airborne allergen content, identify its major and minor allergens, evaluate heterogeneity of patients' IgE response to its allergens and its cross-reactivity with three common *Aspergillus* species viz. *A. flavus*, *A. fumigatus* and *A. niger*.

Materials and methods

Aspergillus extracts

Lyophilized *A. tamarii* extract was procured from a commercial manufacturer (All Cure Pharma Pvt. Ltd., Haryana, India). Besides, *A. flavus*, *A. fumigatus* and *A. niger* extracts were also procured. These extracts were prepared from the strains isolated from Delhi. Fungi were cultured in a synthetic medium for 3 weeks at $28 \pm 2^\circ\text{C}$. Fungal mats were separated from culture medium by filtration, washed with distilled water, lyophilized and finely powdered. The powders were defatted with diethyl ether, extracted in 0.1 M ammonium bicarbonate (pH 7.8), dialyzed and lyophilized. Protein content of the four *Aspergillus* extracts was quantified by modified Lowry's method using phosphotungstic acid reagent (15% in 10% HCl) for protein precipitation (Lowry et al. 1951). Carbohydrate content in each extract was quantified by anthrone method (Scott and Melvin 1953).

Protein profiling of *Aspergillus* extracts

Protein profiles of the four *Aspergillus* extracts were studied by subjecting them to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using vertical slab gel in discontinuous buffer system containing 0.025 M Tris, 0.2 M glycine and 0.1% SDS (Laemmli 1970). The stacking and resolving gels contained 5% and 12% polyacrylamide, respectively. Lyophilized extract was reconstituted in sample buffer containing β -mercaptoethanol followed by boiling at 100°C for 5 min. The sample containing 100 μg of protein was applied in each well and electrophoresis was carried out at 80 volts for 30 min followed by 120 V for 2 h. The bands were detected by staining with 0.1% Coomassie brilliant blue R-250 stain. Protein standards of 19.8–103 kDa (Bio-Rad, CA, USA) were used to determine the molecular weights of allergenic proteins.

Human subjects

A total number of 300 patients suffering with bronchial asthma and/or allergic rhinitis were included in the present study. These

patients were recruited at outpatients department in Viswanathan Chest Hospital, VP Chest Institute. The diagnosis of bronchial asthma and allergic rhinitis was ascertained as per GINA and ARIA guidelines, respectively (Masoli et al. 2004; Bousquet et al. 2008). The detailed history including age, sex, duration of illness, family history of illness, onset of symptoms etc. of each patient was recorded. Besides, 20 non-allergic, healthy volunteers (NHV) with no personal or family history of any allergic disorders were also studied. Phenothiazines and tricyclic antidepressants were discontinued 1–2 weeks before performing diagnostic skin tests. Hydroxyzine, cetirizine and loratadine were discontinued for 3–10 days (Kilinc and Alper 2005). Experimental protocol was approved by Institutional Ethics Committee. Informed, written consent was obtained from each subject.

Skin prick tests (SPTs)

SPT were performed on patients and NHV with *Aspergillus* extracts reconstituted in 50% glycerinated phosphate buffered saline (GPBS; 1:20, w/v) (Agarwal et al. 2003). Histamine (5 mg/ml in GPBS) and 50% GPBS were also tested as positive and negative controls, respectively. Skin wheal diameters were measured after 15 min in two axes (one longest and the other perpendicular to it), excluding any pseudopodia. The two diameters of each test were recorded and average was taken as wheal diameter for grading the cutaneous response. Allergen induced skin wheal response, equivalent to histamine response, 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+ (Agarwal et al. 2003; Kausar et al., 2007). The positive reactions (1+ to 4+) were confirmed by repeating the tests on the same day. Blood samples (15 ml) were collected from each patient and NHV, serum separated and stored at -20°C for performing various clinico-immunologic studies.

Specific IgE estimation

Specific IgE levels against *A. tamarii* were estimated by enzyme linked allergosorbent test (EAST) in the serum of individual patients (Johnstone and Thorpe 1987). Briefly, 1 μg *A. tamarii* protein in 100 μl of carbonate buffer (pH 9.5) was coated in the wells of a 96-well microtitre plate for 16 h at 4°C . After washing the unbound antigen with PBS containing 1% BSA and 0.5% Tween-20 (PBS-BSA-T), unoccupied binding sites were blocked with PBS-BSA (1%) for 3 h at room temperature. This was followed by three washes with PBS-BSA-T and overnight incubation with 100 μl of patient's serum diluted 1:5 (v/v) in PBS-BSA-T at 4°C . The plate was washed three times with PBS-BSA-T. The wells were incubated with 100 μl of diluted alkaline phosphatase-conjugated monoclonal antihuman IgE (Sigma, A3076; 1:1000, v/v in Tris buffered saline; TBS) for 3 h at room temperature. Thereafter, the wells were washed four times with PBS containing 0.05% Tween-20 and the color was developed with 100 μl of substrate solution (1 mg/ml p-nitrophenyl phosphate in 0.1 M diethanolamine; pH 10.3). The reaction was stopped after 30 min with addition of 100 μl of 0.75 N sodium hydroxide and absorbance read at 405 nm using an automated microplate reader (Bio-Rad, CA, USA).

EAST was also performed with the sera of 20 healthy controls. Optical density (OD) 405 values greater than mean OD value obtained with healthy controls + 3 standard deviations were considered positive (Bernstein et al. 2008).

EAST binding assays

For various immunobiochemical studies, pooled patients' sera, PPS (*Ata*) was prepared by pooling equal volumes of hypersensitive

patients' sera ($n=26$), who showed highly positive skin test and EAST response. Sera from 11 non-allergic healthy subjects were pooled to be used as a negative control (NHS). Volume of PPS to be used for performing *A. tamarii* IgE EAST inhibition was ascertained by performing EAST binding assays using increasing amount of PPS (2, 5, 10, 20 and 50 μl). The binding curve was plotted and the amount of PPS (*Ata*) giving an optimum binding (in the linear range) was determined. Besides, binding curves were generated with the sera of ten individual *A. tamarii* hypersensitive patients.

Specific IgE EAST inhibition

To evaluate the specificity of *A. tamarii* EAST, IgE binding was inhibited by pre-incubating PPS (*Ata*) (1:10, v/v in PBS-BSA-T) with 1–1000 ng of *A. tamarii* extract for 16 h at 4°C. The pre-incubated mixture (100 μl) was then added to different wells of a pre-coated microtitre plate coated with *A. tamarii* extract and EAST was performed. PPS (*Ata*) without any inhibitor (100 μl) was used as a positive control and NHS as a negative control. EAST 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). Inhibition of *A. tamarii* 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 to check the specificity of inhibition assays. For cross-reactivity studies, inhibition of *A. tamarii* EAST was also attempted with *A. niger*, *A. fumigatus* and *A. flavus* extracts as liquid phase inhibitors. Besides, inhibition of *A. tamarii* EAST was conducted using sera from *A. tamarii* sensitive individuals ($n=4$).

Quantification of airborne *A. tamarii* allergens

The airborne *A. tamarii* allergens in Delhi metropolitan area were measured as described earlier (Agarwal et al. 1981). A volumetric filtration sampler was installed on the open terrace of Vallabhbhai Patel Chest Institute, Delhi, India at a height of 20 m above the ground unobstructed by buildings or trees. The sampler was operated at a flow rate of 40 liter/min. Airborne particles were collected on a poly-tetra-fluoro-ethylene membrane for 24 h, weekly for 1 year (May 2007–April 2008). The exposed membranes were soaked in sterile 0.1 mol/l ammonium bicarbonate (10 ml) for 72 h with intermittent stirring. The eluates were dialyzed, lyophilized and reconstituted in 1 ml of PBS. The allergen content in these eluates was measured by EAST inhibition assays using *A. tamarii* as solid phase, PPS (*Ata*) as source of IgE antibodies and eluates as liquid phase inhibitors (50 μl aliquots). Besides, a dose-dependent reference inhibition curve was generated with *A. tamarii* extract. On interpolating the values of percent inhibition induced by an eluate in reference curve, amount of allergen extract in 50 μl of eluate was calculated. The amount of *A. tamarii* allergen/ m^3 of air was calculated as

$$v = \text{total air sampled in 24 h (in } \text{m}^3 \text{)}$$

The eluate of 24 h air samples was lyophilized and reconstituted in 1000 μl :

$$\text{amount of allergen/}\text{m}^3 \text{ of air} = \left(\frac{\text{allergen in 50 } \mu\text{l of air sample}}{v \text{ m}^3} \right) \times 20$$

Equal volumes of 11 eluates showing high *A. tamarii* allergen content were pooled. Inhibition of *A. tamarii* EAST was conducted with

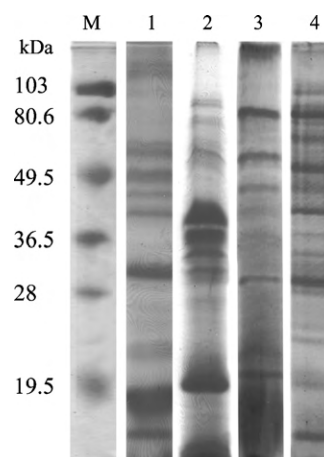


Fig. 1. SDS-PAGE profile of the four *Aspergillus* extracts. Approximately 100 μg protein was loaded in each well. The proteins were electrophoresed at 80 V for 30 min followed by 120 V for 2 h and bands were visualized with Coomassie stain. M, molecular weight markers (in kilo Daltons); 1, *A. flavus*; 2, *A. fumigatus*; 3, *A. niger*; 4, *A. tamarii*.

increasing volume (1.56–100 μl) of this pool. Besides, homologous extract and eluate of a non-exposed filter membrane were used as positive and negative control inhibitors.

IgE immunoblots

Following SDS-PAGE, *A. tamarii* proteins were electrophoretically transferred to nitrocellulose membrane at 30 V in transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol (pH 8.3) (Towbin et al. 1979). Protein markers (19.5–103 kDa; Bio-Rad, CA) were also transferred along with sample proteins. Non-specific sites were blocked with 3% BSA in TBS for 1 h at 37°C. The membrane was washed with TBS and incubated overnight with PPS (*Ata*) or individual *A. tamarii* sensitive patients' sera (1:5, v/v in TBS) at 4°C. As a negative control, a strip of *A. tamarii* proteins was incubated with NHS (1:5, v/v in TBS). After washing four times with TBS containing 0.05% Tween-20 (TBST), monoclonal anti-human IgE-alkaline phosphatase antibody (Sigma; 1:1000, v/v in TBS) was added to the membrane for 3 h at 37°C. The unbound antibodies were removed by washing with TBST and blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium chloride substrate (BCIP/NBT) solution. The blots were washed after 15 min with stop solution (0.5 M ethylenediaminetetraacetic acid in 50.0 ml TBS) to stop the color reaction. As a control, immunoblot experiments with PPS (*Ata*) were performed by substituting *A. tamarii* extract with a non-specific mosquito extract. Besides, immunoblots were also performed with three heterologous *Aspergillus* extracts namely *A. niger*, *A. fumigatus* and *A. flavus* using PPS (*Ata*) to detect any protein(s) in these extracts which bound IgE from sera pool of *A. tamarii* hypersensitive patients.

PPS (*Ata*) incubated with nitrocellulose membrane strip blotted with *A. tamarii* proteins (400 μg) was recovered and used in repeat *A. tamarii* immunoblots to detect inhibition of IgE binding to homologous proteins. Besides, absorbed PPS (*Ata*) samples recovered after incubation with *A. niger*, *A. fumigatus* and *A. flavus* protein strips were also used in repeat *A. tamarii* immunoblots to detect inhibition of IgE binding to heterologous proteins (if any).

Results

The protein contents in the four *Aspergillus* extracts were: *A. tamarii* = 127 $\mu\text{g}/\text{mg}$, *A. flavus* = 140 $\mu\text{g}/\text{mg}$, *A. fumigatus* = 164 $\mu\text{g}/\text{mg}$, *A. niger* = 184 $\mu\text{g}/\text{mg}$. The corresponding

Table 1
Clinical characteristics of 300 patients included in the present study.

Patient groups	Number of patients
Gender	
Male	161
Female	139
Diagnosis	
Bronchial asthma	144
Allergic rhinitis	58
Bronchial asthma with allergic rhinitis	98
Age of onset	
<20 years	104
≥20 years	196
Duration of disease	
<5 years	137
≥5 years	163
Family history of allergic diseases	
Positive	128
Negative	172

carbohydrate contents were 290, 188, 622, 285 µg/mg, respectively. At least 22 distinct proteins (13.3–120 kDa) could be detected in *A. tamarii* extract (Fig. 1). The protein profile was different from that of *A. flavus*, *A. fumigatus* and *A. niger* extracts (Fig. 1).

SPT were performed on 300 patients of bronchial asthma and/or allergic rhinitis with *A. tamarii* extract. The age of patients varied from 14 to 57 years (mean=33.9±13.3 years) and of 20 healthy volunteers from 20 to 38 years (mean=29±5.9 years). The detailed characteristics of patients are presented in Table 1. Positive cutaneous reactivity was observed in 22% (66) patients (Table 2). Only one of 20 NHV showed a 1+ reaction. Markedly positive skin reactions were not observed in healthy volunteers. Significant differences for SPT were observed between patients and healthy volunteers ($P<0.05$). However, no significant difference

Table 2
Skin test reactivity of 300 hypersensitive patients and 20 non-allergic healthy controls to *A. tamarii*.

Allergen extract	Skin prick test response							
	Patients						Healthy controls	
	TTD	1+	2+	3+	4+	1+ to 4+ No. (%)	TTD	1+ No. (%)
<i>A tamarii</i>	300	30	30	5	1	66 (22.0)	20	1 (5%)

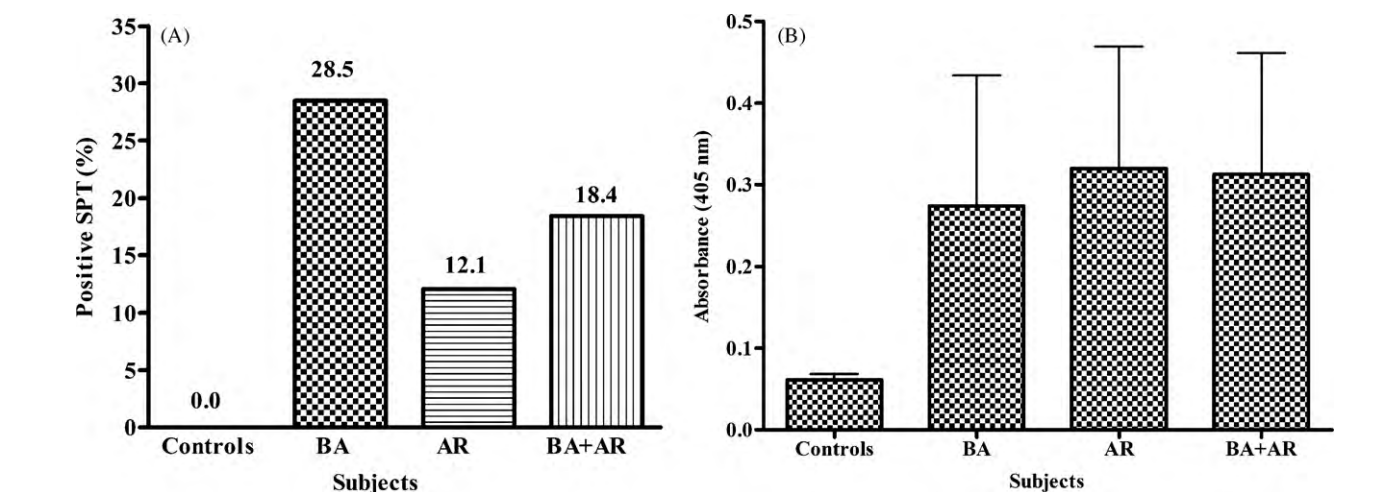


Fig. 2. (A) Comparison of positive SPT results of patients and controls (NHV). (B) OD (mean±SD) values of sera from patients and controls in *A. tamarii* EAST. Significant differences were observed between SPT and EAST results of patients and NHV ($P<0.05$). BA, bronchial asthma; AR, allergic rhinitis; BA+AR, bronchial asthma with allergic rhinitis.

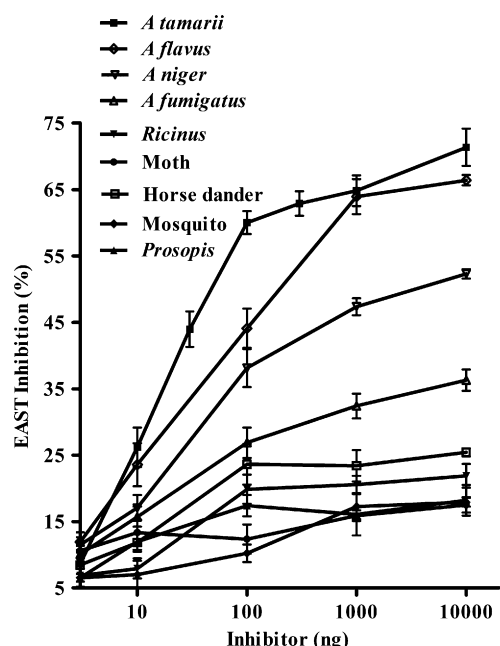


Fig. 3. IgE EAST inhibition of *A. tamarii* extract with homologous, three heterologous *Aspergillus* extracts and five unrelated allergen extracts. Microtitre plates were coated with *A. tamarii* extract (1 µg/well), blocked and incubated with a mix containing 50 µl pooled sera (1:10, v/v) and 50 µl of extract (1–1000 ng). The bound IgE was determined using antihuman IgE-alkaline phosphatase conjugate. The values represent mean ± SD of three independent experiments.

The airborne *A. tamarii* allergen content varied on a daily basis (0.7–1398 ng/m³ of air; Fig. 4) and was highest in February (1398.6 ng/m³) and October (384.0 ng/m³). A pool of 11 air samples showing high *A. tamarii* allergen concentration produced dose-dependent inhibition of *A. tamarii* EAST. Slope of this inhibition line was parallel to that produced by homologous extract ($P > 0.05$). Extract of a non-exposed filter membrane did not show any inhibition in *A. tamarii* EAST.

Immunoblotting of *A. tamarii* extract with PPS (*Ata*) revealed 17 IgE-binding proteins of 13.3–81.5 kDa (Fig. 5, Table 4). Proteins of 13.3, 23, 25, 34, 39.5 and 43 kDa were recognized by more than 50% of individual patients' sera, hence designated as major allergens of *A. tamarii* extract (Larsen and Lowenstein 1996; Fig. 6, Table 4).

To evaluate the heterogeneity of patients' IgE response to major and minor allergens of *A. tamarii* extract, EAST binding, inhibition and immunoblot experiments using multiple patients' sera were conducted. Position and slopes of EAST binding curves obtained

with 10 individual sera varied from patient to patient (Fig. 7A). Similarly, position and slopes of EAST inhibition curves (with homologous extract as inhibitor) using sera from 4 patients also varied, suggesting qualitative as well as quantitative heterogeneity of specific IgE profile against *A. tamarii* proteins (Fig. 7B). In immunoblots performed with 26 sera, the number and molecular weight of IgE-binding proteins detected in *A. tamarii* extract varied among patients, giving conclusive evidence for heterogeneity of IgE response to *A. tamarii* allergens (Fig. 5, Table 4).

To identify the cross-reactivity of *A. tamarii* extract, SPT results were analyzed and EAST inhibition was carried out with PPS (*Ata*) using heterologous *Aspergillus* extracts i.e. *A. flavus*, *A. fumigatus* and *A. niger* extract as inhibitors. A preliminary analysis of SPT results revealed that of the 66 patients who showed positive cutaneous response to *A. tamarii* extract, 66.7%, 63.6% and 48.4% were also SPT positive to *A. flavus*, *A. fumigatus* and *A. niger* extract. In *A. tamarii* EAST, homologous as well as *A. niger* and *A. flavus* extracts induced dose-dependent inhibition, giving evidence for the presence of some *A. tamarii* allergens in these two species (Fig. 3). The amount of *A. tamarii* (homologous) extract required for 50% inhibition of *A. tamarii* EAST was 50 ng as against 152 ng and 4.2 µg of *A. flavus* and *A. niger* extracts, respectively. *A. fumigatus* extract failed to induce any inhibition even with an amount of 10 µg used as inhibitor (Fig. 3).

IgE-binding proteins common in *A. tamarii* and the above *Aspergillus* species were identified in immunoblot inhibition experiments. Immunoblot of PPS (*Ata*) with heterologous *Aspergillus* extracts revealed three IgE-binding proteins of 43, 34 and 13.3 kDa in *A. flavus*, one (34 kDa) in *A. niger* and none in *A. fumigatus* extract (Fig. 8). When the absorbed PPS (*Ata*) from the above experiments was reused in *A. tamarii* immunoblots (IgE immunoblot inhibition): (a) *A. tamarii* absorbed PPS (*Ata*) didn't show any allergenic proteins, (b) *A. flavus* absorbed PPS (*Ata*) detected all the allergenic proteins except 43, 34 and 13.3 kDa proteins, and (c) *A. niger* absorbed PPS (*Ata*) detected all the allergenic proteins except 34 kDa. However, IgE binding to *A. tamarii* proteins was not inhibited by *A. fumigatus* extract. Thus, of the six major allergens of *A. tamarii* extract, three were shared with *A. flavus* and only one with *A. niger*.

Discussion

A few preliminary studies are available on the allergenic significance of *A. tamarii*, a common airborne *Aspergillus* species till date (Aggarwal et al. 2000; Shivpuri and Agarwal 1969). We report a detailed analysis of allergenicity of *A. tamarii*, its major and minor allergens and cross-reactivity with other *Aspergillus* species. The allergenic potential of *A. tamarii* was evaluated by SPT and

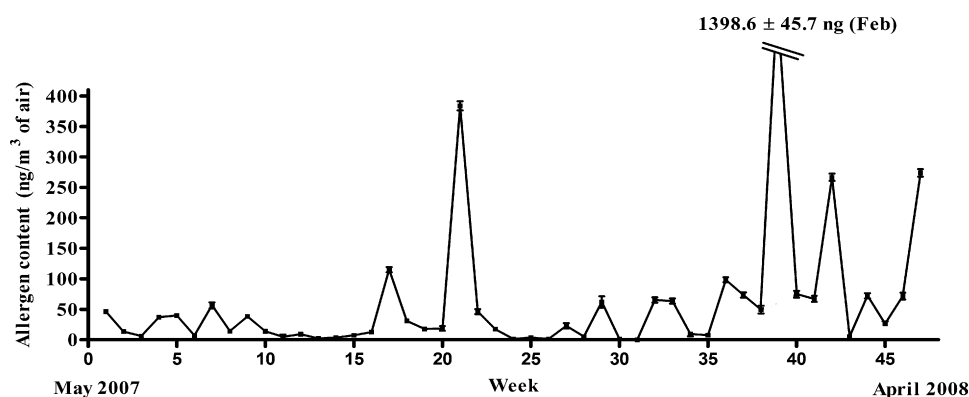


Fig. 4. Airborne *A. tamarii* allergen content in Delhi from May 2007 to April 2008. A volumetric filtration sampler was installed outdoors at 20 m height and 24-h samples (once in a week) were collected on a PTFE membrane (flow rate = 40 l/min). The exposed membranes were extracted in 0.1 mol/l ammonium bicarbonate, dialyzed, lyophilized and reconstituted in PBS. The processed eluates were used as liquid phase inhibitors in *A. tamarii* EAST. Allergen content was calculated from reference inhibition curve generated with homologous extract.

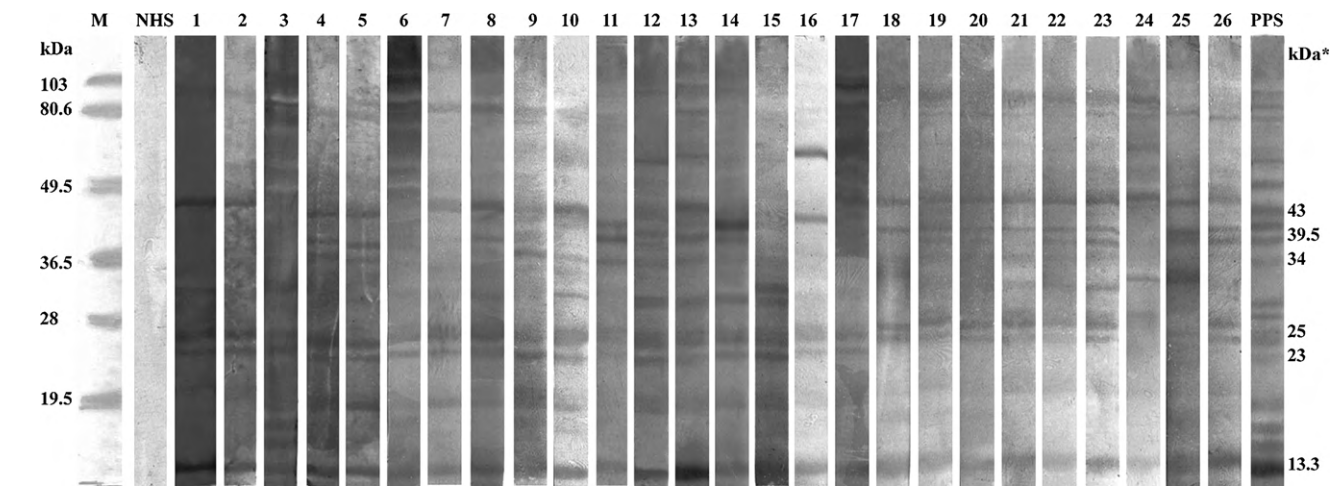


Fig. 5. IgE immunoblot of *A. tamarii* extract using hypersensitive patients' sera. *A. tamarii* proteins (separated by SDS-PAGE) were electrophoretically transferred to a nitrocellulose membrane (0.45 μ m). The protein strips were blocked and incubation with respective serum (1:5, v/v) followed by monoclonal anti-human IgE-alkaline phosphatase antibody. The bound anti-human IgE antibodies were detected with BCIP/NBT substrate. NHS, probed with normal human serum; 1–26, probed with individual patients' sera; PPS, probed with pooled sera; M, Molecular weight markers. *Major allergens.

EAST. Positive cutaneous responses were elicited in 22% of allergic patients. However, in some earlier studies, positive intradermal test responses with *A. tamarii* extract were observed in 16–17% of patients (Aggarwal et al. 2000; Shivpuri and Agarwal 1969). Even though, skin prick tests are less sensitive than intradermal tests, higher incidence of skin prick test reactivity was observed in our study. This variability may occur due to various factors such as differences in patients' population, extent of exposure, characteristics of diagnostic extracts (potency and protein profile), skin testing methods (SPT/intradermal), the site of performance and criterion used for grading. The allergenic potency of *A. tamarii* extract was evaluated by EAST inhibition. Allergen-specific IgE levels were significantly higher in patients, compared to normal controls. Homologous extract induced a dose-dependent response in *A. tamarii* EAST. Besides, non-related heterologous extracts failed to induce any inhibition in *A. tamarii* EAST, further demonstrating the specificity of EAST inhibition.

Direct measurement of fungal allergen concentration in the air has been considered to be a more relevant parameter, as compared to colony counts, to assess their role as inhalant allergens (Esch and Bush 2003). Culture techniques omit non-viable spores that can nevertheless be allergenic. Besides, morphologically non-

identifiable or non-viable fungal hyphae and spore fragments may also possess allergenic activity (Agarwal et al. 1981). Thus, total airborne *A. tamarii* allergen content was quantified by an immunochemical technique. This method was based on (a) collection of airborne particles on a filter membrane, (b) elution of these particles in suitable buffer, and (c) quantification of allergen content in the eluates in EAST inhibition assays using *A. tamarii* solid phase allergens and patients' sera pool as source of *A. tamarii*-specific IgE antibodies. The presence of *A. tamarii* allergen(s) in the air confirmed the exposure of patients to these allergen(s). Although *A. tamarii* allergens were present throughout the year, peak levels were recorded in February and October. The knowledge of peak seasons is important as the onset and severity of symptoms of allergic patients is related to the concentration of specific airborne allergens (Agarwal et al. 1981; Esch and Bush 2003).

Current diagnostic modalities employ crude allergen extracts which only indicate the source of sensitizing allergen (Bhalla and Singh 2008). Immunoblots were performed to identify the precise identity of disease eliciting molecules present in *A. tamarii* extract. Seventeen IgE-binding proteins of 13.3–81.5 kDa were identified. Multiple allergenic proteins have been identified in *Aspergillus* extracts, varying from species to species (Gautam et al. 2007;

Table 4
The frequency of specific IgE-binding proteins in 26 patients sensitized to *A. tamarii*.

MW (kDa)	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z	Freq. %
81.5																											15.4
78																											7.7
72.5																											34.6
55.4																											15.4
47.8																											7.7
43 ^a																											61.5 ^a
39.5 ^a																											50.0 ^a
36.7																											11.5
34 ^a																											57.8 ^a
31.4																											3.8
29.8																											26.9
25 ^a																											88.5 ^a
23 ^a																											92.3 ^a
19.4																											7.7
17.8																											3.8
16.5																											7.7
13.3 ^a																											100.0 ^a

MW, molecular weight of allergenic protein.

^a Major allergens.

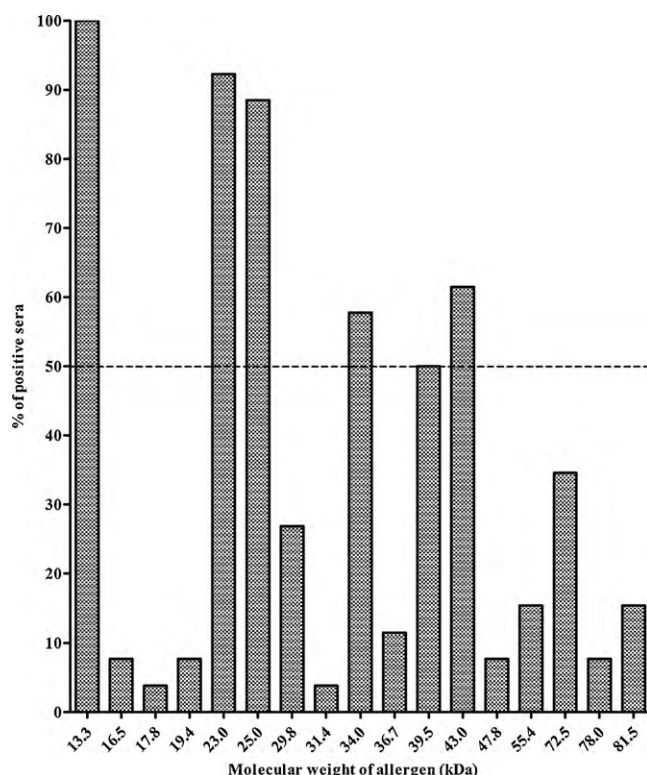


Fig. 6. Major and minor allergens in *A. tamarii* extract. The bars show percentage of hypersensitive patients' sera that bound to a specific allergen in IgE immunoblots of *A. tamarii* extract. Bars greater than 50% indicate major allergens.

Samuelsen et al. 1991; Trompelt et al. 1994; Yu et al. 1999). IgE-binding protein profile of *A. tamarii* extract was different from that of *A. flavus*, *A. fumigatus* and *A. niger*. Further, immunoblot analysis with individual patients' serum demonstrated six proteins of 13.3, 23, 25, 34, 39.5 and 43 kDa as major allergens of *A. tamarii* extract. The 13.3 and 23 kDa allergenic proteins may be considered as most important major allergens as they showed IgE-binding with more than 90% of the patients' sera tested. These major allergens are promising candidate molecules for component resolved diagnosis of hypersensitive patients.

IgE response against various major/minor allergenic proteins of an extract varies from patient to patient. The modern con-

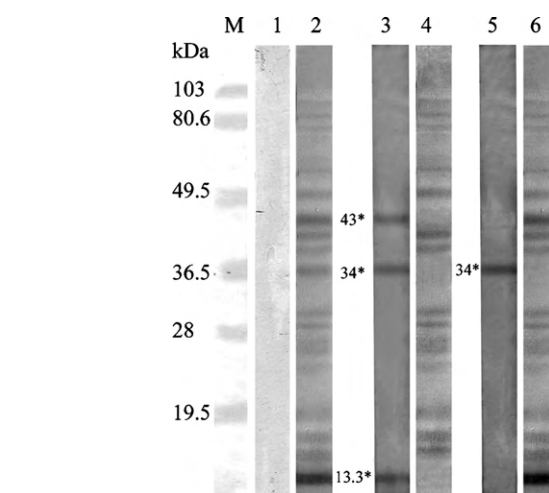


Fig. 8. IgE immunoblots and immunoblot inhibition. Immunoblots of PPS (*A. tamarii*) with heterologous *Aspergillus* extracts were performed. The absorbed PPS (*A. tamarii*) from the above experiments was reused in *A. tamarii* immunoblots. Immunoblot of *A. tamarii* and 1, NHS, Normal human serum; 2, PPS, Pooled sera from *A. tamarii* allergic patients; 3, *A. flavus* extract and PPS (*A. tamarii*); 4, *A. tamarii* extract and absorbed PPS (*A. tamarii*) after incubation with *A. flavus*; 5, *A. niger* extract and PPS (*A. tamarii*); 6, *A. tamarii* extract and absorbed PPS (*A. tamarii*) after incubation with *A. niger*; M, Molecular weight markers. *Cross-reacting allergens.

cept of component resolved diagnosis is based on the fact that the actual clinical sensitivity of a patient to an allergen extract depends on the number and type of its constituent allergenic proteins (Bhalla and Singh 2008). Thus, sera from multiple *A. tamarii* hypersensitive patients were used in EAST binding, EAST inhibition and immunoblot experiments. While dose-dependent binding was obtained in *A. tamarii* EAST with multiple sera ($n = 10$), slopes and position of the binding curves varied from patient to patient. Similarly, slopes and position of *A. tamarii* EAST inhibition curves with six different patients' sera were also different. Besides, in immunoblots, variable IgE-binding profiles were observed with 26 individual sera, giving definitive evidence for heterogeneity of patients' IgE response to different *A. tamarii* allergenic proteins. Gautam and associates also reported varying sensitization profiles of 8 allergic patients to *A. fumigatus* proteins (Gautam et al. 2007).

Cross-reactivity among allergens has been observed among different species/genera of fungi including *Aspergillus* species

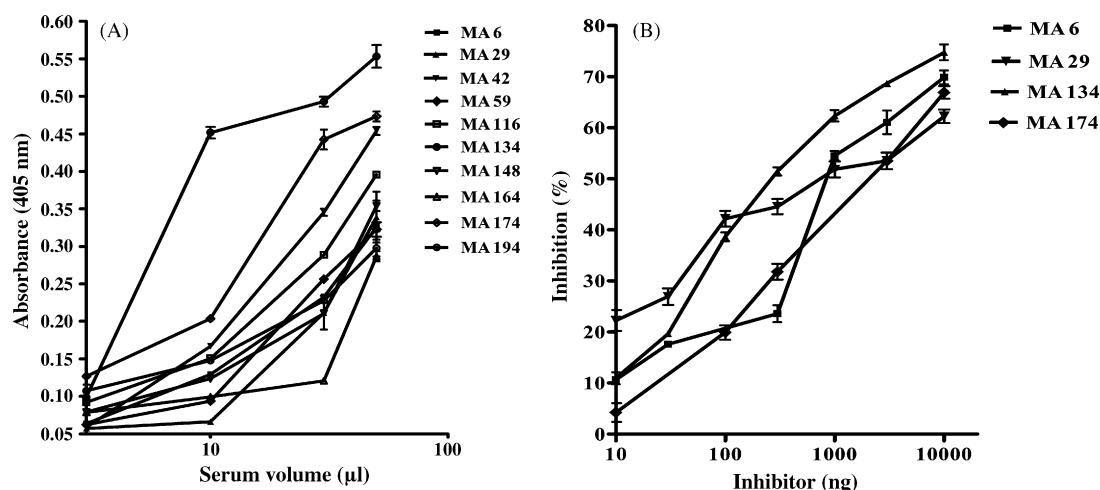


Fig. 7. A. IgE binding in *A. tamarii* EAST using sera from different *A. tamarii* hypersensitive patients. Microtitre plates were coated with *A. tamarii* extract (1 μg/well), blocked and incubated with an increasing amount of individual patients' sera (2, 5, 10, 20 and 50 μl). B. Inhibition of *A. tamarii* EAST with homologous extract using multiple patients' sera. Microtitre plates were coated with *A. tamarii* extract (1 μg/well), blocked and incubated with a mix containing 50 μl of individual patients' sera and 50 μl of homologous extract (1–1000 ng). In both assays, bound IgE was determined using antihuman IgE-alkaline phosphatase conjugate. The values represent mean ± SD of three independent experiments.

(Cramer et al. 2008; Shen et al. 1999, 2007; Simon-Nobbe et al. 2008). The knowledge of shared allergens is helpful in the diagnostics because it reduces the number of allergens for testing. Besides, it is also beneficial in the formulation of immunotherapy vaccines as broad-spectrum effect may be obtained with lesser number of allergens (Lockey et al. 2003). Cross-reactivity among various species of *Aspergillus* has been demonstrated earlier (Aggarwal et al. 2000; Cramer et al. 2008; Kurup and Banerjee 2000; Shen et al. 1999, 2007; Simon-Nobbe et al. 2008). Alkaline and vacuolar-serine proteases (32–34 kDa) from *Aspergillus* species termed as group 13 and group 18 allergens, respectively, have been studied in detail at the molecular level. *A. fumigatus* (Asp f 13 and Asp f 18), *A. flavus* (Asp f 13 and Asp f 18) and *A. oryzae* (Asp o 13) serine proteases share IgE and IgG epitopes with each other (Shen et al. 1999; Simon-Nobbe et al. 2008). In the present study, cross-reactivity of *A. tamarii* with three *Aspergillus* species that are well established sources of airborne allergens, namely *A. flavus*, *A. fumigatus* and *A. niger* was investigated.

Dose-dependent inhibition of *A. tamarii* EAST with *A. flavus* and *A. niger* extracts indicated that some IgE molecules present in the sera pool of *A. tamarii* sensitive patients also reacted with the allergens present in these extracts. There was no significant difference in slopes and position of inhibition curves induced by *A. tamarii* and *A. flavus* extracts, suggesting close identity in the allergenic proteins of the two extracts. In a previous study, cross-reactivity between *A. tamarii* and *A. flavus* has been reported on the basis of IgE radioallergosorbent test inhibition (Aggarwal et al. 2000). In contrast, slope and position of inhibition curve induced with *A. niger* extract was significantly different from the curve with *A. tamarii* extract, indicating a lower degree of cross-reactivity in these extracts. IgE immunoblot inhibition assays demonstrated cross-reactive proteins of 13.3, 34 and 43 kDa in *A. tamarii* and *A. flavus* extract. Presence of three shared allergenic proteins in *A. flavus* and *A. tamarii* may be attributed to a close phylogenetic relationship between these two species i.e. they belong to the same taxonomic group – the *A. flavus-oryzae* group (Raper and Fennel 1965). Phylogenetically related species show high degree of homology in the primary structure of their proteins which results in homologous three-dimensional structures (epitopes) and, potentially, shared allergenic proteins in them (Aalberse 2007). Besides, IgE immunoblot inhibition revealed that only one major *A. tamarii* allergen (34 kDa) was present in *A. niger* extract. The other three major allergens of 23, 25 and 39.5 kDa were specific to *A. tamarii* extract.

In conclusion, our results revealed that airborne *A. tamarii*-derived allergens are present in the air, which might serve as important inhalant allergens in IgE-mediated allergic respiratory diseases. Seventeen different IgE-binding proteins of crude *A. tamarii* extract have been identified with six of them being major allergens. At least 3 *A. tamarii* allergenic proteins (43, 34 and 13.3 kDa) were present in *A. flavus* and one of 34 kDa in *A. niger* extract (cross-reactive allergenic proteins). In future, purification and characterization of clinically important cross-reactive allergens is important for producing better diagnostic as well as effective therapeutic reagents for patients allergic to *A. tamarii* and allergenically related *Aspergillus* species.

Acknowledgements

The authors thankfully acknowledge the financial support (Senior Research Fellowship) from Council of Scientific and Industrial Research, India to one of the co-authors (MV).

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